TECHNISCHE UNIVERSITÄT MÜNCHEN

Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität

Comparative characterization of selected *Saccharomyces* yeast strains as beer fermentation starter cultures

Tim Meier-Dörnberg

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktor-Ingenieurs (Dr.-Ing.)

genehmigte Dissertation.

Vorsitzender: Prof. Dr.-Ing. Ulrich Kulozik

Prüfer der Dissertation:
1. Hon.-Prof. Dr.-Ing. Friedrich Jacob
2. Prof. Dr. rer. nat. habil. Rudi F. Vogel
3. Prof. Dr.-Ing. Dirk Weuster-Botz

Die Dissertation wurde am 04.05.2018 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 16.10.2018 angenommen.

“Yeast is the flavor engine of the brewing industry“

-Tim Meier-Dörnberg-

Acknowledgments

First of all, I would like to thank Prof. Dr. Friedrich Jacob for providing this outstanding topic, his support and trust throughout the last few years. Furthermore, I would like to thank Prof. Dr. Rudi F. Vogel and Prof. Dr. Dirk Weuster-Botz for agreeing to review this dissertation and Prof. Dr. Ulrich Kulozik for acting as chief examiner.

Special thanks go to my mentor, colleague and friend Dr. Mathias Hutzler, who has inspired me with his knowledge and ideas and accompanied me on my scientific journey in word and action.

In addition to collegial cooperation and assistance, I want to thank the “break crew”, who always provided the necessary lightness and enjoyment at work: Florian “Gingi” Mallok, Dominique “Schatzebobbes” Stretz, Dr. Hubertus “Ju” Schneiderbanger, Jennifer Schneiderbanger, Susan Illing, Robert Riedl, Dario Cotterchio, Sebastian Hans, Eva-Maria Kahle, Friedrich Ampenberger, Korbinian Haslbeck, Georg Aymar and Veronika “Vroni” Kaindl and of course my longest friend Dr. Maximilian Michel who has accompanied me all the way since the beginning of our studies. Furthermore, I would like to thank all employees at the Research Center Weihenstephan for Brewing and Food Quality. Special thanks go to Prof. Dr. Mehmet Coelhan, Dr. Martin Zarnkow, Hubert Walter, Tanja Spranger, Markus Schmidt, Sabine Margeth, Anna Kleucker, Margit Grammer, Karl-Heinz Bromig, Sebastian Würzinger, Georgine Widmann, Jutta Betz-Vilser, Sylvia Hubensteiner, Natalie Böck and Josef Pellmeier. Another big thank you goes to my former colleague Dr. Konrad Müller-Auffermann. I also want to thank Prof. Dr. Ronald Steven Wagner as well as Sarah Silva who helped with the quality editing of my English when needed.

A special thank you needs to be expressed to all the students who supported this dissertation with their Diploma, Bachelor, and Master theses. Thank you to Sebastian Eibner, Philipp Sommer, Nils Berg, Sönke Kienitz, Marvin Rabe, and Oliver Ingo Kory.

I would also like to thank Prof. Dr. Rudi F. Vogel, Dr. Jürgen Behr and Alexander Lauterbach of the Chair of Technical Microbiology for the good cooperation and interesting scientific discussions we had to successfully complete our research project AiF 17698 N.

Finally, I would like to thank the Alt-Weihenstephaner-Brauerbund for its support and the friendships I’ve made within its association.

I want to dedicate this work to my family and to my girlfriend Charlotte, who never stopped supporting me, showed great patience, and were always by my side.

Preface and peer-reviewed publications

The results and publications of this thesis were obtained at the Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality from 2014 to 2018.

Parts of this work were funded by the German Ministry of Economics and Technology and the Wifö (Wissenschaftsförderung der Deutschen Brauwirtschaft e.V., Berlin, Germany) in project AiF 17698 N.

This thesis includes the following four peer-reviewed publications with first authorship. A list of all the contributed peer-reviewed papers, non-reviewed papers, oral and poster presentations can be found in Chapter 5.

1. Meier-Dörnberg, T., Michel, M., Wagner, R.S., Jacob, F., Hutzler, M. (2017).
"Genetic and Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale Yeast Isolates."
BrewingScience 70: pp. 9-25. DOI: 10.23763/BRSC16-25MEIER-DOERNBERG

2. Meier-Dörnberg, T., Hutzler, M., Michel M., Methner, F.-J., Jacob F. (2017).
"**The Importance of a Comparative Characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* Strains for Brewing.**"
Fermentation 3, 41: pp. 1-25. DOI: 10.3390/fermentation3030041

3. Meier-Dörnberg T., Jacob, F., Michel M., Hutzler M. (2017).
"**Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry - Cases of contamination with *S. cerevisiae* var. *diastaticus* in the period 2008 to 2017.**"
MBAA Technical quarterly 54, 4: pp. 140-148. DOI: 10.1094/TQ-54-4-1130-01

4. Meier-Dörnberg T., Kory, O.I., Jacob F., Michel, M., Hutzler M. (2018).
"***Saccharomyces cerevisiae* variety *diastaticus* friend or foe? – Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization**."
FEMS Yeast Research 18, 4: pp. 1-27. DOI: 10.1093/femsyr/foy023

Contents

Acknowledgments ***I***
Preface and peer-reviewed publications ***II***
Contents ***III***
List of Tables ***IV***
List of Figures ***IV***
Abbreviations ***V***
Summary ***- 1 -***
Zusammenfassung ***- 3 -***

1 Introduction and motivation **- 5 -**
1.1 The history of brewing yeast - 7 -
1.2 Taxonomy and systematics of *Saccharomyces* brewing yeast - 10 -
1.2.1 Hybridization history of *Saccharomyces pastorianus* and *bayanus* yeast strains - 13 -
1.3 Diversity and potential of brewing-relevant yeast - 16 -
1.3.1 *Saccharomyces* brewing culture yeast strains - 18 -
1.3.2 Non-*Saccharomyces* yeasts in brewing - 21 -
1.4 Phenotypic and DNA-based methods for brewing yeast identification and differentiation - 22 -

2 Results (thesis publications) **- 25 -**
2.1 Summary of results - 25 -
2.2 Genetic and phenotypic characterization of cifferent top-fermenting *Saccharomyces cerevisiae* ale yeast isolates - 26 -
2.3 The importance of a comparative characterization of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* strains for brewing - 44 -
2.4 Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry – Cases of contamination with *S. cerevisiae* var. *diastaticus* in the period 2008 to 2017 - 70 -
2.5 *Saccharomyces cerevisiae* variety *diastaticus* friend or foe? – Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization - 80 -

3 Discussion **- 108 -**

4 References **- 117 -**

5 Appendix **- 132 -**
5.1 Peer-reviewed papers as co-author - 132 -
5.2 Non-reviewed papers - 132 -
5.3 Oral presentations - 133 -
5.4 Poster presentations - 134 -
5.5 Permission of publishers to reproduce publications - 135 -

List of Tables

Table 1: Taxonomic classification of *Saccharomyces* yeast species in the kingdom of fungi (53, 73, 77, 87, 92, 114, 132, 135, 177). .... - 12 -
Table 2: Differences between ale and lager yeast in brewing applications (4, 16, 37, 71, 91, 96, 126, 156, 172). .... - 20 -
Table 3: Selected phenotypic and DNA-based methods for the identification, characterization and differentiation of brewing yeast modified according to HUZTLER 2010 and SAMPAIO 2017 (58, 143); (+, high importance for brewing yeast strains; +/-, medium importance for brewing yeast strains; -, low importance for brewing yeast strains) .... - 23 -
Table 4: Short overview of the four publications with title of the publication, major objective, applied method and main findings. .... - 25 -
Table 5: Main differences of the developed brewing yeast characterization model to former yeast characterization studies of DONHAUSER 1987 and MÜLLER-AUFFERMANN 2015 (31, 32, 111) .... - 110 -

List of Figures

Figure 1: The history of brewing yeast adapted from HUTZLER 2015 (61) .... - 9 -
Figure 2: Current models for the origin of Frohberg and Saaz lineages of *S. pastorianus* adapted from LIBKIND et al. 2011 and GALLONE et al. 2017 (87, 39) .... - 14 -
Figure 3: Multistrain fermentation to achieve specific goals modified according to WHITE and ZAINASHEFF 2010 (181) .... - 16 -
Figure 4: Visualized characterization of top-fermenting brewing strain *S. cerevisiae* LeoBavaricus - TUM 68® (63) .... - 116 -

Abbreviations

%	Percent
°C	Degree Celcius
4-VG	4-vinylguaiacol
AFLP-PCR	Amplified fragment length polymorphism PCR
API 50 C Aux	Analytical profile index
CisH	Chemiluminescence in situ hybridization
DLG	Deutsche Landwirtschafts-Gesellschaft
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
FAME	Fatty acid methyl ester
FDC1	Ferulic acid decarboxylase
FisH	Fluorescence in situ hybridization
FTIR	Fourier transform infrared radiation
GC-TOF MS	Gas chromatography time-of-flight mass spectrometry
GMOs	Genetically modified organisms
hl	Hectoliter
HS-SPME	Head space solid-phase microextraction
IGS2	Intergenic spacer 2
ITS	Internal transcribed spacer
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MEBAK	Mitteleuropäischen Brautechnischen Analysenkommission e. V. (Central European Commission for Brewing Analysis)
mg/l	Milligram per liter
mtDNA	Mitochondrial DNA
NGS	Next generation sequencing
no.	Number

PAD1	Phenylacrylic acid decarboxylase
PCR	Polymerase chain reaction
PCR-DGGE	PCR denaturing gradient gel electrophoresis
PCR-DHPLC	PCR denaturing high performance liquid chromatography
PCR-RFLP	PCR restriction fragment length polymorphism
PCR-TGGE	PCR temperature gradient gel electrophoresis
POF	Phenolic off-flavor
PY-MS	Mass spectrometry
RAPD-PCR	Randomly amplified polymorphic deoxyribonucleic acid PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Real-time polymerase chain reaction
S.	*Saccharomyces*
SAPD-PCR	Specifically amplified polymorphic deoxyribonucleic acid PCR
SO_2	Sulfur dioxide
T	Temperature
WLN	Wallerstein nutrient

Summary

Selecting an alternative or new yeast strain is often very difficult for brewers because of a lack of information that is applicable for practical purposes. The economic pressure to succeed, a lack of capacity, or microbiological uncertainties hamper and prevent the necessary experimentation in many breweries. In contrast to the simple implementation of new hops and malt varieties, introducing a new yeast strain is more challenging in terms of cleaning and disinfection measures. In order to avoid possible cross-contamination, it is necessary to create separate yeast propagation systems and separation the relevant process paths. Statements that can be transferred to practice about the expected spectrum of aromas, the fermentation behavior and how to practically handle individual yeast strains, therefore hold great interest and added value for many breweries.

The overwhelming number of research papers that address yeast properties relevant to brewing do not provide any significant findings for industrial brewing applications because of the different conditions. Frequently, fermentation parameters such as temperatures and yeast pitching cell counts that are too high are used, or key process steps such as maturing to break down undesired young bouquet substances are not considered or not completed. Moreover, a lack of genetic analysis neither guarantees the purity of a strain nor that the studied strains are different. Therefore the origin of a strain is often unclear, genetic drift or population drift over time cannot be excluded, and results that can be transmitted to the brewing industry cannot be guaranteed.

The following paper shows the development and implementation of a model to characterize and differentiate selected *Saccharomyces* yeast strains in terms of their suitability for relevant application in breweries. A plant concept was also established providing transferable and scalable results in the form of 2-liter trial fermentations. Standardized conditions make it possible to monitor diverse phenotypic properties and compare these with each other in a meaningful way for the first time. The strains involved were further genetically classified using species-specific real-time PCR systems and a strain typing method based on PCR-capillary electrophoresis of the partial IGS2 fragment (IGS2-314 PCR-capillary electrophoresis). This guarantees that the strains involved are genetically distinct and continues to maintain a reliable level of quality and product stability. Following genetic characterization, the strains were screened for phenotypic characteristics (e.g. fermentation performance, sugar utilization, amino acid utilization, cell growth, flocculation behavior, change in pH value, phenolic off-flavor, fermentation by-products, sulfur dioxide) and sensory characteristics with the main focus on the flavor and aroma profiles. To investigate the predominant flavor diversity a specially developed tasting scheme was conducted which included: expected beer

type test, DLG scheme for beer a descriptive sensory evaluation and a triangle test. Ten of the most common industrial yeast strains used to produce traditional beer varieties such as German wheat beer, koelsch, alt, trapist, ale and lager beer as well as various unknown *Saccharomyces* and *S. cerevisiae* var. *diastaticus* yeast isolates were analyzed and characterized in terms of their relevance for breweries in order to evaluate and implement the characterization model. The characterization enabled statements to be made on the spoilage potential and the suitability of the investigated *S. cerevisiae* var. *diastaticus* strains for beer manufacture and targeted aroma provision. These were validated in further testing of their potential for super-attenuation and a simple and quick test method was implemented.

The characterization model developed in this thesis provides a precise statement on the practicability of yeast strains in industrial brewing applications. The data collected as part of yeast characterization can be used by brewers for comparative purposes to easily select, in a targeted manner, the yeast strain suitable for their brewing process or beer type.

Zusammenfassung

Die Wahl eines alternativen oder neuen Hefestammes gestaltet sich für den Brauer aufgrund mangelnder und in die Praxis übertragbarer Informationen zumeist sehr schwierig. Wirtschaftlicher Erfolgsdruck, mangelnde Kapazitäten oder mikrobiologische Unsicherheiten hemmen und verhindern die nötige Experimentierfreudigkeit vieler Brauereien. Im Gegensatz zur einfachen Implementierung neuer Hopfen und Malzsorten ist die Einführung eines neuen bzw. weiteren Hefestammes mit einer hohen Herausforderung an Reinigung und Desinfektionsmaßnahmen verbunden. Zur Vermeidung möglicher Kreuzkontaminationen sind separate Hefereinzuchtanlagen sowie die Trennung betreffender Prozesswege nötig. In die Praxis übertragbare Aussagen über das zu erwartende Aromaspektrum, das Gärverhalten und den praktischen Umgang mit einzelnen Hefestämmen, sind daher für viele Brauereien von großem Interesse und Mehrwert.

Die überwiegende Anzahl an Forschungsarbeiten die sich mit braurelevanten Eigenschaften von Hefen beschäftigen liefern aufgrund unterschiedlicher Bedingungen keine aussagekräftigen Erkenntnisse für industrielle Brauanwendungen (Übertragbarkeits- und Up-Scaling-Schwierigkeiten). Zumeist werden Gärungsparameter wie zu hohe Temperaturen und Hefeanstellzellzahlen verwendet oder wichtige Prozessschritte wie die Reifung zum Abbau unerwünschte Jungbukettstoffe nicht berücksichtigt oder nicht abgeschlossen. Fehlende genetische Analysen garantieren weder die Reinheit eines Stammes noch garantieren sie, dass die untersuchten Stämme unterschiedlich sind. Daher ist der Ursprung eines Stammes oft unklar und genetische Veränderungen der Hefepopulation über die Zeit können nicht ausgeschlossen werden, sodass keine in die Brauindustrie übertragbaren Ergebnisse garantiert werden können.

Die nachfolgende Arbeit zeigt die Entwicklung und Implementierung eines Modells zur Charakterisierung und Unterscheidung ausgewählter *Saccharomyces* Hefen hinsichtlich ihrer Eignung für einen brauereirelevanten Einsatz. Durch gleichbleibende und praxisrelevante Bedingungen wurden braurelevante und hefestammspezifische Eigenschaften gesammelt und vergleichend dargestellt. Hierzu wurde ein Anlagenkonzept erarbeitet welches in Form von 2 Liter Versuchsgärungen auf den Großmaßstab übertragbare Ergebnisse liefert. Durch standardisierte Bedingungen können diverse phänotypische Eigenschaften erfasst und erstmalig aussagekräftig miteinander verglichen werden. Durch quantitative Echtzeit-PCR (RT-PCR) und einer Stammtypisierungsmethode basierend auf PCR-Kapillarelektrophorese des partiellen IGS2 Fragments (IGS2-314 PCR-Kapillarelektrophorese) wurden die verwendeten Hefen bis auf Stammebene identifiziert, um die Reinheit und Unterschiedlichkeit der verwendeten Hefestämme zu garantieren und so eine gleichbleibende und reproduzierbare

Produktqualität zu gewährleisten. Neben der genetischen Charakterisierung wurden die Stämme auf phänotypische Merkmale (z. b. Fermentationsleistung, Zuckerverwertung, Aminosäureverwertung, Zellwachstum, Flockulationssverhalten, pH-Wert-Änderung, phenolische off-flavor, Gärungsnebenprodukte, Schwefeldioxid etc.) untersucht. Um die individuellen Aromaeindrücke sowie den sensorischen Gesamteindruck der hergestellten Biere zu beurteilen wurde ein speziell entwickeltes Verkostungsschema verwendet, welches neben dem Biertyp und dem DLG-Schema für Bier ebenfalls eine deskriptive sensorische Bewertung und einen Dreieckstest umfasst.

Zur Evaluierung und Implementierung des Charakterisierungsmodells, wurden zehn der industriell meist eingesetzten Hefestämme zur Produktion klassischer Biersorten wie Weißbier, Kölsch, Alt, Trappist, Ale und Lagerbier sowie verschiedene *Saccharomyces u*nd *S. cerevisiae* var. *diastaticus* Hefeisolate unbekannter Originalherkunft untersucht und brauereirelevant charakterisiert. Durch die durchgeführte Charakterisierung konnten Aussagen über das Schadpotential und die Eignung der untersuchten *S. cer.* var. *diastaticus* Hefestämme zur Bierherstellung und gezielten Aromagebung gewonnen werden. Diese wurden durch neu entwickelte Methoden hinsichtlich ihres Potenzials zur Übervergärung validiert und eine einfache und schnelle Testmethode implementiert. Die im Rahmen der Hefecharakterisierung gesammelten Daten können von Brauereien vergleichend verwendet werden, um gezielt den für ihren Brauprozess oder Biertyp geeigneten Hefestamm auszuwählen.

1 Introduction and motivation

The reduction in volume and market share for classical beer types such as lager and wheat beer has been declining for several years, but the overall beer market has remained almost unchanged because of an increasing interest in beer specialties (185). The consumer trend is shifting from commercial beers with consistent quality and sensory properties to a variety of specially produced beers with different tastes. Following this so-called craft beer boom, increasing numbers of brewers and breweries wish to benefit from the economically profitable specialty beer market. The beer culture in Germany is experiencing a kind of renaissance through this trend. Although beer output has been decreasing in Germany since 1991, the number of German breweries is increasing (28). In 2016, the number of breweries in Germany passed the 1400 mark for the first time. 50 % of the breweries with an output of up to 1000 hL/year are ranked as microbreweries (29, 121). The neighboring country of Austria is also seeing an increase in the number of breweries. There are eleven times more small-scale breweries in Austria today with an annual beer output of up to 20000 hl compared to 1980 (120). Small-scale breweries and microbreweries in particular benefit from the thriving market of craft beers and seize the opportunity to position themselves on the competitive beer market with innovative beer creations (22). There is no limit to the diversity of beer and the interplay of aromas. According to the German brewers' association, German brewers can choose from about 40 different malt varieties (light, dark, smoky malt etc.), 250 different hop varieties (bitter and aroma hops) and over 400 different yeast strains. If you consider the possible combinations of all these raw materials, the resulting beer diversity is virtually unlimited. The choice of a suitable yeast strain is an often-underestimated way of creating innovative beers with appealing tastes. It is now known that beer aroma is primarily determined by fermentation by-products of the yeast (59, 63, 67). Despite controversial discussions that the German purity law restricts beer diversity in Germany, this shows that there is adequate scope for new German beers to be created even without the use of unmalted grains, enzymes or other additives that are customary in other countries (27, 49, 51).

In the past, brewer's yeast was considered purely as a means to an end with the flavor diversity usually achieved using different starting materials (raw materials) and brewing techniques. Today, yeast is no longer regarded just as a necessary agent but as a tool. Every yeast has strain-specific characteristics that impart complex or individual aroma impressions to the beer, thereby ensuring product diversity and a flavor differentiation to rival products. Although brewers now have a variety of yeast strains of different genera and species, only a few cultured yeast strains of the *S. pastorianus* and *S. cerevisiae* species are used to produce

beer based on their proven process and aroma properties (61). As STEWART reports, for example, just four yeast strains are used in Germany to produce beer and it is estimated that 65 % of these originate from just one strain (155).

Economic pressure to succeed, a lack of capacity or microbiological uncertainties mean that brewers revert to these types of domesticated yeast strains with consistent process efficiency and overall quality, thereby limiting the sensory complexity of the beer and beer diversity (154). Statements that can be transferred to practice about the expected spectrum of aromas, the fermentation behavior and how to practically handle individual *Saccharomyces* yeast strains, therefore hold great interest and added value for many breweries. Even non-*Saccharomyces* yeasts classified as foreign and spoilage yeasts, which usually give the finished beer an unfamiliar flavor impression following cross-contamination can, if correctly used, create new beer aromas or accentuate existing aromas (97, 105). For this reason, as part of this dissertation, the results obtained from characterizing selected *Saccharomyces* yeasts are compiled in a uniform data set as brewing starter cultures and summarized for comparison. Consequently, breweries can rely on scientifically based data when selecting a yeast strain appropriate to their brewing process or beer type, irrespective of whether they want to replace their existing yeast strain or wish to introduce another yeast strain to their production. The knowledge of different yeast strain properties can particularly enhance the competitiveness of small and medium-sized breweries and potentially ensure their continued existence. Resources are conserved, and a higher level of operational safety and improved quality standard can also be achieved. STEENSELS highlights this in 2012 in his paper: “Until recently, the appropriate tools and knowledge were lacking to make a well-considered and scientifically founded choice about which strain to pick. This explains why, even today, most beer yeasts are used according to historical rather than scientific reasons” (152).

1.1 The history of brewing yeast

The use of yeasts to produce beer can be traced back thousands of years to prehistoric times (40, 125, 125). Mesopotamian records from more than 5000 years ago show that beer production was one of the oldest technologies in human history (103). Even at that time it is likely that *Saccharomyces* yeasts were used to create palatable beverages from substrates or prepared substrates (e.g. coarse grain-water blend, fruit pulps etc.) with an extended 'shelf life' and a euphoric effect (ethanol) (40, 154). The fact that yeasts of the *Saccharomyces* genus would be responsible for this process was first discovered in the 17th century following the development of microscopy in research by ANTONI VAN LEEUWENHOEK (1680), SCHWANN and CAGNIARD-LATOUR (1837-1838) and PASTEUR (1860) (10, 20, 157). Until then, the driving force of fermentation had not been clearly identified and the fermentation process was initiated by spontaneous fermentation or by deliberately adding bark, part-fermented fruits or chewed grains. Fermentation often started spontaneously without the specific addition of starter cultures or their carriers. Particles containing microorganisms enter the substrate through the air or other vectors. Suitable microorganisms and their control were not known, so fermentation and the resulting products were left to chance (61). Even today, spontaneous fermentation is still used to manufacture specialty beers such as Lambic, Geuze or Berliner Weiße, whereby the precise composition (varying concentrations and time spans) of all the microorganisms involved in the fermentation is not entirely clear (18, 24, 102, 165). The first step towards reproducing the fermentation process was achieved by unintentionally continuing to use the yeast culture, whereby presumably the foam or the yeast head, the sediment or the dregs or the partly fermented beverage were used to inoculate new preparations. One example of this is the Egyptians refilling the yeast trub of a beer fermentation with a new brew (61, 88). Today, brewing represents the only major fermentation process that recycles its yeast culture from one fermentation to another (155). The discovery of microscopy enabled fermentations to be microscopically tracked for the first time, and made it possible to identify - though not prevent or influence (only by changing process parameters) - mixed populations or contaminations with bacteria such as lactic acid bacteria and acetic acid bacteria. Only the discovery/development of pure culture yeast at the end of the 19th century by EMIL-CHRISTIAN HANSEN paved the way for fermentation with biologically pure yeast as a starter culture for beer manufacture. The first step of the microbiological process or quality control for reproducible fermentation processes with pure starter cultures was taken. Inefficient fermentations and insufficient product quality due to mixed populations or spontaneous fermentation based on unforeseeable changes in the number and type of existing microorganisms soon became a thing of the past. However, brewing scientists have long disagreed whether purebred beers are comparable to mixed fermentations regarding the flavor profile of the final beer (4).

Over time, increasing numbers of yeast strains were isolated, propagated and selected according to their performance and process suitability. Pioneer: the bottom-fermenting yeast isolated in 1883 by HANSEN and cultivated as a pure culture three years later in the Carlsberg brewery "Carlsberg Yeast no. 1", is known as a separate species today under the name *Saccharomyces pastorianus* (18, 50, 167). Since then, the use of bottom-fermenting pure yeast cultures to manufacture beer has become common practice and, together with lager beers, delivers the most commonly produced beer varieties to date (44, 157). However, ale brewers did not initially recognize this pioneering innovation and saw it only as a means of reducing infection from wild yeasts and bacteria (157). According to HOUGH, in 1959 just a third of 39 analyzed British pitching yeast cultures were used as a pure culture, with the rest consisting of a mixed population with two to five yeast strains (55). Gradually, the use of pure yeast cultures was also established in the top-fermenting field and today, the majority of industrial beer production is based on the use of defined starter cultures. Brewery yeast management became increasingly standardized and optimized on the basis of the fermentation and propagation properties of a few selected yeast strains. Subsequently, adapted and well-researched high-performance yeast strains were increasingly used for beer production, replacing the formerly available variety with "house yeast cultures" (61). Commercial interests resulted in further targeted selection and propagation of yeast strains with process and aroma-optimized properties, and the so-called culture yeasts became industrially established (44). Figure 1 sums up the history of brewing yeast from random or inoculated spontaneous fermentation to today's selection and breeding techniques to the selective use of various strains (61).

Interest in beer began to grow following the world wars and during the economic upswing in the 1950s and 60s. To obtain as large a share as possible of this promising, profitable beer market, beer should be made accessible to a broad spectrum of the population (103). This objective should be achieved by offering beers of consistent quality and universal flavor. As a consequence, just a few cultured yeast strains of the *Saccharomyces pastorianus* (bottom-fermenting yeasts) and *Saccharomyces cerevisiae* (top-fermenting yeasts) species are used in the brewing industry, while product diversity and flavor differentiation between competing breweries are being reduced (18, 61, 99, 156).

Today, the perception of beer has shifted from a mass product to a luxury beverage in many countries. Over the course of the craft beer movement, the consumer trend is shifting from commercial beers of consistent quality and sensory properties to a range of specially produced beers with different tastes. Consumers are becoming more aware of the complex craft of brewing beer and innovative beer creations are capturing the flourishing beer market (99). Across the world, new experimental breweries are invigorating the beer market by reviving

old beers or creating new beer styles and beers (18). The hop industry has adapted to the new demand and expanded its range of specialty hops for new flavor directions. As the yeast strain used accounts for more than 80 % of all aroma-active substances in beer, the demand for new process- and aroma-suitable yeast strains for producing beer is also increasing, offering brewers around the world scope for new beer creations (63).

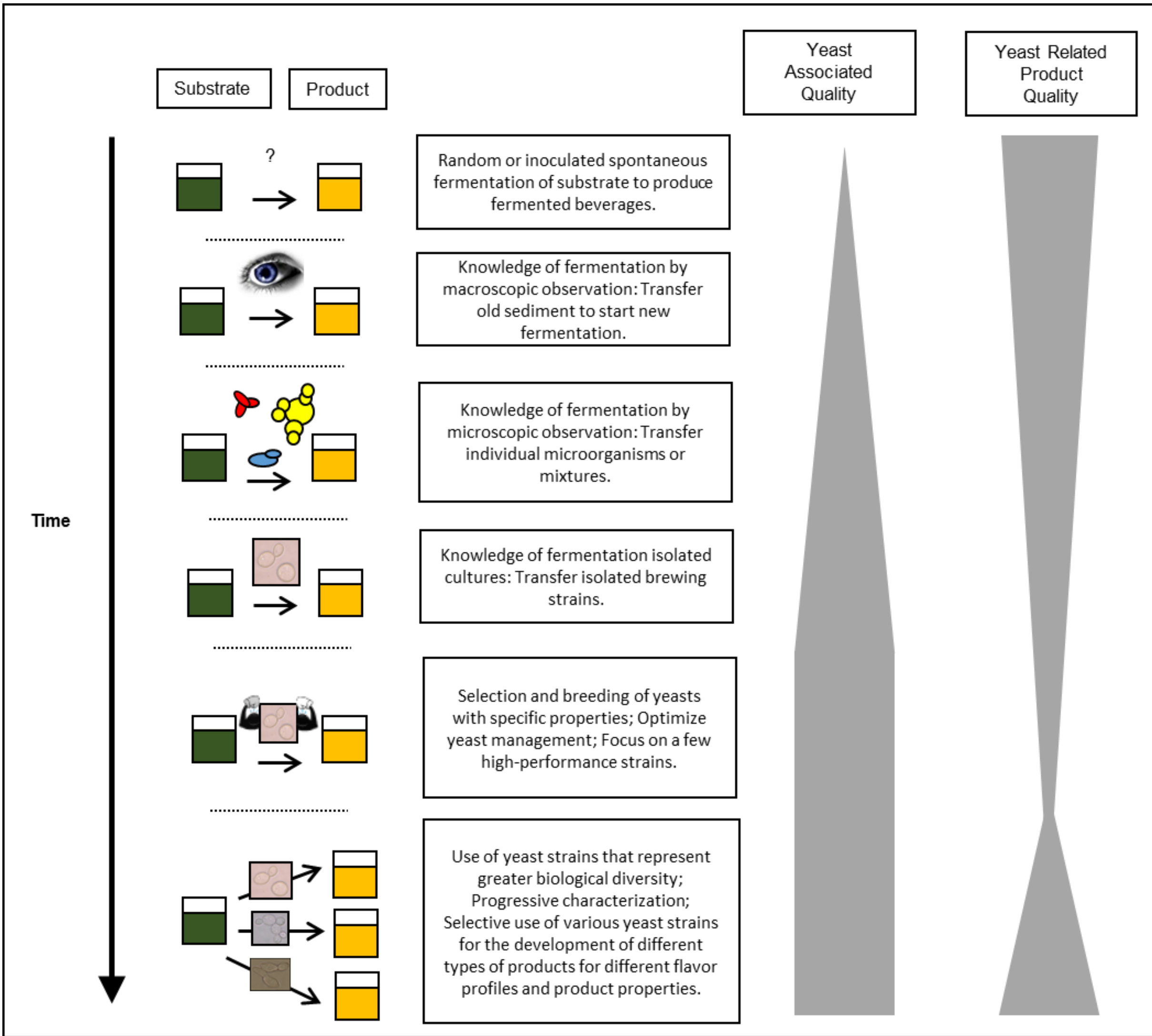


Figure 1: The history of brewing yeast adapted from HUTZLER 2015 (61)

1.2 Taxonomy and systematics of *Saccharomyces* brewing yeast

The taxonomy and systematics of brewing yeast have been a matter of debate and controversy since the early days of microbiology. Up to now, the nomenclature and classification of species is not always uniform in the literature (35, 156). Researchers used morphological characters and physiological traits to distinguish and classify species resulting in numerous species synonyms and misclassifications (143). Only the introduction of molecular biological methods in 1980 led to precise taxonomic classification of brewing yeast.

Brewer's yeasts are generally eucaryotic organisms in the kingdom of fungi. They belong to the group of Ascomycota and together with the Basidiomycota group they form the fungi subkingdom of called Dikarya (53). The Ascomycota phylum differs from the Basidiomycota phylum in its form of sporulation. Within the phylum of the asci spore-producing Ascomycota, brewing yeasts are classed as *Saccharomycetales* yeasts, which is the only order of fungi in the *Saccharomycetes* class. This class, as part of the subdivision *Saccharomycotina,* forms one of the three subdivisions of the ascomycetes. *Saccharomycotina* includes fungi able to reproduce by budding and unable to produce ascocarps (fruiting bodies) (80). Brewing yeasts further belong to the genus of *Saccharomyces,* the family of *Saccharomycetaceae* (136, 167). The *Saccharomyces* genus can be separated into the *Saccharomyces sensu stricto* and the *Saccharomyces sensu lato* complex according to the relevance of their species for the fermentation industry (136). The *S. sensu stricto* complex includes *S.* species, which are strictly associated with the fermentation industry, and represents the main reference group when selecting yeasts to be used in beer production (23). The *S. sensu lato* complex contains *Saccharomyces* species that are only distantly related to *S. cerevisiae*. The *S. sensu lato* complex is no longer common and their species are now attributed to other genera such as *Kazachstania*, *Naumovia* or *Lachancea* (73), (78), (136).

The main *Saccharomyces* yeasts used as starter cultures in the brewing industry are represented by different strains of the top-fermenting yeast *S. cerevisiae* (often referred to as "ale yeast") and the bottom-fermenting yeast *S. pastorianus* (often referred to as "lager yeast"). The *S. sensu stricto* complex also includes the species *S. bayanus* (118, 122, 132), *S. cariocanus* (115), *S. kudriavzevii* (115), *S. mikatae* (115), *S. paradoxus*, *S. arboricolus syn. arboricola* (42, 116, 177) and according to recent findings based on genetic analysis, additional species such as *S. uvarum* (117, 118, 132, 135), *S. eubayanus* (87) and *S. jurei* (114). As time passes, it can be expected that the taxonomic grouping of *S. sensu stricto* yeasts will continue to evolve and change in accordance with the yeast culture classification system due to increasingly advanced genetic analysis methods. Table 1 simply shows the recent taxonomic classification of the single *Saccharomyces* yeast species in the kingdom of fungi.

While the top-fermenting brewery yeasts are associated without doubt with the *S. cerevisiae* species, the bottom-fermenting brewery yeast *S. pastorianus* has undergone many taxonomic classifications during past decades. In 1908 HANSEN described *S. carlsbergensis* as an independent species. In 1970 this was transferred to the species *S. uvarum* and in 1990 then reclassified as the *S. cerevisiae* species (9, 149). The brewing yeast *S. cerevisiae var. carlsbergensis*, often then listed as a subspecies, was further classified as the bottom-fermenting brewing yeast *S. pastorianus* we know today (18, 167). However, the species names *S. carlsbergensis* and *S. monacensis* are sometimes used as synonyms in the present literature (122). The ongoing development and improvement of genetic sequence analysis such as DNA–DNA hybridization has enabled identification at strain level as well as the assignment of complex multi-*Saccharomyces* species hybrids such as the bottom-fermenting brewing culture yeast *S. pastorianus* (83, 156).

Table 1: Taxonomic classification of *Saccharomyces* yeast species in the kingdom of fungi (53, 73, 77, 87, 92, 114, 132, 135, 177).

KINGDOM
Fungi
SUBKINGDOM
Dikarya
PHYLUM
Ascomycota, *Basidiomycota*
SUBPHYLUM
Pezizomycotina, ***Saccharomycotina***, *Taphrinomycotina*
CLASS
Saccharomycetes
ORDER
Saccharomycetales
FAMILY
Ascobotryozyma,Ascoideaceae, Cephaloascaceae, Debaryomycetaceae, Dipodascaceae, Endomycetaceae, Eremotheciaceae,Hyphopichia, Kodamaea, Lipomycetaceae, Metschnikowiaceae, Nakazawaea, Phaffomyces, Pichiaceae, ***Saccharomycetaceae****, Saccharomycodaceae, Saccharomycopsidaceae, Starmera, Starmerella, Trichomonascaceae, Yamadazym*
GENERA
Ashbya, Brettanomyces, Candida, Citeromyces, Cyniclomyces, Debaryomyces, Hansenula, Issatchenkia, Kazachstania, Kloeckera, Kluyveromyces, Komagataella, Kuraishia, Lachancea, Lodderomyces, Nakaseomyces, Nakazawaea, Naumovia, Naumovozyma, Pachysolen, ***Saccharomyces****, Spathaspora, Tetrapisispora, Torulaspora, Vanderwaltozyma, Williopsis, Zygosaccharomyces, Zygotorulaspora*
SPECIES
arboriculus, bayanus, cariocanus, cerevisiae, eubayanus, jurei, kudriavzevii, mikatae, paradoxus, pastorianus, uvarum

1.2.1 Hybridization history of *Saccharomyces pastorianus* and *bayanus* yeast strains

Yeast strains of the species *Saccharomyces pastorianus* are allopolyploid genetic hybrids of the species *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (11, 87). *S. eubayanus* is thought to be an early ancestor of the *S. pastorianus* lager yeast and to have given yeast the capacity to ferment at cold temperatures (156). It has also been discussed that the *S. cerevisiae* subgenome confers efficient fermentation, including the use of maltotriose (39). *S. eubayanus* was first identified/isolated in the Patagonian native forests of Argentina (South America) (87). Later on, some *S. eubayanus* isolates were also found in East Asia, North America and New Zealand, and it was shown that the East Asian isolate found on the Tibetan plateau shows the closest genetic similarity to the *S. eubayanus* subgenome of *S. pastorianus* found so far (12, 42, 123). Also in 2011, NGYUYEN et al. identified a new species and proposed the name *Saccharomyces lagerae* (119). It was found that the genome of *S. lagerae* corresponds with the genome of *S. eubayanus* found by LIBKIND et al. (119). However, according to GIBSON et al., no known extant strain seems to be the direct ancestor of lager-brewing yeasts (43). Different strains of the lager yeast *S. pastorianus* are divided in two main distinct lineages, most commonly referred to as 'Saaz' (Type 1) and 'Frohberg' (Type 2) (35, 39, 119, 122). In contrast to Saaz-type strains, Frohberg-type strains can ferment maltotriose, the second main sugar content in beer wort, resulting in greater fermentation performance and higher apparent attenuations (45, 100). The two *S. pastorianus* hybrid groups are genetically distinct from one another (35, 90). PÉREZ-TRAVÉS et al. showed that the Saaz and the Frohberg-lager strains possess different allele for the same genes, *BREfeldin A5 (BRE5)* and *BASal 1 (BAS1*) (122). However, the precise ancestry and evolution of the two *S. pastorianus* lineages remains controversial (107).

Figure 2 shows the hybridization hypothesis according to LIBKIND et al. and GALLONE et al. (87, 39). In 2011 LIBKIND et al. proposed the evolution of *S. pastorianus* and *S. bayanus* under domestication caused by strong positive selection imposed by the brewers or by the competitive brewing environment itself (Figure 2a). According to LIBKIND et al., a wild *S. eubayanus* from Patagonia hybridized with a domesticated ale-type yeast within the brewing environment and formed a 50:50 hybrid, which suffered a domestication process involving the genetic inactivation of less efficient sulfate transporters, the addition of extra copies of *cerevisiae* genes related the assimilation of maltose, and various chromosome rearrangements including the loss of several ones. In 2017 GALLONE et al. reported that three main hypotheses for the origin of *S. pastorianus* have been proposed (39). The most widespread hypothesis based on recent comparative genome hybridization studies showed that *S. pastorianus* lager yeast strains were a result of at least two completely independent

hybridization events, each involving a different domesticated ale-type *S. cerevisiae* and different wild *S. eubayanus* strains and resulting in the Saaz and Frohberg groups of yeasts (Figure 2b). Other genetic analyses lead to the hypothesis of a single hybridization event caused by several breakpoints within the subgenomes of *S. cerevisiae* and *S. eubayanus*, which are identical within both lineages (Figure 2c). As GALLONE reported, the recent phylogenetic and genetic analysis are not clear and can indicate a shared hybridization event prior to the divergence into the distinct lager lineages (Figure 2d).

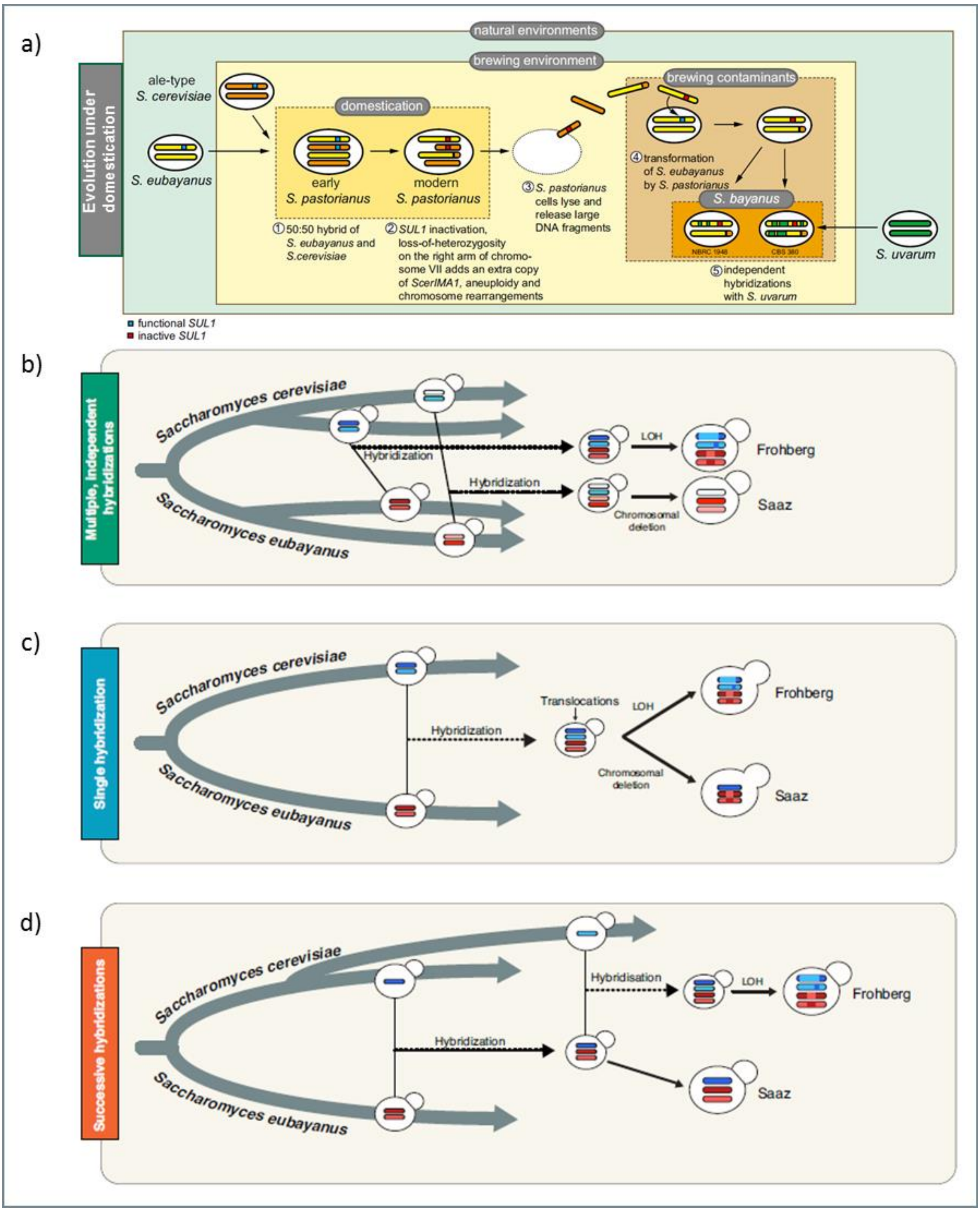


Figure 2: Current models for the origin of Frohberg and Saaz lineages of *S. pastorianus* adapted from LIBKIND et al. 2011 and GALLONE et al. 2017 (87, 39)

The other controversial *Saccharomyces* taxon is the species *S. bayanus*. The assignment and classification of strains belonging to this species is unclear and controversial due to the fact that this taxon includes genetically diverse lineages of pure and hybrid strains (122). Some authors have subdivided *S. bayanus* into two varieties: *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum*, whereas other authors classified *S. bayanus* and *S. uvarum* as different species of the *Saccharomyces* genus (118, 122, 132).

Recent sequencing studies have shown that *S. bayanus* is a triple hybrid yeast strain containing sequences of *S. eubayanus*, *S. uvarum* and *Saccharomyces cerevisiae* (119, 156). As BORNEMAN and PRETORIUS in 2015 reported, the *S. bayanus*-type strain CBS380^T comprises almost equal genomic contributions from *S. eubayanus* and *S. uvarum* with a minor input from *S. cerevisiae* (17). The genomic portion of *S. cerevisiae* seems to be responsible for the ability of such *S. bayanus* strains to ferment maltotriose, a property that is lacking in *S. uvarum* strains (17). Therefore *S. uvarum* (*S. bayanus* var. uvarum) strains represent a pure lineage that contains very little genetic input from other *Saccharomyces* species (17). These investigations have shown that there are two clearly defined groups within the *S. bayanus* species, classified by PÉREZ-TRAVÉS as the molecularly and physiologically heterogeneous group of strains belonging to *S. bayanus* var. *bayanus*, and the homogenous group of strains pertaining to *S. bayanus* var. *uvarum* (122). In conclusion, it follows that *S. bayanus* and *S. uvarum* are different species (17). According to HUTZLER, it is very likely that the use of the strain CBS380^T has led to misinterpretations and confusing conclusions (58, 136).

In summary, industrial strains that belong to hybrid species within the *Saccharomyces* genus are often linked to historical long-term usage by humans in specific human-made artificial environments and related domestication. The investigation of the real genetic relationships and genetic comparisons of hybrid strains in the context of the historical strain history is very challenging. Whole genome sequencing and digging deeper into documented brewing strain histories can provide a better insight into current brewing strains.

1.3 Diversity and potential of brewing-relevant yeast

The selection of yeast strains for beer production is no longer limited to culture strains of the species *S. cerevisiae* and *S. pastorianus*. In addition to the widespread and established high-performance strains of both species, there are a variety of other yeast strains, species or even genera. Biodiversity means that the range of potential yeasts is almost unlimited. According to an extrapolation, there are currently about 669,000 different types of yeast species of which only 1,500 are known (62, 75, 170). Therefore, it is not surprising that more and more research papers and projects are also looking at alternative brewing yeasts in the sector of non-*Saccharomyces* or *Saccharomyces* wild yeasts (43, 64, 105). The main goal is to influence the aroma profile of the finished beer in a targeted manner and to make the brewing process more efficient by using yeasts with process-optimized properties. For this purpose, a huge variety of strains can be used as pure cultures or in combination with other yeast strains or microorganisms in so-called mixed fermentations, resulting in beers with a variety of aroma impressions and intensities (59). Varying the pitching time can also greatly influence the finished product. Figure 3 shows the multistrain fermentation types to achieve specific goals modified according to WHITE and ZAINASHEFF (181).

Fermentation type	Goal	Yeast strains	Pitching time
Additive fermentation	Maintaining house flavor and achieving higher attenuation	One yeast strain for flavor and one yeast strain for attenuation	Pitch with “flavor-yeast" then add “attenuation-yeast“ during last third of fermentation
Mixed fermentation	Complex flavor profile or unique flavor(s)	Two or more yeast strains	Pitch with all yeast strains
Post fermentation	Unique flavor(s) or desired yeast turbidity	One yeast strain for flavor or one yeast strain for turbidity	Bottle fermentation/-maturing

Figure 3: Multistrain fermentation to achieve specific goals modified according to WHITE and ZAINASHEFF 2010 (181)

The variety of different yeasts as beer fermentation starter cultures continues to increase as a result of today's technological and scientific progress. The latest genetics and molecular biology techniques and approaches were used to change yeast genetics and create new strains with special selected or desired characteristics. The improvement in ethanol tolerance, fermentation speed, attenuation (e.g. sugar metabolism), yeast flocculation, foam stability, flavor production, the reduction of diacetyl or an increased flavor stability represents only a

selection of modern genetic goals (82, 86, 152, 178, 179). The use of genetically modified yeast strains with special characteristics has not found its way into today's brewing practice. The use of genetically modified organisms (GMOs) is not accepted by breweries and consumers and is prohibited by German law.

Adaptive or experimental evolution represents the oldest method in the history of cultivation techniques to select yeast strains with specific characteristics (21). Yeast populations have been specifically adapted to a wide variety of environmental conditions such as high sugar concentration, low temperatures, high ethanol concentration, and selected by serial repitching in order to achieve the desired expression (30, 36). This evolutionary natural route to force the optimization of phenotypic traits is lengthy, subject to microbiological risks and follows the principle of trial and error. For this reason, recent researchers are interested in different ways of achieving faster and more reliable methods and techniques to improve yeast strains. Breeding (41), mutagenesis (182), sexual hybridization (e.g. mating) (100), asexual hybridization (e.g. protoplast fusion and cytoduction) (25) or genetic modifications by changing and cloning DNA in yeast cells (plasmid transfusion or fixed integration) (34, 145) are recent research projects. One yeast strain that combines the gene of the yeast *Schizossaccharomyces pombe*, which is responsible for the transportation of malate, and one gene of *Oenococcus oeni*, is already used in industrial wine fermentations in the USA and Canada. This genetically modified yeast strain is used to save the time-consuming malolactic post-fermentation by converting malate into lactate during the main fermentation (57, 56). However, the use of GMOs in food production is prohibited by law in most European countries and their future use remains controversial (153).

The prevailing variety of methods and opportunities will result in greater yeast diversity and will also create a revolution of the beer market outside the German purity law by offering custom-designed yeast strains. Despite the variety of yeast strains and their estimated potential, it is difficult to generalize on the yeast strains used in industrial beer production, as they are generally poorly characterized and few comparative studies have been reported (126). Comparable investigations regarding the phenotype of yeast strains are therefore necessary before it is possible to tell if the desired phenotype is available for the discovered or created yeast strain.

1.3.1 *Saccharomyces* brewing culture yeast strains

To ensure a reliable level of quality and product stability, beer yeasts have been domesticated by enduring growth in man-made fermentation environments (39). The strong selective pressure imposed over many generations has contributed to the emergence of desirable phenotypes (45). The focus was placed on yeast properties that have a critical impact on fermentation performance such as flocculation, osmotic pressure, ethanol tolerance, oxygen requirements, carbohydrate as well as nitrogen metabolism and are described by many researchers (2, 91, 155, 156, 173). Important characteristics that appear to have been selected during brewing yeast domestication are the ability to ferment maltotriose and the reduced production of phenolic off-flavors (POF) (39, 43). Maltotriose is generally not found in significant concentrations in natural yeast environments, but as the second most abundant sugar in beer wort (15-20 %) it is crucial for beer production (39). In contrast, most of the naturally occurring yeast strains (*Saccharomyces* wild yeast) are able to form POF due to the presence of two active POF genes *PAD1* and *FDC1* (48). Occurring as an unpleasant flavor in lager beers, mutations in these genes have been found in some brewing yeasts, suggesting that the selection of POF-negative yeast strains has favored the spread of domesticated lager beer yeasts unable to produce these off-flavors (39, 48).

Domestication has led to the use of a limited number of strains today. Brewing yeast is originally classified based on flocculation behavior into two major groups: top-fermenting ale yeasts (*S. cerevisiae*) and bottom-fermenting lager yeast strains (*S. pastorianus*) (91). Accordingly, beers are classified as ale or lager beers, each produced by a unique fermentation process that results in different aroma profiles. Ale beers were produced by different *S. cerevisiae* brewing strains resulting in different beer types such as German wheat beer, ale, stout, koelsch and alt, Belgian special beer styles (witbeer, trapist beer) or African indigenous beer styles. Lager yeast strains of *S. pastorianus* were used for lager beers such as lager, pilsener, export, bottom-fermented special beers and bottom-fermented low alcohol beer (62). Table 2 sums up the main differences between ale and lager yeast in brewing applications and represents the most common differences even if they can vary between single strains of both species.

Compared with lager yeasts, ale yeasts are genetically and phenotypically more diverse, resulting in aroma intensive and fruity beers (91). Several ale and wine yeast strains were recently identified as hybrids of *S. cerevisiae* and the cryotolerant, strong-aroma-producing species *S. kudriavzevii* (154). Some ale strains produce beer with spicy, clove- and band-aid like flavor notes due to the presence of active POF genes. These phenolic off-flavors are especially desired in German wheat beers and therefore more often classified as wheat beer yeast strains within the group of ale yeast strains.

Lager beer is the most popular and widespread beer style in the world because of its clean flavor profile with a relatively low level of fruit or floral flavors compared with ale (12, 39). The limited aromatic diversity can be due to the *S. eubayanus* subgenome of *S. pastorianus*. According to MERTENS, *S. eubayanus* is characterized by a relatively modest production of acetate and ethyl esters and higher concentrations of fusel alcohols (100). The use of *S. eubayanus* strains for beer production has so far only been investigated for the type strain of the species (45, 72, 100, 175). Beers produced with *S. eubayanus* were described as having strong sulfur-like flavors without any detectable phenolic flavors, even if the strains were found to be POF positive (100). The first commercial product exclusively brewed with *S. eubayanus* has recently been released by Heineken and was sold as a so-called "wild lager" as a limited edition (43).

In addition, some yeasts of the species *Saccharomyces* that are classified as spoilage yeasts may be purposefully used for the industrial production of beer. *S. cerevisiae* var. *diastaticus* strains often occur as secondary contaminants in the filling process and cause abnormal attenuations due to its ability to ferment residual carbohydrates in beer. Spoilage yeasts generally include yeast species and strains that directly or indirectly spoil the finished product, whereas yeasts often referred to as wild strains include species and strains that are not identical to the used starter culture and do not necessarily have any spoilage potential for the finished product. A classification of spoilage yeasts that occur in beer and beer-mixed beverages is provided by HUTZLER, subdivided according to their spoilage potential into fermentative, fermentable, low-fermentation and non-fermentable or respiratory yeasts (58).

In this thesis, *S. cerevisiae* var. *diastaticus* was investigated with regard to its spoilage and brewing potential and it could be shown that some strains are suitable for beer production.

Table 2: Differences between ale and lager yeast in brewing applications (4, 16, 37, 71, 91, 96, 126, 156, 172).

Criteria	Lager yeast	Ale yeast
Species	*S. pastorianus*	*S. cerevisiae*
Fermentation type	bottom fermenting	top fermenting
Cell form and compartments	no difference	
Budding	single or in pairs	chain-forming (approx. 8 cells)
Optimal growth temperature	T_{opt} = 27-30°C	T_{opt} = 30-34°C
Maximum growth temperature	T_{max} = 31-34°C	T_{max} = 37-40°C
Typical fermentation temperature	T_{ferm} = 8-14°C	T_{ferm} = 18-24°C
Temperature sensitivity	growth and fermentation at low temperatures	sensitivity <10°C, sedimentation
Typical industrial fermentation time	7-14 days	4-6 days
Drying of cultures	not easily	easier than lager strains
Sporulation	low	high
Flocculation	flocculent	non-flocculent (powdery)
Cropping	bottom	top (depended on brewery processes some S. cerevisiae strains are also cropped from the bottom)
Repitching	5 – 8 times	up to 20 times or more
Raffinose utilization	completely	raffinose to 1/3, no melibiose, no galactosidase
Maltotriose utilization	better than ale strains	strain dependent
Phenolic off-flavor (POF) ability	negative	positive
Fermentation by-products	less intense fruity and aroma (lower amount of higher alcohols and esters)	Intensely fruity and aroma (high amount of higher alcohols and esters)
Diacetyl production/reduction	more α-acetolactate more diacetyl, same reduction rate	lower production, same reduction rate
SO_2-production	high, SO_2 >4 mg/L	low, SO_2 <2 mg/L
Vitamin B5 and pantothenic acid	self synthesis	no self-synthesis, must be provided by the fermentation substrate

1.3.2 Non-*Saccharomyces* yeasts in brewing

Before the domestication of today's brewing culture strains of the species *Saccharomyces*, it is most likely that all beers included non-*Saccharomyces* yeast strains. Previously regarded as spoilage microorganisms, brewer's interest today in using non-*Saccharomyces* yeasts as potential beer fermentation starter cultures has started to grow (105). Belgian brewers in particular have already recognized the potential and have used wild yeasts and mixed fermentations that often contain non-*Saccharomyces* yeasts such as *Brettanomyces bruxellensis* to produce special beers. Within the huge diversity of non-*Saccharomyces* yeast strains, the focus is on the potential of traditional beer styles and the production of low-alcohol or alcohol-free beers. Species such as *Schizosaccharomyces pombe* are used in traditional African beers, *Dekkera bruxellensis* in Belgian beers and German Berliner Weiße, or many other species for the production of spontaneously fermented beer types such as lambic and American coolship ales, which represent just a few examples (15, 151). In the production of low-alcohol and alcohol-free beers in particular, species such as *Scheffersomyces shehatae* (85), *Wickerhamomyces anomalus* (174), *Pichia kluyveri* (140) and *Zygosaccharomyces rouxii* (38) have been the subject of recent studies (43). The most common yeasts considered for this purpose include the species *Saccharomycodes ludwigii,* which is already used in industrial brewing applications as a result of its unique characteristics. The low performance of this species in fermenting maltose and maltotriose means that it can produce alcohol-free beers that still retain some of the aromatic complexity of standard beers (95). This example shows how such alternative yeasts can improve existing beer styles and produce beers that deviate from the known flavor profiles. The use of non-*Saccharomyces* yeasts is a natural way to introduce diversity to beers on the market and there is a large set of yeast strains with potential suitability as beer fermentation starter cultures that have not yet been investigated. MICHEL recently studied the brewing abilities of *Torulaspora delbrueckii* and was able to show that strains of this species are capable of producing high levels of fruity flavors and are resistant to stresses associated with industrial brewery handling (105, 104). However, since non-*Saccharomyces* yeasts represent mostly undomesticated strains, their introduction for brewing applications must be carefully assessed. Each microorganism has unique fermentation characteristics and can develop a range of process adaptations in contact with different substrates and/or conditions. The diverse enzymatic apparatus as well as the diverse bioconversion abilities of different species or genera can result in undefined consistency and quality of the produced beer which is deemed essential for brand image and customer loyalty (155). Nevertheless, consumers are becoming more aware of how variable beer can be and the demand for craft-produced beers with special flavors is increasing. The rising demand for traditional beer styles, alternative flavors and low-alcohol beers stimulates further research and studies into the potential benefits of alternative yeasts (43).

1.4 Phenotypic and DNA-based methods for brewing yeast identification and differentiation

Phenotypic detection methods have been established to count, characterize and identify individual cultures. In the brewing industry in particular, the use of pure yeast cultures is critical for a consistent and reproducible quality of the product. Despite the latest technology and hygienic guidelines, it is not possible to exclude infection and contamination with undesired microorganisms in the most diverse process steps. (Trace) detection of microorganisms in complex mixed cultures is therefore essential for early identification and monitoring of possible contamination. In comparison with traditional phenotypic detection and identification methods, molecular biological methods also provide evidence and a way of identifying microorganisms that are difficult or impossible to cultivate at a species and strain level. Contrary to phenotypic methods, identification using molecular biological methods is virtually independent of the culture and detection media used as well as of the mutations arising due to long storage periods or repeated inoculation (65). Nevertheless, in many molecular biological methods, precultivation in a suitable nutrient media is indispensable for upstream target germ enrichment. It is possible to identify unknown microorganisms by comparing their DNA with the DNA of reference strains. Identification is hereby defined as assigning an unknown microorganism to a specific genus/species, whereas differentiation relates to a distinction between two organisms – irrespective of the taxonomic level (129). Current phenotypic and DNA-based methods used for the identification and differentiation of brewing yeast are shown in Table 3. This table also presents the individual methods and their importance for brewing strains as well as references to corresponding publications on brewing yeasts.

Even if the characterization of yeast strains is based on phenotypic and genetic methods, most of them do not provide any information on the genomic or proteomic background and the beer type to which the yeast strain is most suited. To give reliable results about the specific phenotypic brewing properties and the suitable beer type, different phenotypic and genetic methods were combined in a characterization platform, developed using pilot scale fermentation trials. The results of the yeast strain characterization were presented in the following thesis publications.

Table 3: Selected phenotypic and DNA-based methods for the identification, characterization and differentiation of brewing yeast modified according to HUZTLER 2010 and SAMPAIO 2017 (58, 143); (+, high importance for brewing yeast strains; +/-, medium importance for brewing yeast strains; -, low importance for brewing yeast strains)

Method	Relevance for brewing yeast	References regarding brewing yeast
Phenotypic methods		
Amino acid uptake pattern	+/-	(68, 111, 130, 131)
Decarboxylation of coumaric acid, cinnamic acid and ferulic acid and production of 4-vinylphenol, styrene and 4-vinylguaiacol (POF)	+/-	(98, 101, 104, 109, 166)
Ethanol production	+	(104)
Ethanol resistance	+	(14, 104)
Fermentation kinetics in standardized wort analyzing cell concentration, pH and ethanol	+	(98, 96, 111)
Fermentation performance utilizing high gravity worts (osmotic stress)	+	(26, 84, 108)
Flocculation behavior	+	(150, 158, 164, 169)
Flow cytometry and FACS	+/-	(110, 148, 175)
FTIR-spectroscopy (chemotaxonomic fingerprint)	+/-	(161, 180)
Glucoamylase activity plate-based tests	+/-	(67, 142)
Growth and colony appearance on specific culture media (e.g. Melibiose-Agar, WLN-Agar)	+	(7, 58)
Growth at 37 °C	+	(5–7, 66, 138, 58, 184)
HS-SPME for ester production	+/-	(137, 139)
MALDI-TOF MS, GC-TOF MS, PY-MS and other mass-spectrometry-based methods (protein fingerprint)	+/-	(46, 83, 127, 161)
Maltose fermentation at 28 °C	+	(44, 146, 171, 184, 62)
Maltotriose fermentation at 28 °C	+	(171, 44, 62, 146)
Microarray platforms	+/-	(52)
Protein fingerprinting (e.g. 2D protein map)	-	(1, 69)
Propagation characteristics under standardized aeration in standardized wort (generation times during propagation)	+	(106, 148)
Screening for flocculation	+	(98, 96, 111)
Screening of fermentation by-products after fermentation in standardized wort	+	(98, 96, 111)
Screening of fatty acids after fermentation in standardized wort	+	(106, 104)
Sensory beer analysis after fermentation in standardized wort	+	(98, 96, 111)
Total fatty acids analysis (determination of FAME compounds)	-	(160)
Yeast viability and vitality methods	+/-	(112)

Method	Relevance for brewing yeast	References regarding brewing yeast
Genetic methods (DNA-based)		
AFLP-PCR	-	(147)
FisH/CisH	-	(159)
Karyotyping	+	(70, 162)
Microsatelitte PCR	+/-	(144)
Multiplex PCR	+/-	(70)
NGS-based methods (whole genome sequencing)	+	(40),
Partial IGS2 rDNA capillary electrophoresis (IGS2-314 rDNA)	+	(58)
PCR-DGGE, PCR-TGGE	+/-	(47, 93)
PCR-DHPLC	+/-	(60)
PCR-RFLP of the 5.8s ITS region and other genes	+/-	(70, 133, 163),
RAPD-PCR	+/-	(8, 70, 144)
Real-time PCR (specific primers and targets)	+	(19, 58)
RFLP mtDNA	+/-	(70)
rRNA gene sequencing	+	(160, 163)
SAPD-PCR	+/-	(13)
Sequencing of mtDNA	+/-	(134)
Sequencing of specific genes/house-keeping genes	+	(74)
Standard PCR (specific primers and targets)	+	(79, 19, 76, 78, 74)
δ-Sequence PCR	+/-	(144)

2 Results (thesis publications)

2.1 Summary of results

The thesis publications are each summed up in the following subchapters 2.2 to 2.5. A description of the authorship contribution and full copies of the publications are given. Table 4 gives a short overview of the publications. Publisher permission for the reproducing these publications can be found in Section 5.5.

Table 4: Short overview of the four publications with title of the publication, major objective, applied method and main findings.

Publication Title			
Publication 1 Genetic and Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale Yeast Isolates	**Publication 2** The Importance of a Comparative Characterization of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* Strains for Brewing	**Publication 3** Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the Beverage Industry: Cases of Contamination, 2008 - 2017	**Publication 4** *Saccharomyces cerevisiae* variety *diastaticus* friend or foe? – Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization
Major objective			
Development and evaluation of a characterization platform combining different genetic and phenotypic methods to distinguish *Saccharomyces* brewing yeast strains and determine their suitability and application potential for brewing.	Comparable characterization of 10 common brewing culture strains regarding brewing and sensory properties.	Collection and evaluation of positive PCR analyses on *S. cerevisiae* var. *diastaticus* incidences in the beverage industry between 2008 and 2017 (statistical data evaluation of RCW BLQ microbiological database).	Investigation of the spoilage potential (super-attenuating and sporulation) and brewing properties of different *S. cer.* var. *diastaticus* yeast isolates from beer and beer-mixed beverages.
Applied methods / investigations			
Genetic analysis: Real-time PCR, PCR-DNA sequencing (publication 1), PCR-capillary electrophoresis Brewing trials: Fermentation performance, sugar utilization, amino acid utilization, cell growth, flocculation behavior, change in pH value, phenolic off-flavor, fermentation by-products, sulfur dioxide Sensorial testing: DLG scheme for beer, beer type differentiate test, descriptive sensory evaluation, triangle test.		Evaluation of the data according to: Total number of companies categorized in type and operating site (country), total number of positive and negative incidences and the related matrix as well as the type of contamination (primary or secondary).	In addition to publication 1: Microscope images and determination of inter-mediate cell size, sodium acetate agar test for sporulation behavior, modified durham tube test with fermented beer medium and starch and dextrin agar plate test for spoilage potential
Main findings			
The developed yeast characterization platform in 2 L fermentation vessels is a broadly based standardized tool to find the right yeast strain for distinct brewing aims. All investigated ale yeast strains differ in their brewing properties and the resulting sensory profile of the final beers.	All investigated lager, kölsch, alt, ale and wheat beer yeast strains differ in their brewing properties, their sensory profile and the recommended beer style. One lager strain showed considerably higher SO_2 concen-trations, one ale strain was shown to be maltotriose negative.	126 cases from a total of 52 companies from 15 countries in Europe were evaluated. 62 of them were positive for *S. cer.* var. *diastaticus*, which mostly occurred as secondary contaminants. From 2015 the incidence increased with most cases occurring in the third quarter of each year.	Clear differences in the super-attenuating properties and brewing potential could be verified. No direct correlation between the glucoamylase gene and the spoilage potential could be demonstrated. All beers had a good flavor having one *diastaticus* strain with no spoilage potential.

Part 1 Page 27 - 43	**2.2 Genetic and phenotypic characterization of different top-fermenting *Saccharomyces cerevisiae* ale yeast isolates**

The craft beer movement encourages brewers to use more and more aroma-intense ale strains to create special, innovative beers. Breweries that want to replace their yeast strain or introduce a second strain for other styles or specialty beers need comparable, reliable and practical information regarding the characteristics of individual strains. In order to obtain adequate strain-specific information, different phenotypic methods are required, which are often not directly relevant for industrial brewing applications. In this study a characterization platform was developed that combines these phenotypic methods to distinguish brewing yeasts and determine their suitability and potential for brewing purposes. To enable quality safety and product stability, a genetic classification scheme using molecular methods was developed in order to distinguish five commercially available top-fermenting *S. cerevisiae* ale-yeast isolates and confirm genetic differences between them. Real-time PCR and sequencing of the ITS and 26s rDNA region were used to identify yeast strains at a species level and a PCR system IGS2_314 combined with capillary electrophoresis to differentiate at a strain level. Following genetic characterization, the genetically different strains were screened for phenotypic characteristics, fermentation performance, flavor, and aroma profiles by using controlled and identical brewing conditions. To simulate industrial brewing conditions experiments were carried out in 2 L stainless steel fermentation vessels with a height to diameter ratio of 2:1 and a head pressure of 0.5 bar (imitating the hydrostatic pressure in industrial vessels). Brewing attributes were measured according to MEBAK methods at regular intervals of 24 hours during the primary fermentation and maturing. The final beer was also measured in terms of fermentation performance, sugar utilization, amino acid and free amino nitrogen utilization, cell growth, flocculation behavior, change in pH value, phenolic off-flavor, fermentation by-products, and sulfur dioxide. Furthermore, the results of sensory analysis using organoleptic descriptions were compared with those of the unfiltered products. The obtained yeast isolates were confirmed as belonging to the species *S. cerevisiae*, representing different strains with different brewing properties and flavor characteristics. There was considerable variation in the fermentation dynamics, maltotriose utilization, flocculation behavior and the overall beer flavor.

Authors/Authorship contribution:

Meier-Dörnberg, T.: Literature search, data creation, writing, conception and design; **Michel, M.**: Critical review of draft; **Wagner, R.S.**: Drafted article for English language and content; **Jacob, F.**: Supervised the project; **Hutzler M.**: Design of genetic methods and selected physiological tests, critical revision, revised the conception and manuscript.

9 January / February 2017 (Vol. 70) BrewingScience

Meier-Dörnberg, T., Michel, M., Wagner, R. S., Jacob, F. and Hutzler, M.

Genetic and Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale Yeast Isolates

Brewing yeast plays a pivotal role in determining the flavor and quality of beer. Different process techniques and fermentation conditions can interact with each yeast strain to create a wide variety of different flavor profiles. The craft beer movement encourages brewers to use more and more aroma-intense ale strains to create special, innovative beers. Breweries either maintain individual brewing strains or they order yeast strains from yeast strain providers or culture collections. To ensure a reliable level of quality and product stability it is necessary to genetically classify the strains involved. The origin of a strain is often unclear, and genetic drift or population drift over time cannot be excluded. Some isolates represent very close strains or the same strain. Whether two yeast strains are the same, similar or different, this does not provide any information on their phenotypic (brewing) properties. To determine these properties, genetic and phenotypic characterization methods were used, which distinguished brewing yeasts and determined their suitability and application potential for brewing. The five yeast strains *Saccharomyces cerevisiae* TUM 210, 211, 213, 506, 511 were characterized using a broad spectrum of genetic and phenotypic methods with a focus on brewing properties and sensorial performance. Sequencing ribosomal genes and spacer regions revealed that the strains belong to *Saccharomyces cerevisiae* and showed some polymorphisms. DNA fingerprinting techniques demonstrated that all strains were genetically different. Phenotypic characterization revealed that the brewing properties (e.g. fermentation performance, sugar utilization, amino acid utilization, cell growth, flocculation behavior, change in pH value, phenolic off-flavor, fermentation by-products, sulfur dioxide) and the sensorial characteristics of each strain were unique. The developed yeast characterization platform using special 2 l fermentation vessels is a broadly based, standardized tool to find the right yeast strain for distinct brewing aims.

Descriptors: *Saccharomyces cerevisiae*, brewing yeasts, ale yeast, top-fermenting yeast strains, yeast characterization

1 Introduction

Beer brewing is one of the oldest technological processes in human history [1]. The Sumerians produced beers about 5,000 – 6,000 years ago [2]. The earliest beers produced by humans relied upon natural or spontaneous fermentation. It wasn't until 1838 when the French engineer Charles Cagniard de Latour (1838) reported that a living organism, 'yeast', was necessary and responsible for fermentation [3]. In the late 19th century, it was possible to select yeast strains. Emil Christian Hansen developed a technique for breeding specific cultures [3]. The most commonly used brewing yeast strains include the *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* species, with the exception of a few spontaneously fermented beers [4]. The selection of strains of *S. cerevisiae* and *S. pastorianus* by brewers for fermentation consistency, flavor and aromatic profile, and quality have led to the use of a limited number of strains in brewing [5]. The result of this selection and subsequent adaptation of yeasts for the physiological conditions preferred by yeasts has resulted in strains being used that have little variation in flavor and quality and a reduction in the production of beer styles [6].

Overall, the consumption trend for industrially produced beers is declining, while more recently there has been increased global interest in the production of craft beers with distinctive flavors and a diversity of specialty beers [7, 8]. Different starting materials (e.g. malts, hops) and brewing techniques are being used to produce these diverse beers [9]. Yeasts contribute greatly to the flavor profile of beers, for example, some *S. cerevisiae* strains produce esters and phenolic flavors that give the beers banana and clove flavors. Other *S. cerevisiae* strains are more restrained and allow more wort-like and grain flavors to predominate [10]. Approximately 1,500 yeast species are currently described [11] and estimations indicate that an additional 669,000 extant yeast species have not yet been described [12]. Many of these strains may be useful to produce different beers. The choice of an individual yeast

Authors

Tim Meier-Dörnberg, Maximilian Michel, Fritz Jacob, Mathias Hutzler, Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany; Ronald Steven Wagner, Central Washington University, Washington, USA; corresponding author: m.hutzler@tum.de

strain is often underestimated as an opportunity to develop new beer types and styles, especially within the German purity law. Recently, several non-*Saccharomyces* yeasts have been used in commercial production such as strains like *Saccharomycodes ludwigii* to produce low-alcohol and alcohol-free beers and *Dekkera bruxellensis* to produce Belgian beers or German Berliner Weiße [10; 13]. Non-*Saccharomyces* yeast strains are able to generate distinct fruity aromas and can be used to produce top-fermented wheat beers with many special flavors [8]. However, there is great interest among craft brewers and traditional breweries for using new *S. cerevisiae* yeast strains. Much of their current equipment and processes are developed to accommodate top-fermenting ale strains. Furthermore, some ale yeast strains resemble bottom-fermenting beer yeast fermentation in several key properties which allows traditional bottom-fermenting breweries to easily adopt these strains [14]. There is great biodiversity within *S. cerevisiae* and choosing and evaluating a new strain for beer production can be difficult. Breweries are often economically constrained and/or have a lack of capacity for the necessary microbiological experimentation to implement a new strain.

Yeast strain providers or culture collections offer a wide variety of brewing yeasts to supply the increasing demand. The origin of some yeast strains and their physiological properties is often unclear and cannot be traced back in the majority of cases. Physiological and genetic properties can deviate significantly. Yeast strains with genetically identical patterns do not need to have equal brewing characteristics. In terms of the prevailing brewery conditions and further adaptation over time with responses to stress such as high gravity brewing or different cropping techniques to change the flocculation behavior, the physiological characteristics of the yeast will change. However, brewers aim for consistent brewing quality. Therefore yeast can only be used several times by repitching before the yeast needs to be repropagated using a pure culture. The pure culture should be genetically checked after a certain storage period. Depending on storage conditions, genetic drifts and/or population drifts can occur over time and may also change the brewing properties of the yeast. In order to ensure a reliable level of quality and product quality to distinguish genetically similar yeast strains or even identify and characterize unknown yeast isolates, yeast should be first taxonomically and phenotypically classified.

Many research studies have focused on the impact of different fermentation conditions and the influence of application techniques on the behavior of brewing yeast [15–17]. In 2015 *Parker* and *James* compared five British ale yeast strains under controlled fermentation conditions to investigate variation in their specific flavor profiles and attempted to relate this variation to strain origins [18]. To do this, Parker and James first determined the species identity of the British ale strains by large subunit (LSU) ribosomal DNA (rDNA) and internal transcribed spacer (ITS) sequence analysis. To examine and determine the hybrid nature of all five strains, they used species-specific primers and the PCR-RFLP method. After confirming the species identity, they fermented 1 liter of pilsner wort with an original extract of 12 °P at 20 °C at an inoculation rate of 6 million cells/mL. In terms of the variation at 24, 48, 72 and 240 hours of fermentation, Parker and James analyzed the samples for specific gravity, attenuation, pH and for six flavor compounds (vicinal diketones, acetaldehyde, iso-amyl acetate, ethyl acetate, amyl alcohols and 1-propanol), to see if there was any variation in flavor characteristics.

Table 1 Ale yeast isolates with TUM identifier and the used reference strains (Bavarian wheat beer type and lager type)

Yeast isolates (TUM identifier)	Industrial application
TUM 210	ale production
TUM 211	ale production
TUM 213	ale production
TUM 506	ale production
TUM 511	ale production
LeoBavaricus – TUM 68®	wheat beer production
Frisinga – TUM 34/70®	lager beer production

However, as a result of different conditions most research studies do not provide comparable and transferable results for breweries to use in practice. Besides species confirmation, brewing yeast should also be distinguished at the strain level to ensure quality reliability and product stability. Parker and James investigated the flavor profile, focusing on the overall amount in the final beer and the peak production level over the observed time intervals. Every brewer's ultimate goal is the final desirable taste of the produced beer, which is influenced by different fermentation by-products and the prevailing synergistic effects. Therefore, the individual flavor and main flavor impression of the final beer are the main focus of this study. In addition to the flavor profiles, phenotypic characteristics of fermentation are also analyzed in greater detail to provide information on the suitability and application potential of the yeast strain for industrial brewing. All this typical information will help the brewer to replace their yeast strain, introduce a second, or develop a specialty beer with particular properties. Therefore, this paper provides methods to identify and characterize strains for use in brewing.

To assess and compare yeasts, different yeast strains were isolated and molecular methods were used to confirm strains were genetically different and taxonomically related to the top-fermenting yeast group, *S. cerevisiae*. This genetic identification step is very important because many strains have been mischaracterized in yeast banks and some isolates that are labeled differently may represent very close strains or even the same strain [19].

Traditionally, yeast strain identification and characterization has been based on different morphological traits and physiological properties [20]. Large cell formations or large star-like clusters are not formed by all *Saccharomyces cerevisiae* strains, whereas for top- and bottom-fermenting yeast the strains can hardly be determined by cell morphology [10]. Different culture conditions or repitching by means of bottom cropping from cylindroconical tanks may lead to a loss of some of their vigorous top-fermenting character [10]. Nowadays, molecular microbiological techniques such as polymerase chain reaction (PCR) -based DNA techniques are commonly used for rapid and reliable yeast identification and differentiation. *Hutzler* gave an overview of the additional characteristics and microbiology methods that differentiate bottom-fermenting and top-fermenting yeast [10]. In this approach real-time polymerase chain

reaction (RT-PCR) and ITS1-5.8S-ITS2 and D1/D2 26S ribosomal rRNA gene PCR-sequencing were used to distinguish between *S. cerevisiae* and *S. bayanus*, *S. pastorianus* and *S. cerevisiae var. diastaticus* and a PCR system IGS2-314 combined with capillary electrophoresis to distinguish at strain level.

Following genetic characterization, the strains were screened for fermentation characteristics, flavor, and aroma profiles by using controlled and identical conditions. Brewing trials were conducted to determine the phenotypic characteristics of fermentation, aroma, and flavor parameters: sugar utilization, amino acid utilization, flocculation, drop in specific gravity, change in pH value, phenolic off-flavor, fermentation by-products, sulfur dioxide and individual flavor impressions. The approach taken in this study can be broadly applied to the characterization of isolates from yeasts to rapidly determine their distinctive genetic characters and fermentation properties, flavor, and aroma profiles.

2 Materials and methods

2.1 Yeast Isolates and Strains

Five different ale yeast isolates with unknown origin were obtained as isolates in agar slants from the Yeast Center of the Research Center Weihenstephan for Brewing and Food Quality (BLQ). All isolates were given a TUM identifier (Table 1). We refer to these initial cultures as isolates until species confirmation and confirmation that they represent different strains. We define a strain as being genetically distinct and/or physiologically distinct.

2.2 Genetic Isolate Identification and Strain Determination

The genetic distinctiveness of each Yeast Center TUM isolate was determined by real-time polymerase chain reaction (RT-PCR), ITS1-5.8S-ITS2 and D1/D2 26S ribosomal rRNA gene PCR-sequencing, and a strain typing method based on a PCR-capillary electrophoresis of partial intergenic spacer 2 (IGS2) fragment (IGS2-314 PCR-capillary electrophoresis). The RT-PCR and sequencing methods were used to identify if the isolate belonging to *S. cerevisiae*.

2.2.1 DNA extraction

To isolate the DNA from each investigated yeast isolate, cultures were taken from wort agar slants using an inoculation loop, transferred to a 1.5 mL tube, and mixed with an aliquot of 200 µL InstaGeneTM Matrix solution (Biorad, Munich, Germany). Each tube was vortexed for ten seconds and incubated at 56 °C for 30 minutes, followed by another ten seconds of vortexing and incubation at

Table 2 **Primer sequences of real-time PCR systems to identify *Saccharomyces* species related to brewing [10]**

			PCR		
Target-specificity	**Primer**	**Probe**	**System name**	**Primer sequence (5` - 3`)**	**Reference**
S. cerevisiae *S. pastorianus* *ssp.carlsbergensis*	Sc-GRC-f	Sc-GRC	Sc-GRC3	CACATCACTACGAGATGCATATGCA	[23], [20]
	Sc-GRC-r			GCCAGTATTTTGAATGTTCTCAGTTG	
S. cerevisiae	Sc-f	Scer	Sce	CAAACGGTGAGAGATTTCTGTGC	[21]
S. pastorianus *ssp. carlsbergensis,* *S. paradoxus* *S. cariocanus*	Sc-r			GATAAAATTGTTTGTGTTTGTTACCTCTG	
S. cerevisiae	TF-f	TF-MGB	TF-COXII	TTCGTTGTAACAGCTGCTGATGT	[21]
	TF-r			ACCAGGAGTAGCATCAACTTTAATACC	
S. bayanus, *S. pastorianus*	Sbp-f	Sbp	Sbp	CTTGCTATTCCAAACAGTGAGACT	[23], [20]
	Sbp-r1			TTGTTACCTCTGGGCGTCGA	
	Sbp-r2			GTTTGTTACCTCTGGGCTCG	
S. pastorianus, *S. bayanus (partially)*	BF-LRE-f	BF-LRE	BF-LRE1	ACTCGACATTCAACTACAAGAGTAAAATTT	[21]
Main target: Bottom-fermenting culture yeast	BF-LRE-r			TCTCCGGCATATCCTTCATCA	
S. pastorianus, *S. bayanus (partially)*	BF300E	BF	BF-300	CTCCTTGGCTTGTCGAA	[23]
Main target: Bottom-fermenting culture yeast	BF300M			GGTTGTTGCTGAAGTTGAGA	
S. cerevisiae *var. diastaticus*	Sd-f	Sdia	Sdia	TTCCAACTGCACTAGTTCCTAGAGG	[23], [24]
	Sd-r			GAGCTGAATGGAGTTGAAGATGG	

96 °C for eight minutes. The incubation steps occurred in a Thermomix 5436 (Eppendorf, Hamburg, Germany). After incubation, the tubes were centrifuged at 13,000 × g for two minutes then a 100 µL aliquot of the DNA-containing supernatant was transferred to a new 1.5 mL tube [21]. The DNA concentration was adjusted to 25 ng/µL after being measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

2.2.2 Real-time polymerase chain reaction (RT-PCR)

RT-PCR (Light Cycler® 480 II. Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used to taxonomically classify the isolates. The primer and TaqMan®probe sequences used are listed in table 2 and the RT-PCR procedure followed that of *Hutzler* [21; 22]. All RT-PCR systems listed in table 2 are compatible and were performed with 10 µL 2x Mastermix (Light Cycler® 480 Probe Master, Roche, Germany), 1.4 µL ddH_2O PCR water, 0.8 µL (400 nM) of each primer (Biomers, Ulm, Germany), 0.4 µL (200 nM) probe (Biomers, Ulm, Germany; MGB probe from ThermoFisher scientific, Applied Biosystems®, USA), 0.5 µL IAC135-f (250 nM), 0.5 µL IAC135-r (250 nM), 0.4 µL IAC135-S (HEX) (200 nM), 0.1 µL IAC135 (dilution 1: 10^{-13}), 0.1 µL IAC135 rev (dilution 1: 10^{-13}) and 5 µL template DNA with a total reaction volume of 20 µL, using the same temperature protocol: 95 °C / 10 min; 40 cycles of 95 °C / 10 s, 60 °C / 55 s; 20 °C . IAC135 was developed by *Riedl* at the Research Center Weihenstephan for Brewing and Food Quality of the Technical University Munich. IAC (internal amplification control) is a control to confirm that the PCR reaction itself took place. If IAC is negative the reaction has to be repeated. The yeast strains *S. cerevisiae* (LeoBavaricus – TUM 68®) and *S. pastorianus* (Frisinga – TUM 34/70®) were used as a positive and negative control according to the RT-PCR system tested.

2.2.3 PCR-Sequencing of the D1/D2 domain of the 26S rRNA gene and and the ITS1-5.8S-ITS2

To amplify the D1/D2 domain of the 26S rRNA gene the primers NL1 (5` -GCATATCAATAAGCGGAGGAAAAG– 3`) and NL4 (5` -GGTCCGTGTTTCAAGACGG- 3`) were used according to *Kurtzman* [25]. PCR was performed with 25 µL RedTaq Mastermix 2x (Genaxxon bioscience GmbH, Ulm, Germany), 16 µL ddH_2O PCR water, 2 µL of each primer having a concentration of 400 nM (Biomers, Munich, Germany), and 5 µL template DNA with a total reaction volume of 50 µL, using the temperature protocol according to Hutzler [21]: 95 °C / 5 min; 35 cycles of 95 °C / 30 s, 52 °C / 60 s; 72 °C / 60 s; 72 °C / 10 min.

To amplify the ITS1-5 8S-ITS2, the primers ITS1 (5` -TCCGTAGG-TGAACCTGCGG–3`) and ITS4 (5` -TCCTCCGCTTATTGATATGC-3`) were used according to *White* [26]. PCR was performed with 25 µL RedTaq Mastermix 2x (Genaxxon bioscience GmbH, Ulm, Germany), 15 µL ddH_2O PCR water, 2.5 µL of each primer having a concentration of 500 nM (Biomers, Munich, Germany), and 5 µL template DNA with a total reaction volume of 50 µL, using the temperature protocol according to Hutzler [21] 95 °C / 5 min; 40 cycles of 95 °C / 30 s, 55.5 °C / 60 s; 72 °C / 60 s; 72 °C / 10 min.

Table 3 **TaqMan® probe sequences and reporter and quencher combination of real-time PCR systems used to identify *Saccharomyces* species related to brewing [10]**

Probes (Reporter and Quencher)				
Probe name	**Reporter**	**Quencher**	**Sequence (5` - 3`)**	**Reference**
Sc-GRC	FAM	BHQ1	TCCAGCCCATAGTCTGAACCACACCTTATCT	[21]
Scer	FAM	BHQ1	ACACTGTGGAATTTTCATATCTTTGCAACTT	[23]
TF-MGB	FAM	BHQ1	ATGATTTTGCTATCCCAAGTT	[21]
Y58	FAM	BHQ1	AACGGATCTCTTGGTTCTCGCATCGAT	[23]
BF-LRE	FAM	BHQ1	ATCTCTACCGTTTTCGGTCACCGGC	[21]
BF	FAM	BHQ1	TGCTCCACATTTGATCAGCGCCA	[23]
Sdia	FAM	BHQ1	CCTCCTCTAGCAACATCACTTCCTCCG	[23]

Table 4 **Primer, probe and target DNA sequences of the internal amplification control system (IAC135) used for real-time PCR systems**

Internal amplification control (IAC135)		
System name	**Primer**	**Primer sequence (5` - 3`)**
IAC135	IAC135-f	TGGATAGATTCGATGACCCTAGAAC
	IAC135-r	TGAGTCCATTTTCGCAGATAACTT
	Probe	**Probe sequence (5` - 3`)**
	IAC135-S	HEX-TGGGAGGATGCATTAGGAGCATTGTAAGAGAG-BHQ1
	Target DNA	**DNA sequence (5` - 3`)**
	IAC135	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGGGAGGATGCAT-TAGGAGCATTGTAAGAGAGTCGGAAGTTATCTGCGAAAATGGACTCATTC-GAGTGGCCTATTGACGGTCGCCCAAGGTGTCGCA
	IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCATTTTCG-CAGATAACTTCCGACTCTCTTACAATGCTCCTAATGCATCCTCCCACTAGTTC-TAGGGTCATCGAATCTATCCATTCTCTAGCA

Amplified fragments were purified using a QIAquick® Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer`s recommendations. The quality of amplicons was subsequently checked by capillary gel electrophoresis (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA). The DNA concentration of the purified amplicons was adjusted to 25 ng/µL after being measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The sequencing reaction was assigned to GATC Biotech AG (Konstanz, Germany). For this reason, sanger sequencing for PCR amplificons was chosen.

Each sequence was subsequently trimmed and analyzed with MEGA6 (Molecular evolutionary Genetics Analysis Software). The D1/D2 26S rDNA as well as the ITS1-5.8S-ITS2 rDNA nucleotide sequences were identified for each yeast isolate separately using the GenBank Basic Local Alignment Search Tool (BLAST) of the NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike, Bethesda MD, USA) [27]. Afterwards, sequences were compared with the sequences of the reference strains (Frisinga – TUM 34/70®, LeoBavaricus – TUM 68®) and the type strain *S. cerevisiae* CBS 1171 via ClustalW alignment using MEGA6. Phylogenetic trees were built using MEGA6. The evolutionary history was inferred using the UPGMA method [Sneath P.H.A. and Sokal R.R. (1973). Numerical Taxonomy. Freeman, San Francisco.]. The tree was drawn to scale, with branch lengths of the same units as those of the evolutionary distances that were used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [28] and the units correspond to the number of base substitutions per site. The analysis involved 8 nucleotide sequences of D1/D2 26S rDNA (TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, Frisinga – TUM 34/70®, LeoBavaricus – TUM 68® and CBS 1171 GenBank accession nos. AF528077; AY046146) and except TUM 213 and TUM 511, 6 nucleotide sequences of the D1/D2 domain of the 26S rRNA gene. Codon positions included were 1st+2nd+3rd+Non-coding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [29]. Nucleotide sequence polymorphism was shown for the D1/D2 26S rDNA as well as the ITS1-5.8S-ITS2 rDNA compared with the *S. cerevisiae* CBS 1171 yeast strain (CBS 1171 GenBank accession nos. AF528077/AY046146).

2.2.4 DNA Fingerprinting (PCR-capillary electrophoresis of the IGS2-314 fragment)

In order to determine if isolates represented different or identical strains, genetic fingerprints were generated using the IGS2-314 method [21]. The IGS2 is a spacer region within of the ribosomal cluster. To a partial sequence of the intergenic spacer 2 (IGS2-314) the specific primers IGS2-314f (5'-CGGGTAACCCAGTTCCTCACT-3') and IGS2-314r (5'-GTAGCATATATTTCTTGTGTGAGAAAGGT-3') (Biomers GmbH, Ulm, Germany) [30] were used at a concentration of 600 nM as described by Hutzler [22].

PCR was performed with 22.5 µL RedTaq Mastermix (2x) (Genaxxon, Ulm, Germany) and 2.5 µL template DNA with a total reaction volume of 25 µL. The Mastermix contained 12.5 µL buffer solution (RedTaq Mastermix), 7.0 µL DNA-free PCR water and 1.5 µL of each primer (Biomers, Munich, Germany).

Table 5 Starting wort composition used for propagation and brewing trials (12.4 °P wort)

Wort composition	
Parameter	Amount
Original gravity (°P)	12.40
pH	5.19
Spec. weight SL 20/20 ºC	1.05
Zinc (mg/L)	0.15
FAN (mg/100 mL)	25.00
Total AS (mg/100 mL)	203.22
Total sugar (g/L)	83.78
EBC-Bittering units (EBC)	20.20
Glucose (g/L)	11.46
Fructose (g/L)	2.57
Saccharose (g/L)	1.12
Maltose (g/L)	53.65
Maltotriose (g/L)	14.98

Cycling parameters were: A pre-denaturing step at 95 °C for 300 s, then 35 cycles for denaturing at 95 °C for 30 s, for annealing and elongation at 54 °C for 30 s and 72 °C for 40 s and for final elongation at 72 °C for 300 s. PCR was performed using a SensoQuest LabCycler48s (SensoQuest GmbH, Gottingen, Germany).

Amplified fragments were analyzed using a capillary electrophoresis system (Agilent DNA 1000 kit) following the manufacturer`s recommendations (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).

2.2.5 Phylogenetic analysis of the IGS2-314 fingerprint patterns using Bionumerics Software 7.6

Based on the specific capillary electrophoresis IGS2-314 rDNA patterns, a dendrogram was built using the Bionumerics program 7.6 (Applied Maths, Belgium) to show the relationship between the investigated yeast isolates and reference strains (Fig. 4, see page 18). To create the dendrogram, a curve-based cluster was analyzed using a Pearson correlation with an optimization degree of 0.5 % and a band-based cluster was analyzed using a Jaccard correlation with an optimization of 0.5 % and a tolerance set of 1 %.

2.3 Brewing trials

2.3.1 Wort

The wort specifications used for propagation and the brewing trials are shown in table 5. The wort was based on hopped barley malt concentrate (N53940; Döhler GmbH, Darmstadt, Germany). To achieve an original gravity of 12.4 °P, wort concentrate was diluted with distilled water and boiled for 5 min to guarantee sterile conditions. The same wort batch preparation was used for the propagation and brewing trials to ensure constant wort composition. Free alpha-amino nitrogen was quantified using the MEBAK II. 2.8.4.1 method. Sugar composition was determined using the HPLC MEBAK II. 3.2.2.1.2 method.

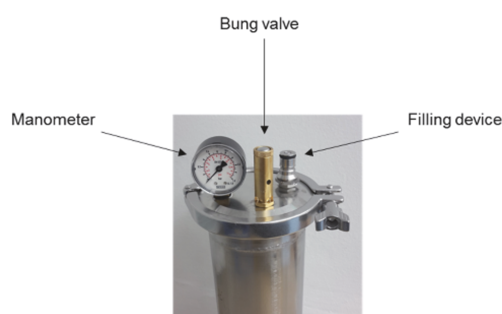


Fig. 1 Modified fermentation vessels

2.3.2 Propagation

In order to propagate yeasts, isolates were inoculated from agar slants (yeast pure culture) into 60 mL of sterile wort medium in an 100 mL Erlenmeyer flask and incubated for 72 h at ambient temperature (20 °C) and pressure, and agitated at 80 rpm using a WiseShake 207 orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, yeasts were transferred to 4 kg of sterile wort medium and further propagated at the same conditions for an additional 72 hours. After allowing six hours for sedimentation, the supernatant was decanted and 2 kg of sterile wort medium at pitching temperature (20 °C) was added to the yeast sediment in each container. Yeast concentration was determined in cells/g using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area per square of 0.00025 m^3 (Brand GmbH&Co.KG, Wertheim, Germany).

2.3.2 Fermentation

Laboratory-scale brewing trials were performed using stainless steel vessels with dimensions of 10 cm diameter x 33 cm height (2.5 liters) with 20 % headspace and clamped down lids according to *Müller-Auffermann* [31]. The vessels were placed in a tempered cooling chamber (2023 Minicoldlab, LKB-Produkter AB, Bromma, Sweden) to guarantee a constant fermentation temperature. To imitate industrial brewery conditions during fermentation, a head pressure of 0.5 bar was applied to simulate a liquid height of 10 m (median hydrostatic pressure). In contrast to Müller-Auffermann, the lid of each vessel was modified to include a manometer and a bung valve (TÜV SV 15-2055 safety valve, spring loaded, 0.5 bar) to achieve practical conditions without affecting each other in a series connection (see Fig. 1).

Table 6 Sensory triangle test assay to determine flavor differences between the final beers produced with the ale yeast isolates TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511

Triangle test assay	
Odd sample	**Non-anomalous sample**
TUM 210	TUM 211
TUM 210	TUM 213
TUM 210	TUM 506
TUM 210	TUM 511
TUM 211	TUM 213
TUM 211	TUM 506
TUM 211	TUM 511
TUM 213	TUM 506
TUM 213	TUM 511
TUM 506	TUM 511

Brewing trials were evaluated by pitching 8.5 L wort per yeast isolate/strain. Each batch was then divided into 4 fermentation vessels. By having 4 vessels, samples could be taken daily from one of the 4 vessels to estimate the specific gravity, cells in suspension and pH, while the other three vessels remained undisturbed. Yeast isolates were added at an inoculation rate of 15 million cells/g of homogeneous mixed wort medium. The wort was not oxygenated. Primary fermentation was maintained at 20 °C and considered complete after the specific gravity remained constant for two consecutive days. An additional five days for maturation was given following primary fermentation at same temperature of 20 °C, and seven days for lagering at 0 °C. The beers were then removed from the fermentation vessels, homogenized, and collected in sterile bottles. The specific gravity and pH of samples were determined from the filtered fermentation samples using a DMA 35N (Anton-Paar GmbH, Graz, Austria) for specific gravity and a pH3210 (WZW, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) for pH measurement. The samples were filtered using a Whatman® folded filter paper with a diameter of 320 mm (GE Healthcare Europe GmbH, Freiburg, Germany).

2.4 Analytical methods

After lagering, the finished beers were analyzed for physical and chemical attributes, which included the following parameters: ethanol, pH, specific gravity, degree of attenuation, free amino nitrogen, amino acid composition, sugar composition, total SO_2, free and total dimethylsulfide, free vicinal diketones and the concentration of fermentation by-products.

Ethanol, pH, specific gravity, and degree of attenuation were measured using an Anton Paar DMA 5000 Density Meter with Alcolyzer Plus measuring module, pH measuring module, and Xsample 122 sample changer (Anton-Paar GmbH, Ostfildern, Germany). Free amino nitrogen and amino acid composition were quantified using the HPLC MEBAK II (2.8.4.1) method. Residual sugar composition was determined using the HPLC MEBAK II (3.2.2.1.2) method. Total SO_2, free and total dimethylsulfide, and free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, USA) with a headspace unit and Elite 5 60 m 1.5DF column using a 2,3-hexandione internal standard. The final concentrations of fermentation by-products (e.g. acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols, 4-vinylguajacol, diacetyl, 2,3-pentandione) were measured according to the MEBAK II (3.2.21) methods using a gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 µm column (Perkin-Elmer, USA).

2.4.1 Determining the cell count (cells in suspension and total cell count)

Cell counts for pitched yeast, cells in suspension until lagering, and

total cell count after lagering were determined using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area per square of 0.00025 m³ (Brand GmbH&Co.KG, Wertheim, Germany).

Cells in suspension were analyzed every 24 h up to the start of lagering. To ensure cell count accuracy during fermentation and maturation, 20 mL of green beer was removed from the middle of the fermentation vessel by using a 10 mL volumetric pipette mounted on a stand. Prior to sampling, the head pressure in the vessel was released very slowly so that the cells in suspension were not affected by a pressure surge.

The total cell count was determined after the lagering phase. Beers were removed from the fermentation vessels and the decanted yeast masses were collected by suspending the yeast cells in a total of 50 g distilled water. The yeast cells were washed by centrifugation twice with 50 g distilled H_2O (5 min at 3000 U) and resuspended with distilled water up to a total of 100 g. Afterwards, distilled water was added to 1 g of the homogenous yeast suspension to make up to 100 mL. Total cell counts were determined in cells/g using the Thoma cell counting chamber.

2.4.2 Phenolic off-flavor test (POF-test)

TUM yeast culture isolates were taken from wort agar slopes and spread on a YM-agar plate containing one of the aroma active compounds: ferulic acid, cinnamic acid and coumaric acid. After 3 days of incubation at 24 °C, the three single agar plates per yeast isolate were evaluated by sniffing to detect any of the following aromas: ferulic acid becomes 4-vinylguajacol (4-VG, clove-like), cinnamic acid becomes 4-vinylstyrene (4-VS, styrofoam-like) and coumaric acid becomes 4-vinylphenol (4-VP, medicinal-like). *S. cerevisiae* LeoBavaricus - TUM 68® and *S. pastorianus* Frisinga - TUM 34/70® were used as positive and a negative control, respectively [22].

For the YM-agar plates a YM-media was made by adding distilled water to 3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone, 11.0 g glucose monohydrate and 20.0 g agar to 1000 mL and autoclaved. After autoclaving, an aliquot of the following stock solutions was added to the YM-media at 45-50 °C under sterile conditions. For the stock solution of coumaric acid, 100 mg of the instant was dissolved in 10 mL of 96 % [v/v] ethanol. The stock solution of ferulic and cinnamic acid was made by dissolving 1 g in 20 mL of 96 % [v/v] ethanol. 10 mL coumaric acid, 2 mL ferulic acid or 2 mL cinnamic acid stock solution was added for 1000 mL YM-media.

2.5 Sensory evaluation

Four single sensory tests were conducted which included: expected beer type test, DLG-scheme for beer, descriptive sensory evaluation, and a triangle test. All beer samples were tasted and evaluated by a sensory panel of 7 DLG-certified tasters (Deutsche Landwirtschafts-Gesellschaft) with long-standing experience in the sensory analysis of beer at the Weihenstephan Research Center for Brewing and Food Quality. Accredited sensory evaluations were performed according to DIN EN 17025. Sensory evaluations were performed in individual walled tasting stations under controlled environmental conditions. Samples were provided in triplicate sets for all beers in dark glasses, each with a three digit code. All samples were served at 12 °C to guarantee optimal conditions to investigate the predominant flavor diversity. At first the panelists associated the beer samples with their expected beer type (e.g. ale, wheat-, Kölsch-, Alt-, stout, Berliner Weisse, porter-, lager-; Bock-; Märzen-, Rauch-, Schwarz-, Dunkles-, malt beer) followed by an examination of the beer samples according to the DLG-scheme for beer. Secondly, a descriptive sensory evaluation was conducted during which trained panelists described specific flavors. Seven main categories were described (e.g. sweet, tropical fruity, fruity (other fruits), citric, spicy, floral and other flavors). Every category was evaluated from 0, meaning not noticeable, to 5, extremely noticeable. Finally a triangle test was performed to determine if there was a difference in the flavor of the final beers produced with the investigated *S. cerevisiae* ale isolates to suggest if there was any difference between the yeasts. The sensory panel evaluated a three sample triangle set per yeast isolate to identify which beer sample differed from the other two equal beer samples. Each yeast isolate was therefore tested against each other in the following order, see table 6.

3 Results and discussion

3.1 Genetic analysis

3.1.1 Real-time PCR assays and PCR-DNA sequencing (D1/D2 26S rRNA gene and ITS)

Based on the real-time PCR (RT-PCR) results, all of the selected TUM ale yeast isolates from the TUM Yeast Center were positive for the Sc-GRC3 and Sce loci. The RT-PCR systems Sc-GRC3 and Sce have positive signals when *S. cerevisiae* DNA is measured

Table 7 Qualitative results of the real-time PCR systems used for the investigated ale yeast isolates and the reference strains to differentiate *Saccharomyces* sensu stricto species; positive (+), negative (–)

		RT-PCR-System						
Species	**Yeast isolates / reference strains**	**Sc-GRC3**	**Sce**	**TF-COXII**	**Sbp**	**BF-LRE1**	**BF-300**	**Sdia**
S. cerevisiae	TUM 210	+	+	+	–	–	–	–
	TUM 211	+	+	+	–	–	–	–
	TUM 213	+	+	+	–	–	–	–
	TUM 503	+	+	+	–	–	–	–
	TUM 506	+	+	+	–	–	–	–
	TUM 511	+	+	+	–	–	–	–
	LeoBavaricus – TUM 68®	+	+	+	–	–	–	–
S. pastorianus	Frisinga – TUM 34/70®	+	+	–	+	+	+	–

Table 8 D1/D2 26S rRNA gene sequence polymorphisms of the investigated yeast isolates compared with *S. cerevisiae* CBS 1171 Access. No. AF528077 by sequence alignment (MEGA6 ClustalW-Alignment)

D1/D2 26S rDNA sequence polymorphisms of the investigated yeast isolates compared to *S. cerevisiae* CBS 1171 Access.No. AF528077		
Yeast strain sequence	D1/D2 26S rDNA polymorphism	
S. cerevisiae TUM 210	A	T
S. cerevisiae TUM 211	A	T
S. cerevisiae TUM 213	A	T
S. cerevisiae TUM 506	A	T
S. cerevisiae TUM 511	–	C
S. cerevisiae LeoBavaricus – TUM 68®	A	T
S. pastorianus Frisinga – TUM 34/70®	A	T
S. cerevisiae CBS 1171	A	T
Number of base pairs *S. cerevisiae* CBS 1171	45	197

or DNA of hybrid strains that contain these DNA loci. In addition, they were positive for the TF-COXII locus suggesting that they belong to the *S. cerevisiae*. In contrast, they were negative for loci that correlate with the PCR systems Sbp, BF-LRE1 and BF-300, which detect *S. bayanus/S. pastorianus* strains. They were also negative for the RT-PCR system Sdia which detects *S. cerevisiae var. diastaticus* strains. Reference strain patterns of TUM 34/70 and TUM 68 were according to the proposed patterns. Table 7 shows the tested RT-PCR systems and the obtained results for all strains. The results indicate that all the investigated strains belong to *S. cerevisiae*. In brewing, *S. cerevisiae* belong to the technical category of top-fermenting brewing yeasts. The results obtained by RT-PCR were confirmed by sequence analysis of the D1/D2 26S and ITS1-5.8S-ITS2 ribosomal DNA. Evolutionary analyses were conducted in MEGA6 [29]. Nucleotide sequence polymorphism was shown for the D1/D2 26S rRNA gene as well as the ITS1-5.8S-ITS2 region in contrast to the *S. cerevisiae* CBS 1171 yeast strain (CBS 1171 GenBank accession nos. AF528077/AY046146) in table 8 and table 9. Table 8 shows the D1/D2 26S rRNA gene sequence polymorphism of all yeast isolates compared with *S. cerevisiae* CBS 1171.

With the exception of yeast strain TUM 511, the D1/D2 26S rDNA sequences are exactly the same as the D1/D2 26S rDNA sequence of *S. cerevisiae* type strain CBS 1171 (GenBank accession No. AF528077). The yeast isolate TUM 511 shows sequence polymorphisms at the base pair position 45 (gap) and 197 (thymine) compared with the *S. cerevisiae* type strain CBS 1171 with adenine (A) and thymine (T). Figure 2 shows the results for the D1/D2 26S rDNA nucleotide sequences visualized in a phylogenetic tree that was built using MEGA6. According to the settings used, the optimal phylogenetic tree shown has a total branch length of 0.00217580 and a total of 462 positions in the final dataset. Additionally, table 9 shows the ITS1-5.8S-ITS2 sequence polymorphism compared with *S. cerevisiae* CBS 1171 except for TUM 213 and TUM 511.

Sanger sequencing for PCR amplificons of the TUM 213 and TUM 506 ITS1-5.8S-ITS2 region delivered short nucleotide sequences which could not be used for reliable genetic analysis (compare with ITS1-5.8S-ITS2 and IGS2-314 PCR-Capillary electrophoresis pattern of the PCR amplicons; chapter 3.1.2). The ITS1-5.8S-ITS2 sequences of all the investigated yeast strains are different to the ITS1-5.8S-ITS2 sequence of *S. cerevisiae* type strain CBS 1171 (GenBank accession No. AY046146). Yeast isolates TUM 210, TUM 211, Frisinga – TUM 3470® and LeoBavaricus – TUM 68® have a total of five and TUM 506 has four sequence polymorphisms compared with *S. cerevisiae* type strain CBS 1171. Within Parker and James study [18], they found that each British ale yeast strain in the investigation had one overall dominant poly-A/T tract of variable length, and this was located at the 5` end of the ITS1 region (length variant of 11 to 12 Ts, nucleotide positions 28-34, based on S288c ITS1 numbering) [18]. The most abundant length variant in this study is 9 Ts in TUM 210 and TUM 506 and 10 Ts in TUM 211 (nucleotide positions 598-607, based on CBS 1171 numbering). Figure 3 shows the results obtained for the ITS1-5.8S-ITS nucleotide sequences visualized in a phylogenetic tree that was built using MEGA6. According to the settings used, the optimal phylogenetic tree is shown to have a total branch length of 0.00568571 and a total of 705 positions in the final dataset.

3.1.2 ITS1-5.8S-ITS2 and IGS2-314 PCR-Capillary electrophoresis

Figure 4 shows the banding patterns of the PCR product of the ITS1-5.8S-ITS2 region for TUM 213 and TUM 511. As figure 4 shows,

Table 9 ITS1-5.8S-ITS2 sequence polymorphisms of the investigated yeast isolates compared with *S. cerevisiae* CBS 1171 Access.No. AY046146 by sequence alignment (MEGA6 ClustalW-Alignment)

ITS1-5.8s-ITS2 rDNA sequence polymorphism of the investigated yeast isolates compared to *S. cerevisiae* CBS 1171 Access.No. AY046146								
Yeast strain sequence	ITS1-5.8s-ITS2 rDNA polymorphism							
S. cerevisiae TUM 210	C	T	A	C	C	T	–	TT
S. cerevisiae TUM 211	C	–	C	T	C	T	T	--
S. cerevisiae TUM 506	C	T	A	C	A	T	-	TT
S. cerevisiae LeoBavaricus – TUM 68®	T	T	A	C	C	T	T	--
S. pastorianus Frisinga – TUM 34/70®	C	T	C	C	C	T	T	--
S. cerevisiae CBS 1171	C	–	A	C	A	–	–	--
Number of base pairs *S. cerevisiae* CBS 1171	271	280	493	525	532	606	607	691–692

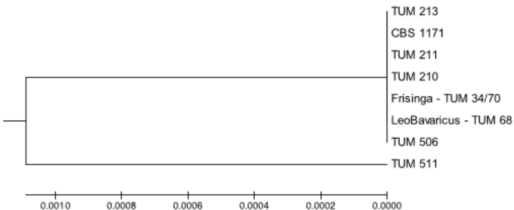


Fig. 2 **Phylogenetic tree of the trimmed single D1/D2 26S rDNA sequences after ClustalW alignment with MEGA6**

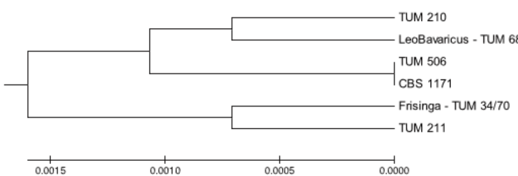


Fig. 3 **Phylogenetic tree of the trimmed single ITS1-5.8S-ITS2 rDNA sequences after ClustalW alignment with MEGA6**

the patterns do not give a clear banding pattern at a specific base pair length. This suggests that each rDNA domain does not have the same and specific nucleotide sequence in the TUM 213 and TUM 511 genome. These nucleotide sequences differ with their different loci in the genome of the yeast isolate and resulted in an unclear and smeared capillary electrophoresis banding pattern. The TUM 213 and TUM 511 ITS1-5.8S-ITS2 nucleotide sequences could not therefore be used for genetic analysis across all yeast isolates (e.g. Real-time PCR assays and PCR-DNA sequencing 3.1.1).

Taking it one step further to Parker and James [18], the PCR of the IGS2-314 locus was used to investigate if different isolates represented different strains by amplifying amplicon fragments of different sizes. Each isolate was compared with two reference strains: The yeast strains *S. cerevisiae* LeoBavaricus – TUM 68®, a top-fermenting, and *S. pastorianus* Frisinga – TUM 34/70® a bottom-fermenting strain. The results showed unique banding patterns suggesting that each isolate represents a genetically different strain (Fig. 5).

3.1.3 Phylogenetic analysis of the IGS2-314 patterns using Bionumerics Software 7.6

Based on the specific capillary electrophoresis IGS2-314 patterns, a curve-based (Fig. 6) and a band-based (Fig. 7) cluster analysis were performed using the Bionumerics program 7.6 (Applied Maths, Ghent, Belgium). Dendrograms were built to visualize the genetic relationship between the investigated yeast isolates and reference strains. Figure 6 and figure 7 show that all the investigated yeast isolates are genetically different. Within the curve-based cluster analysis shown in figure 6, the banding patterns of the yeast isolate TUM 210 and TUM 213 have the highest similarity of all isolates with a similarity of 92.7 %. Variation within the curve-based genetic relationship was below 56.1 % for all of the isolates. Figure 8 shows a similarity of 36.8 % between LeoBavaricus – TUM 68® and Frisinga - TUM 34/70® and no similarity at all for the ale yeast isolates. A genetic relationship to the top-fermenting or to the bottom-fermenting reference strain LeoBavaricus – TUM 68® and Frisinga – TUM 34/70® could not be determined either by means of curve-based or band-based cluster analysis of the IGS2-314 patterns.

3.2 Brewing trials

Brewing trials were conducted to evaluate the phenotypic differences between the isolates/strains and characterize the flavor and aroma profiles. Strains were evaluated for fermentation and maturation parameters which included sugar utilization, amino acid utilization, flocculation, drop in specific gravity, change in pH value, phenolic off-flavor, fermentation by-products, sulfur dioxide and individual flavor impressions. Furthermore, these parameters were used to provide fermentation kinetic curves and validate the performance of each investigated yeast strain.

3.2.1 Sugar utilization

As table 10 shows, all of the strains were able to metabolize the major wort sugars (e.g. glucose, fructose, sucrose, maltose, maltotriose). Variation in glucose, fructose, and sucrose utilization was above 98 % for all of the strains. However, there was considerable variation in the utilization of maltotriose which ranged from 26.66 % for TUM 211 to 83.91 % for TUM 511. TUM 211 also had the lowest utilization rate for maltose, which was 87.23 %, while all other strains were >98.89 %. The results suggested that TUM 211, TUM 213 and TUM 506 do not utilize maltotriose completely. TUM 211 also does not utilize maltose completely. In contrast, TUM 210 and TUM 511 fermented almost all wort sugars.

3.2.2 Amino acid utilization

Table 11 shows the mean amino acid uptake in the finished beers after lagering by the investigated *S. cerevisiae* ale yeast strains. The commonly accepted amino acid uptake classification is indicated

Table 10 **Mean percentage of total wort sugar utilization in beer, measured in triplicate after lagering; confidence level 95 %**

	Sugar utilization (%)				
	TUM 210	**TUM 211**	**TUM 213**	**TUM 506**	**TUM 511**
Glucose	99.16 ± 0.05	98.55 ± 0.47	99.13 ± 0.00	98.23 ± 0.12	99.42 ± 0.12
Fructose	99.35 ± 0.21	98.57 ± 0.21	98.83 ± 0.00	98.05 ± 0.36	98.05 ± 0.00
Sucrose	98.51 ± 0.48	98.51 ± 0.48	98.21 ± 0.00	99.11 ± 0.00	95.54 ± 0.00
Maltose	98.89 ± 1.23	87.23 ± 0.82	99.39 ± 0.07	98.48 ± 0.93	99.28 ± 0.09
Maltotriose	75.01 ± 8.39	26.66 ± 0.26	38.96 ± 0.46	59.28 ± 0.81	83.91 ± 0.71

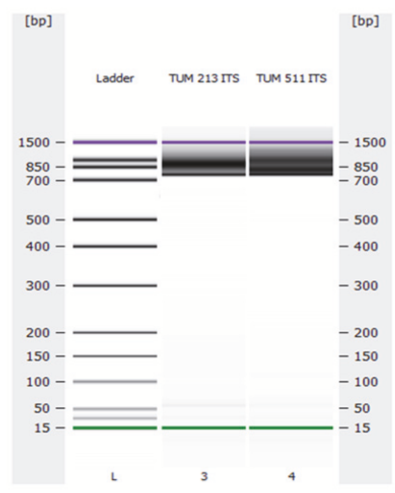
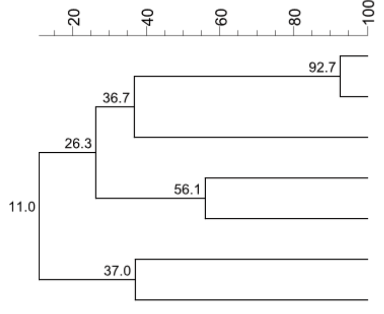


Fig. 4 Capillary electrophoresis ITS1-5.8S-ITS2 patterns for yeast isolates TUM 213 and TUM 511

Fig. 5 Capillary electrophoresis IGS2-314 rDNA patterns of all yeast isolates (TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, LeoBavaricus – TUM 68® and Frisinga – TUM 34/70®)



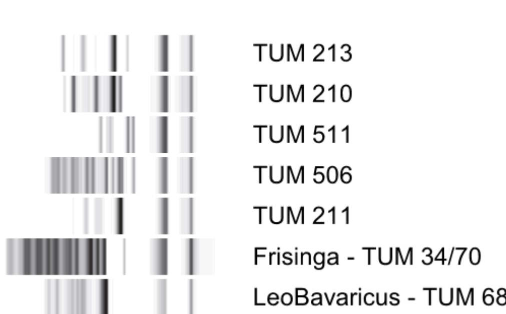


Fig. 6 IGS2-314 rDNA curve-based genetic relationship in percentage between TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, LeoBavaricus – TUM 68® and Frisinga – TUM 34/70® (dendrogram built with Bionumerics 7.6)

Fig. 7 IGS2-314 rDNA band-based genetic relationship in percentage with capillary electrophoresis patterns between TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, LeoBavaricus – TUM 68® and Frisinga – TUM 34/70® (dendrogram built with Bionumerics 7.6)

Table 11 Mean amino acid uptake of all tested yeast strains after lagering measured in the finished beers (Group A = light gray, Group B = dark gray, Group C = no shading); confidence level 95 %

TUM 210	TUM 211	TUM 213	TUM 506	TUM 511
Threonine 98.21 ± 0.18	Threonine 95.39 ± 0.81	Threonine 92.95 ± 2.88	Threonine 94.51 ± 0.44	Serine 95.29 ± 1.00
Methionine 96.84 ±0.72	Serine 93.18 ± 0.94	Lysine 92.64 ± 1.39	Serine 92.02 ± 0.61	Threonine 93.58 ± 0.58
Serine 96.82 ± 0.95	Asparagine 92.62 ± 0.36	Serine 89.66 ± 4.08	Asparagine 91.11 ± 0.23	Lysine 89.29 ± 1.60
Asparagine 96.63 ± 0.57	Histidine 91.57 ± 1.35	Methionine 87.19 ± 3.07	Lysine 90.83 ± 1.23	Asparagine 83.51 ± 0.88
Histidine 94.86 ± 0.60	Methionine 90.04 ± 0.87	Histidin 85.25 ± 4.18	Histidine 89.89 ± 1.04	Arginine 82.86 ± 2.23
Lysine 94.77 ± 0.45	Lysine 89.40 ± 0.92	Isoleucine 84.93 ± 3.88	Methionine 84.02 ± 1.60	Methionine 80.58 ± 2.69
Isoleucine 91.89 ± 4.07	Arginine 83.29 ± 2.09	Asparagine 84.55 ± 4.43	Leucine 82.97 ± 0.05	Leucine 76.50 ± 1.64
Arginine 91.50 ± 1.11	Tryptophan 81.64 ± 0.98	Arginine 84.62 ± 6.13	Arginine 75.25 ± 1.91	Histidine 75.92 ± 3.61
Leucine 87.33 ± 3.95	Isoleucine 80.18 ± 0.69	Tryptophan 82.49 ± 4.40	Isoleucine 73.28 ± 0.79	Isoleucine 69.56 ± 3.01
Aspartic-acid 84.94 ± 6.22	Leucine 79.08 ± 0.74	Glutamic-acid 82.07 ± 5.66	Aspartic-acid 71.93 ± 0.10	Phenylalanine 61.81 ± 2.66
Glutamic-acid 73.82 ± 1.34	Aspartic-acid 78.85 ± 1.04	Leucine 73.66 ± 4.07	Tryptophan 69.26 ± 2.71	Glutamic-acid 61.36 ± 1.15
Tryptophan 72.96 ± 4.16	Glutamic-acid 75.01 ± 0.79	Aspartic-acid 64.68 ± 8.79	Glutamic-acid 67.28 ± 1.79	Tryptophan 57.97 ± 6.79
Phenylalanine 69.07 ± 8.21	Phenylalanine 64.98 ± 1.47	Phenylalanine 60.66 ± 4.47	Phenylalanine 64.06 ± 0.20	Valine 51.93 ± 1.35
Valine 64.87 ± 9.14	Valine 60.76 ± 1.77	Valine 56.71 ± 4.85	Valine 51.89 ± 0.36	Aspartic-acid 48.31 ± 2.11
Alanine 60.71 ± 0.22	Tyrosine 52.92 ± 2.52	Alanine 44.25 ± 6.96	Tyrosine 48.70 ± 0.56	Tyrosine 43.56 ± 3.09
Tyrosine 48.30 ± 10.44	Alanine 48.56 ± 2.24	Tyrosine 38.71 ± 6.43	Alanine 39.18 ± 0.30	Glycine 18.81 ± 2.68
Glycine 43.73 ± 2.08	Glycine 43.92 ± 2.58	Glycine 23.87 ± 7.53	Glycine 28.81 ± 1.02	Alanine 17.02 ± 2.64
Gamma-amino butyric acid 29.80 ± 1.66	Gamma-amino butyric acid 35.58 ± 1.91	Gamma amino butyric acid 21.27 ± 8.55	Gamma amino butyric acid 27.75 ± 0.48	Gamma amino butyric acid 13.67 ± 5.77
Norvaline 0.00 ± 0.00	Norvaline 0.00 ± 0.00	Norvaline 0.00 ± 0.00	Norvaline 0.00 ± 0.00	Norvaline 0.00 ± 0.00
Glutamine -54.36 ± 2.57	Glutamine -39.20 ± 3.27	Glutamine -19.45 ± 3.83	Glutamine -14.44 ± 0.54	Glutamine -9.96 ± 0.72

with shading according to *Jones* and *Pierce* [31–33]. As shown in table 11, threonine and serine are the most metabolized amino acids for each yeast strain. The tested yeast strains all metabolized glutamine by the lowest amount. The other amino acids were utilized in the order indicated. The exact course of absorption and the sequence varies even if specific amino acids were preferred by the yeast. Therefore the amino acid utilization follows no defined process and is different for each observed yeast strain.

Figure 8 and figure 9 show the FAN and the total amino acid (AS) utilization of each yeast strain in comparison with the corresponding residual contents. The utilization rate of FAN and AS is correlated for the same yeast strain but different across strains. The total uptake efficiency is in descending order as follows: TUM 210, TUM 211, TUM 506, TUM 213 and TUM 511.

3.2.3 Fermentation dynamics

Figure 10 shows the drop in specific gravity during fermentation by the investigated yeast strains. As shown in figure 10, TUM 210 has the quickest drop in specific gravity and reached the final gravity after 96 hours of fermentation. TUM 211 has the lowest drop and took 144 hours more to reach the final gravity. However, the fermentation performance/rate of the yeast strains TUM 210 and TUM 213 as well as TUM 211 and TUM 511 was very close. The biggest differences can be seen when reaching the apparent attenuation (Table 12). TUM 506 ferments the wort slower than the other strains but does so continuously until it reached a apparent attenuation of 77.37 % after 216 hours of fermentation. In comparison, TUM 211 was quicker to start fermentation but took 24 hours more to reach the apparent attenuation of 66.13 %. Table

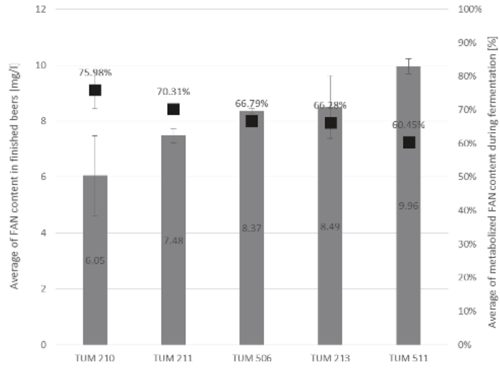


Fig. 8 **Average of metabolized and free amino nitrogen (FAN) content in finished beers produced with yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

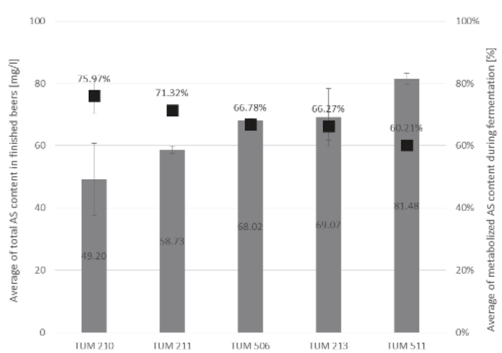


Fig. 9 **Average of metabolized and total amino acid (AS) content in finished beers produced with yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

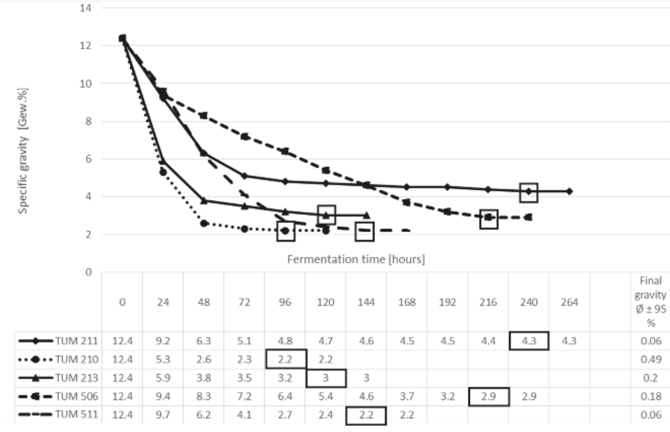
12 shows the apparent attenuation compared with the fermentation time required by the isolated strains. The different fermentation rates and degrees of apparent attenuation are due to their ability to ferment maltose and maltotriose (see Table 10).

3.2.4 Flocculation (Cell count)

A flocculent yeast strain accumulates to flocs and settles at the bottom of the fermentation vessel when the nutrients present in brewers wort are largely consumed [3]. As figure 11 shows, all investigated yeast strains are largely dispersed and remain in a suspension that is close to the pitching concentration, even once they have reached their apparent attenuation. According to *Bühlingen*, the strains exhibited a non-flocculent ("powdery") behavior [34]. However, there was a wide variation in the maximum suspension and final flocculation among the different yeast strains. For example, TUM 211 remained in suspension markedly longer than the other yeast strains and never reached a flocculation similar to that of the other strains. In contrast to TUM 506, TUM 211 remained in suspension markedly longer according to the slower drop in specific gravity. In addition, TUM 211 exhibited a more "powdery" or non-flocculent behavior, followed by strain TUM 506 (Table 13). Yeast strains TUM 210, TUM 213 and TUM 511 sediment out more rapidly after reaching their apparent attenuation after 96, 120 and 144 hours of fermentation respectively.

3.2.5 Change in pH value

Table 14 shows the drop in pH during the first 96 hours of primary fermentation, the pH value after maturation phase, and the average in pH value of the final beer. As shown in table 14 (see page 22), all investigated yeast strains reached their minimum pH value for primary fermentation after 48 hours. With the exception of TUM 511, the used yeast strains recorded a pH value increase of 0.1 after the maturation and lagering phase. The increase after maturation might be due to the excretion of yeast metabolites and the uptake and metabolization of pyruvate. According to *Annemüller* and *Manger* [35], this effect is common if an increase does not exceed 0.1. So that the other vessels are not disturbed by sampling, the system is kept closed and yeast cells remain in the fermentation vessel until the lagering phase is complete. Therefore the further increase by 0.1 after the lagering phase to a final beer pH of between 4.6 and 4.7 is indicated by cell autolysis [35]. TUM 511 exhibited the strongest capacity for acidification (ΔpH 0.8) compared with the other yeast strains.



	0	24	48	72	96	120	144	168	192	216	240	264		Final gravity Ø ± 95 %
TUM 211	12.4	9.2	6.3	5.1	4.8	4.7	4.6	4.5	4.5	4.4	4.3	4.3		0.06
TUM 210	12.4	5.3	2.6	2.3	2.2	2.2								0.49
TUM 213	12.4	5.9	3.8	3.5	3.2	3	3							0.2
TUM 506	12.4	9.4	8.3	7.2	6.4	5.4	4.6	3.7	3.2	2.9	2.9			0.18
TUM 511	12.4	9.7	6.2	4.1	2.7	2.4	2.2	2.2						0.06

Fig. 10 **Drop in specific gravity measured in a single reference vessel compared with the average in final gravity (marked with box) measured in triplicate for the tested yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

Table 12 **Apparent attenuation (AA %) of the final beer compared with specific time for primary fermentation for the investigated yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

	Apparent attenuation (AA %) of the final beer				
	TUM 210	**TUM 211**	**TUM 213**	**TUM 506**	**TUM 511**
AA (%)	82.73 ± 3.52	66.13 ± 0.51	74.77 ± 0.38	77.37 ± 1.34	82.7 ± 0.42
Fermentation time (hours)	96	240	120	216	144

3.3 Flavor characterization

3.3.1 Phenolic off-flavor

Table 15 shows the results of the POF-tests evaluated by sniffing. As shown in table 15, see page 22, not all of the investigated yeast strains are capable of building phenolic flavors. The panelists could only detect aroma active components formed by TUM 213 and TUM 511. For both yeasts, all three corresponding POF-flavors were detected by sniffing. TUM 210, TUM 211 and TUM 506 are POF-negative. These three yeast strains cannot decarboxylate any of the precursor acids. Therefore the PAD or/and FDC activity might be inactive or blocked [36–38].

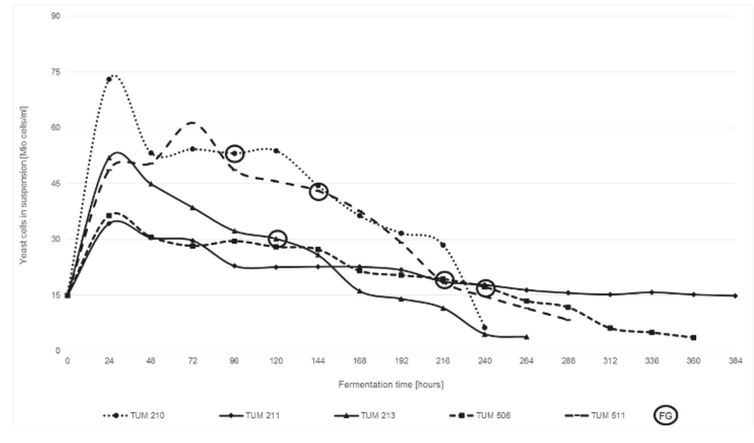


Fig. 11 **Yeast cells in suspension during the main fermentation and maturing phase. The circle marks the specific final gravity of the investigated yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511**

Figure 12 shows the concentrations of 4-vinylguajacol measured in the finished beers after lagering. According to the evaluation by sniffing, TUM 213 and TUM 511 are POF-positive with detected concentrations of 2.77 mg/L and 3.33 mg/L of 4-vinylguajacol, respectively (Fig. 10). Both concentrations are above the individual threshold for 4-vinylguajacol of 0.3 mg/L [39].

3.3.2 Fermentation by-products

There was a variation in the production of fermentation by-products for all of the yeast strains (Table 16, see page 23). Except for yeast strain TUM 211, the concentration of higher alcohols is above 100 mg/L. TUM 210 has the highest concentration of higher alcohols (159.73 ± 12.31 mg/L) but also the lowest ester concentration (34.37 ± 4.09 mg/L). The highest level of esters was detected in the beer produced by TUM 511 with a concentration of 57.33 ± 0.65 mg/L. The quantity of acetaldehyde as well as the sum of diacetyl and 2.3-pentanedione (vicinal diketones) are below their individual thresholds. The concentration of diacetyl is mostly taken by brewers as an indication that the maturation phase is complete and a butter or butterscotch flavor impression is imparted above their threshold. Brewers use the ratio of diacetyl to pentanedione to indicate whether elevated diacetyl concentrations are due to contaminants or fermentation by-products. [40]. Acetaldehyde is also associated with unmatured, so-called green beer and the flavor reminiscent of 'grassy' off-flavors and green apples [39].

3.3.3 Sulfur dioxide

SO_2 in beer is a natural antioxidant, acting as a reducing agent and oxygen scavenger by reacting with stale-tasting carbonyls (acetaldehyde and trans-2-nonenal) to form flavor-inactive carbonyl-sulfite adducts [40]. Therefore SO_2 has an extending and positive effect on the flavor stability of a beer. 80 % of the flavor stability potential is affected by its concentration in the final beer. Many breweries therefore reach higher SO_2 concentrations by adjusting and improving the process control. However, it has been shown that the level of SO_2 formation is mainly influenced by the yeast strain. Identical fermentation conditions can produce differences of between 2 and 10 mg/L SO_2 in the finished beer [3; 41]. If the concentrations exceed 5 mg/L SO_2, each additional mg/L SO_2

Table 13 **Difference in maximum yeast cell concentration during primary fermentation and yeast cell concentration by reaching the specific final gravity (FG) and the flocculation behavior of TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511**

Yeast cell sedimentation at the end of primary fermentation				
Yeast strain	**Max. Cell conc.**	**Cell conc. FG**	**Difference**	**Flocculation behaviour**
TUM 210	73.07	53.20	–19.87	More flocculent
TUM 211	34.30	17.80	–16.50	Most powdery
TUM 213	51.97	30.20	–21.77	Most flocculent
TUM 506	36.45	19.36	–17.09	More powdery
TUM 511	61.30	43.07	–18.23	Less powdery

Table 14 Change in pH value during primary fermentation, after the maturation and lagering phase, rounded to two decimal figures, confidence level 95 %

pH value decrease during primary fermentation					
	TUM 210	TUM 211	TUM 213	TUM 506	TUM 511
0 h	5.2	5.2	5.2	5.2	5.2
24 h	4.6	4.5	4.5	4.6	4.6
48 h	4.4	4.4	4.5	4.5	4.4
72 h	4.4	4.4	4.5	4.5	4.4
96 h	4.4	4.4	4.5	4.5	4.4
After primary fermentation	4.4	4.4	4.5	4.5	4.4
After maturation	4.5	4.5	4.5	4.6	4.4
Final Beer (after lagering)	4.6 ± 0.01	4.7 ± 0.01	4.6 ± 0.01	4.7 ± 0.01	4.4 ± 0.01
∆pH	0.6	0.5	0.6	0.5	0.8

Table 15 POF results of the investigated yeast strains

	POF-test/Sniffing perception of				
Product/ Precursor	TUM 210	TUM 211	TUM 213	TUM 506	TUM 511
4-vinylguajacol/ ferulic acid	–	–	+	–	+
4-vinylphenol/ coumaric acid	–	–	+	–	+
4-vinylstyrene/ cinnamic acid	–	–	+	–	+

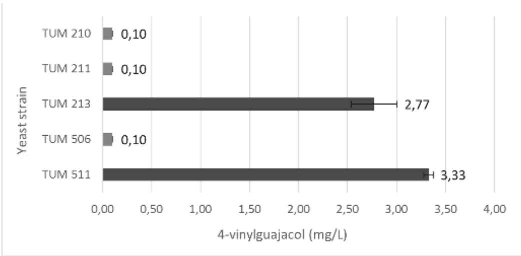


Fig. 12 Phenolic off-flavor ability of the investigated yeast strains; confidence level 95 %

prolongs the flavor stability of beer by about 1 month [*Back* 2015 [42]]. All of the investigated yeast isolates form sulfur dioxide (SO_2) during fermentation. Table 17(see page 23) shows the SO_2 concentration of the finished beers produced by yeast strain. As shown in table 17, TUM 210 and TUM 213 produced the highest quantity of SO_2. The beer brewed with TUM 511 has the lowest concentration of sulfur dioxide (0.50 mg/L), which is four times lower than the second lowest concentration of 2.23 mg/L in the finished beer brewed with TUM 506.

3.3.4 Sensory Evaluation

Sensory analysis of the beers was conducted after maturation and lagering. In terms of the descriptive sensory evaluation, the following figure 13 shows the average of each flavor intensity judged by all seven panelists and summarized according to the main flavor categories.

Figure 13 shows that beers prepared using TUM 211 and TUM 511 are very balanced in all flavor categories, with the exception of spicy flavors, while citric and fruity flavors are perceived as being slightly more distinct using TUM 511. TUM strain 210 produced more floral and citric beers in comparison with the beers brewed using TUM 506, which are very fruity, particularly within the tropical fruit category. TUM 213 is an exception and produced mostly spicy and other (yeasty) flavors. In the beer differentiate test, 85.71 % of the panelists also referred to that beer as a wheat beer. This is confirmed by the POF-test and the detected concentration of 4-VG above the threshold. TUM 511 also produced a concentration of 4-VG above the threshold (3.33 mg/L), but the flavor wasn't recognized by the panelists (flavor intensity of 0.14 e.g. Fig. 11), which may have been caused and suppressed by synergistic effects. According to the results obtained, the top-fermenting yeast strain TUM 511 seems to be particularly suitable for brewing a "Bavarian ale". This beer style has a well-balanced flavor profile, with the fruitiness of an ale style brewed beer and underlined by the slightly spicy and yeasty flavors of a wheat beer. In conclusion, 28.5 % of the panelists could not clearly assign this beer to a wheat nor to an ale style. With the exception of the beers produced using strains TUM 213 and TUM 511, all the used brewing yeast beers were an ale beer style (TUM 210 57.14 %, TUM 211 85.71 % and TUM 506 42.85 %). The sweet flavor impressions are caused by the low final attenuation and may correspondingly decrease by repitching the yeast strains in order to adapt the utilization of maltotriose. Figure 14 indicates which differences were perceived by at least two of the seven trained tasters (e.g. 28.57 %) as significant and shows the most pronounced flavors within the main flavor categories. As the results of the sensory evaluation show, the different yeast strains alter the profile of the product in an unpredictable or multi-faceted way. For this reason a triangle test was set up to determine if there is a difference between the investigated *S. cerevisiae* ale strains. By keeping all brewing and fermentation conditions identical, the potential differences are limited to just one aspect of the product's profile.

Two yeast strains could not be clearly distinguished by the tasters (Table 18, see page 24). Analyzing the numbers of the 70 tasters given the non-anomalous samples shows that 63 of them correctly identified the odd sample. In other words, 90 % of the time, a taster could correctly identify the beer made with the different yeast. That seems to be very significant compared with the expectations of random chance (e.g. 33 %). Particularly striking in this case is that the POF-positive yeast isolates could be clearly distinguished from the POF-negative yeast isolates. Contrary to the results obtained in the POF-test, the panelists could not detect spicy flavors in the beer produced with TUM 511. The clear distinction of this yeast strains seems to be due to a high level of citric flavors compared with the other strains. When tasting the fruity yeast strains TUM 211 and TUM 506 against one another, no significant difference could be detected. Only four of seven tasters could distinguish between TUM 211 and TUM 506 (e.g. 57.1 %). Therefore the correct allocation of three tasters can be regarded as random/an accidental occurrence. The tasters did not detect any difference in the flavor

Table 16 Average of important fermentation by-products (FBP) measured in triplicate of the final beers produced with TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %

	Fermentation by-products (mg/L)					
	TUM 210	TUM 211	TUM 213	TUM 506	TUM 511	Threshold (Lager beers)*
Isoamyl acetate	2.40 ± 0.33	2.03 ± 0.05	4.77 ± 0.35	1.53 ± 0.11	2.93 ± 0.05	1.6
Ethyl acetate	31.97 ± 3.76	37.93 ± 0.11	51.57 ± 3.35	22.57 ± 1.31	54.40 ± 0.61	30
∑ Ester (E)	34.37 ± 4.09	39.97 ± 0.14	56.33 ± 3.66	24.10 ± 1.36	57.33 ± 0.65	n.v
n-Propanol	27.30 ± 3.53	18.30 ± 0.16	26.00 ± 1.49	20.67 ± 1.01	20.77 ± 0.53	800
i-Butanol	31.77 ± 4.05	16.20 ± 0.37	13.80 ± 0.96	20.90 ± 1.40	13.13 ± 0.28	200
Amyl alcohols	100.67 ± 5.65	59.27 ± 1.32	75.97 ± 7.30	88.50 ± 5.90	74.97 ± 1.24	70
∑ Higher alcohols (HE)	159.73 ± 12.31	93.77 ± 1.83	115.77 ± 9.05	130.07 ± 8.12	108.87 ± 1.23	n.v.
4-Vinylguajacol	0.10 ± 0.00	0.10 ± 0.00	2.77 ± 0.23	0.10 ± 0.00	3.33 ± 0.05	0.3
Diacetyl	0.09 ± 0.01	0.10 ± 0.01	0.07 ± 0.00	0.12 ± 0.01	0.06 ± 0.00	0.15
2,3-Pentanedione	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.9
∑ Vicinal diketones	0.11 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.14 ± 0.01	0.07 ± 0.00	n.v.
Acetaldehyde	2.53 ± 0.93	4.60 ± 0.48	5.60 ± 1.61	5.93 ± 0.93	4,03 ± 0.46	25
Ratio (∑E : ∑HE)	1 : 4.65	1 : 2.35	1 : 2.06	1 : 5.40	1 : 1.90	

* [39]

Table 17 SO_2 concentration of the final beers produced with TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %

	SO_2 concentration of the finished beers (mg/L)				
	TUM 210	TUM 211	TUM 213	TUM 506	TUM 511
SO_2	2.63 ± 1.64	2.60 ± 0.98	2.63 ± 1.521	2.23 ± 1.02	0.50 ± 0.00

of either beer even if the tasters described them as having different flavors. Therefore the test tentatively indicates that the different yeast strains makes no perceptual difference.

4 Conclusion/Summary

Nowadays, consumption trend is turning away from industrially produced beers that are similar in quality and taste, towards a diversity of specialty beers with distinctive flavors. Individual and non-traditional brewing yeast strains can be ordered from different yeast strain providers or culture collections to meet this increasing demand. Due to the high biodiversity, the diversity of the strains and the different flavor profiles, it is difficult to choose the appropriate yeast strain. Many craft- and microbreweries are presently interested in *S. cerevisiae* yeast strains, also known as ale or top-fermenting yeasts. Reliable and practical information regarding the characteristics of individual strains is required and collecting comparable data of brewing yeast strains will help brewers around the world to adapt existing or new yeasts in order to create novel products for the beer market. To continue and maintain a reliable quality and product stability, it is necessary to perform a genetic and phenotypic characterization as well as a proper organoleptic description comparing the unfiltered products. This paper presents a comparison of five commercially available top-fermenting *S. cerevisiae* ale yeast strains. The used strains were tested under identical fermentation conditions. For this purpose, fermentation plants were designed to provide conditions comparable to those found in large industrial tanks. For this purpose, five ale yeast isolates were obtained from the Yeast Center of the Research Center Weihenstephan of the Technical University of Munich. The first species identification of the investigated yeast isolates was determined by using specific polymerase chain reaction systems. Regardless of their phenotypic characteristics, all strains were positive for the Sc-GRC3, Sce and TF-COXII loci, which indicated that these isolates belong to the species *S. cerevisiae*. The results obtained by RT-PCR were confirmed by sequence analysis of the D1/D2 26S rRNA gene and ITS1-5.8S-ITS2 region with 0.5 % nucleotide differences. Compared to the *S. cerevisiae* type strain CBS 1171 (GenBank accession nos. AF528077/AY046146) that was used as a reference strain, all ale yeast isolates were identical except TUM 511 with two gaps compared with the nucleotide sequence of the D1/D2 26S domain of the rRNA gene. Greater differences of 4 to 5 gaps could be seen in the sequencing alignment of the ITS1-5.8S-ITS2 region. The ITS1-5.8S-ITS2 of TUM 213 and TUM 511

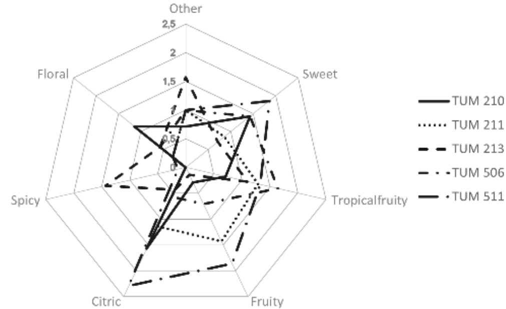


Fig. 13 Comparison of the flavors grouped according to the main categories

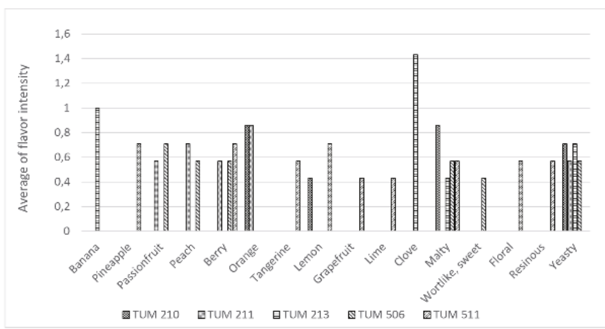


Fig. 14 Average of main flavor intensity of each yeast strain

could not be taken into account, as a result of different polymorphic repeats in the genome and therefore not be unambiguously sequenced. This was confirmed by capillary electrophoresis of the corresponding PCR amplificons. All of the investigated ale yeast isolates belong to the *S. cerevisiae* species and could also be classified as being genetically different strains by means of PCR of the IGS2-314 loci combined with capillary electrophoresis of the amplificon fragments. Following genetic characterization, the strains were screened for phenotypic characteristics, fermentation performance, flavor, and aroma profiles by using controlled and identical brewing conditions carried out in 2 L pilot fermentation vessels. The obtained yeast isolates were confirmed as belonging to *S. cerevisiae*, representing different strains with different brewing properties and flavor characteristics. There was considerable variation in fermentation dynamics, the utilization of maltotriose, flocculation behavior and the beer flavor. The flavor ranged from floral (TUM 210) and fruity (TUM 506) to phenolic off-flavors reminiscent of German wheat beer (TUM 213). As top-fermenting *S. cerevisiae* brewing yeast strains can be very heterogeneous at the end of the main fermentation phase and in the final beer, both phenotypic and genetic tools are essential to determine the brewing potential of a distinct strain and the phylogenetic position

Table 18 Triangle test of the investigated yeast strains: Evaluation (successful identification needed for 7 tasters according to DLG): n.s. =significant p=>0.05 (<5), s. =significant p=≤0.05 and >0.01 (5), h.s. =highly significant p=≤0.01 and 0.001 (6), v.h.s. =very highly significant p=≤0.001 (7) [43; 44]

Triangle Test					
Yeast Isolate		Tasters	Successful Identification's		
Single	Double	n	n	%	Significance
TUM 210	TUM 211	7	5	71.4	s.
TUM 210	TUM 213	7	7	100.0	v.h.s
TUM 210	TUM 506	7	7	100.0	v.h.s.
TUM 210	TUM 511	7	7	100.0	v.h.s.
TUM 211	TUM 213	7	7	100.0	v.h.s
TUM 211	TUM 506	7	4	57.1	n.s.
TUM 211	TUM 511	7	7	100.0	v.h.s
TUM 213	TUM 506	7	7	100.0	v.h.s
TUM 213	TUM 511	7	7	100.0	v.h.s.
TUM 506	TUM 511	7	7	100.0	v.h.s

within *S. cerevisiae*. The approach presented in this study can be widely applied for the characterization of isolates from yeasts to rapidly determine their distinctive genetic characters and fermentation properties, flavor, and aroma profiles. Whether two yeast strains are the same, similar or different, this does not provide any information on their phenotypic (brewing) properties. Future work will assess if genetically equal yeast strains have different phenotypic brewing characteristics or even similar or the same. The isolation and characterization of different yeast isolates for application in breweries is an underestimated opportunity to develop new beer styles or create new interesting flavors without violating the German purity law.

Acknowledgement

Parts of this work were funded by the German Ministry of Economics and Technology and the Wifö (Wissenschaftsförderung der Deutschen Brauwirtschaft e.V., Berlin, Germany) in project AiF 17698 N.

5 Literature

1. Piskur, J.; Rozpedowska, E.; Polakova, S.; Merico, A. and Compagno, C.: How did *Saccharomyces* evolve to become a good brewer?, Trends in Genetics, **22** (2006), no. 4, pp. 183-186.
2. Hornsey, I.-S.: A history of beer and brewing (RSC paperbacks), Royal Society of Chemistry, Cambridge, 2003.
3. Annemüller, G.; Manger, H.-J. and Lietz, P.: Die Hefe in der Brauerei – Hefemanagement, Kulturhefe - Hefereinzucht, Hefepropagation im Bierherstellungsprozess (VLB-Fachbücher), 3rd ed., VLB, Berlin, 2013.
4. Cocolin, L.; Campolongo, S.; Gorra, R.; Rolle, L. and Rantsiou, K.: *Saccharomyces cerevisiae* Biodiversity During the Brewing Process of an Artisanal Beer: A Preliminary Study, J. Inst. Brew, 3 (2011), no. 117, pp. 352-358.
5. Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, 2016, Technische Universität München. Available online at www.blq-weihenstephan.de, checked on 11/15/2016.
6. Hutzler, M. and Jacob, F.: Immer bessere Kontrolle - Hefe im Wandel der Zeit, Brauindustrie (2015), no. 100, pp. 76-80.
7. Canonico, L.; Comitini, F. and Ciani, M.: Dominance and influence of selected *Saccharomyces cerevisiae* strains on the analytical profile of craft beer refermentation, J. Inst. Brew, **120** (2014), no. 3, pp. 262-267.
8. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Methner, F.-J.; Wagner, R.-S. and Hutzler, M.: Review: Pure non-*Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications, J. Inst. Brew., **122** (2016), no. 4, pp. 569-587.
9. Jespersen, L.; van der Aa Kühle, A. and Petersen, K.-M.: Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeasts, International Journal of Food Microbiology, **60** (2000), no. 1, pp. 43-53.
10. Hutzler, M.; Koob, J.; Riedl, R.; Schneiderbanger, H.; Müller-Auffermann, K. and Jacob, F.: Yeast identification and characterization (Chapter 6). In: Hill, A. E. (Ed.): Brewing microbiology – Managing Microbes, Ensuring Quality and Valorising Waste (Woodhead Publishing Series in Food Science, Technology and Nutrition, 289), Elsevier, 2015, pp. 65-105.
11. Rosa, C. and Gábor, P. (Eds.): Biodiversity and ecophysiology of yeasts, The Yeast Handbook, Springer, Berlin, London, 2006.

12. Verstrepen, K.-J.; Chambers, P.-J. and Pretorius, I.-S.: The Development of Superior Yeast Strains for the Food and Beverage Industries: Challenges, Opportunities and Potential Benefits (Chapter 13). In: Querol, A., Fleet, G.-H. (Eds.): Yeasts in Food and Beverages (The Yeast Handbook), Springer, Berlin, Heidelberg, 2006, pp. 399-444.
13. Meier-Dörnberg, T.; Hutzler, M.; Jacob, F. and Schneiderbanger, H.: Geschmacklich ansprechend Mit der richtigen Hefe anregende alkoholfreie Biere produzieren, Brauindustrie (2015), no. 7, pp. 12-15.
14. Leskošek, I. and Stojanović, M.: A possible application of ale brewery strains of *Saccharomyces cerevisiae* in lager beer production, World journal of microbiology & biotechnology, **9** (1993), no. 1, pp. 70-72.
15. Saerens, S.-M.-G.; Verbelen, P.-J.; Vanbeneden, N.; Thevelein, J.-M. and Delvaux, F.-R.: Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast, Appl. Microbiol. Biotechnol., **80** (2008), no. 6, pp. 1039-1051.
16. Vidgren, V.; Multanen, J.-P.; Ruohonen, L. and Londesborough, J.: The temperature dependence of maltose transport in ale and lager strains of brewer's yeast, FEMS Yeast Research, 10 (2010), no. 4, pp. 402-411.
17. Hiralal, L.; Olaniran, A. O. and Pillay, B.: Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions, J. Biosci. Bioeng., **117** (2014), no. 1, pp. 57-64.
18. Parker, N.; James, S.; Dicks, J.; Bond, C.; Nueno-Palop, C.; White, C. and Roberts, I.-N.: Investigating flavour characteristics of British ale yeasts: techniques, resources and opportunities for innovation, Yeast, **32** (2015), no. 1, pp. 281-287.
19. Rittmann, R., 2016: Hier lagern die Schätze der deutschen Brauer. Edited by WeltN24 GmbH, Axel Springer SE (148093400). Available online at http://www.welt.de/sonderthemen/bierreport/article148093400/Hier-lagern-die-Schaetze-der-deutschen-Brauer.html 20.05.2016 artikel vom 27.10.15, checked on 5/20/2016.
20. Josepa, S.; Guillamon, J.-M. and Cano, J.: PCR differentiation of *Saccharomyces cerevisiae* from *Saccharomyces bayanus/Saccharomyces pastorianus* using specific primers, FEMS microbiology letters (2000), no. 193, pp. 255-259.
21. Hutzler, M.: Entwicklung und Optimierung von Methoden zur Identifizierung und Differenzierung von getränkerelevanten Hefen, dissertation, Technische Universität München, Weihenstephan, Lehrstuhl für Technologie der Brauerei 2, 2009.
22. Hutzler, M.; Geiger, E. and Jacob, F.: Use of PCRDHPLC (Polymerase Chain Reaction Denaturing High Performance Liquid Chromatography) for the Rapid Differentiation of Industrial *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* Strains, J. Inst. Brew, **116** (2010), no. 4, pp. 464-474.
23. Brandl, A.: Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien, dissertation, Technische Universität München, Weihenstephan, Lehrstuhl für Technologie der Brauerei 2, 2006.
24. Scherer, A.: Entwicklung von PCR-Methoden zur Klassifizierung industriell genutzter Hefen, dissertation, Technische Universität München, Weihenstephan, Lehrstuhl für Technologie der Brauerei 2, 2003.
25. Kurtzman, C.-P. and Robnett, C.-J.: Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses, FEMS Yeast Research, **3** (2003), no. 4, pp. 417-432.
26. Innis, M.-A.: PCR protocols – A guide to methods and applications, Academic Press, San Diego, 1990.
27. Altschul, S.-F.; Madden, T.-L.; Schäffer, A.-A.; Zhang, J.; Zhang, Z.; Miller, W. and Lipman, D.-J.: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, NuleicAcids Research, **25** (1997), no. 17, pp. 3389-3402.
28. Tamura, K.; Nei, M. and Kumar, S.: Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences of the USA, **30** (2004), no. 101, pp. 11030-11035.
29. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S.: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0, Molecular biology and evolution, **30** (2013), no. 12, pp. 2725-2729.
30. Büchl, N.-R.; Hutzler, M.; Mietke-Hofmann, H.; Wenning, M. and Scherer, S.: Differentiation of probiotic and environmental *Saccharomyces cerevisiae* strains in animal feed, Journal of Applied Microbiology, **109** (2010), no. 3, pp. 783-791.
31. Müller-Auffermann, K.; Caldera, A.; Jacob, F. and Hutzler, M.: Characterization of Different Bottom Fermenting *Saccharomyces pastorianus* Brewing Yeast Strains, BrewingScience – Monatschrift für Brauwissenschaft, **68** (2015), no. 3-4, pp. 46-57.
32. Jones, M. and Pierce, J.-S.: Absorption of amino acids from wort by yeasts, J. Inst. Brew, **2** (1964), no. 70, pp. 307-315.
33. Procopio, S.; Brunner, M. and Becker, T.: Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation, Eur Food Res Technol, **239** (2014), no. 3, pp. 421-439.
34. Bühligen, F.; Sträuber, H.; Ondruschka, J.; Harms, H. and Müller, S.: Monitoring von Bruch- und Staubhefeanteilen in Brauprozessen – Prozessoptimierung, BRAUWELT (2010), no. 1-2, pp. 18-21.
35. Annemüller, G. and Manger, H.-J.: Gärung und Reifung des Bieres - Grundlagen – Technologie – Anlagentechnik (VLB-Fachbücher), 2nd ed., VLB, Berlin, 2013.
36. European Bioinformatic Institut Cambridge, 2010. Available online at http://www.ebi.ac.uk/interpro/IEntry?ac=IPR004507, checked on 11/15/2016.
37. Richard, P.; Viljanen, K. and Penttilä, M.: Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*, AMB Express, **5** (2015), no. 12, pp. 2-5.
38. Mukai, N.; Masaki, K.; Fujii, T. and Lefuji, H.: Single nucleotide polymorphisms of PAD1 and FDC1 show a positive relationship with ferulic acid decarboxylation ability among industrial yeasts used in alcoholic beverage production, J. Biosci. Bioeng., **118** (2014), no. 1, pp. 50-55.
39. Meilgaard, M.-C.: Flavour chemistry of beer. Part 2. Flavour and threshold of 239 aroma volatiles, Technical Quarterly Master Brewers Association of the Americans, **12** (1975), no. 3, pp. 151-168.
40. Lodolo, E.-J.; Kock, J.-L.-F.; Axcell, B.-C. and Brooks, M.: The yeast *Saccharomyces cerevisiae* – the main character in beer brewing, FEMS Yeast Research, **8** (2008), no. 7, pp. 1018-1036.
41. Swiegers, J.-H. and Pretorius, I.-S.: Modulation of volatile sulfur compounds by wine yeast, Appl. Microbiol. Biotechnol., **74** (2007), no. 5, pp. 954-960.
42. Back, W.: Bedeutung der Hefetechnolgie für die Bierqualität, 48. Technologisches Seminar Weihenstephan, Technische Universität München, Weihenstephan, 2015.
43. Busch-Stockfisch, M.: Praxishandbuch Sensorik in der Produktentwicklung und Qualitätssicherung, Behr, Hamburg, 2002.
44. Ptach, C.: Statistische Methoden in der Sensorik (Teil 1): „Analytische Prüfungen", DLG-Arbeitsblätter Sensorik (2011), no. 1, pp. 1-4.

Received 16. December 2016, accepted 5. February 2017

Part 2 Page 45 - 69	2.3 The importance of a comparative characterization of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* strains for brewing

The beer aroma is primarily influenced by the yeast strain used. The yeast's specific metabolism means that it can produce about 500 different flavor and flavor compounds (181). For decades, only a few domesticated yeast strains with selected phenotypic brewing properties have been used for beer production worldwide. In addition to these established and widespread culture yeast strains, approximately 300 brewing yeast strains can be obtained from different providers and these strains differ in their flavor profile and their phenotypic properties, which influences the brewing process. Comparable and reliable results in terms of key technological and sensory properties of individual strains are necessary to improve brewing efficiency and beer diversity. The results can be used to offer brewers a targeted and simplified selection of brewing yeasts adapted to their technological and product-specific needs. For this purpose, ten commercial brewing culture strains of the Technical University of Munich (TUM), including eight top-fermenting ale strains and two bottom-fermenting lager strains, were investigated using the developed characterization platform presented in publication 1 (see pages 28-45).

All ten TUM yeast strains showed different fermentation rates and degrees of apparent attenuation, which can be explained by their different ability to ferment maltotriose. Further differences between single strains could be shown in their total amino acid utilization, the ability to build phenolic off-flavors, the production of fermentation by-products, and the resulting flavor composition in the finished beers. All strains showed a specific flocculation behavior and not every top-fermenting yeast strain demonstrated powdery behavior. Frisinga-TUM 34/70® and LeoBavaricus-TUM 68® showed the best phenotypic characteristics and stood out from the other investigated yeast strains.

Authors/Authorship contribution:

Meier-Dörnberg, T.: Designed and performed the experiments and analyzed the data; **Hutzler, M.**: Contributed reagents/materials/analysis tools, designed primer and probes for real-time PCR and DNA-based strain differentiation; **Meier-Dörnberg T. and Hutzler, M.**: Wrote the paper; **Michel, M. and Methner, F.-J.**: Revised the conception and manuscript, and agreed on submission; **Jacob, F.**: Supervised the project.

Article

The Importance of a Comparative Characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* Strains for Brewing

Tim Meier-Dörnberg [1,†], Mathias Hutzler [1,*,†], Maximilian Michel [1], Frank-Jürgen Methner [2] and Fritz Jacob [1]

[1] Research Center Weihenstephan for Brewing and Food Quality, Technische Universität München, Alte Akademie 3, 85354 Freising, Germany; timmd@tum.de (T.M.-D.); m.michel@tum.de (M.M.); f.jacob@tum.de (F.J.)
[2] Technische Universität Berlin, Seestraße 13, 13353 Berlin, Germany; frank-juergen.methner@tu-berlin.de
* Correspondence: m.hutzler@tum.de; Tel.: +49-8161-71-3100
† These authors contributed equally to this work.

Received: 1 August 2017; Accepted: 17 August 2017; Published: 21 August 2017

Abstract: The volume and market share loss for classical beer types such as pils beer and wheat beer has been declining for several years, but the overall beer market remains almost unchanged as a result of the increasing interest in beer specialties Due to high biodiversity, the diversity of the strains, and the different flavor profiles, reliable and practical information regarding the characteristics of individual brewing strains is required to help brewers to find the right strain for their brewing purposes. This paper presents a comparison of 10 commercially available Technical University of Munich (TUM) brewing yeast strains. The strains were screened for genetic and phenotypic characteristics. After confirming the genetic distinctiveness by using species-specific real-time polymerase chain reaction (RT-PCR) systems and a strain typing method based on PCR-capillary electrophoresis of the partial intergenic spacer 2 (IGS2) fragment (IGS2-314 PCR-capillary electrophoresis), the strains were tested regarding phenotypic characteristics under controlled and identical fermentation conditions in small-scale brewing trials. Besides the fermentation performance, flocculation behavior, sugar metabolism and other phenotypic characteristics, the main focus was on the flavor and aroma profile of each investigated TUM yeast strain.

Keywords: *Saccharomyces pastorianus*; *Saccharomyces cerevisiae*; brewing yeasts; yeast characterization; top-fermenting yeast strains; bottom-fermenting yeast strains; culture yeast; brewing trials

1. Introduction

The Bavarian purity law of 1516 only permits barley, hops and water to be used to produce beer [1]. In 1993, the purity law was further clarified by the German "vorläufiges Biergesetz" (German preliminary beer law) and expanded in accordance with public perception that beer can only be produced using malt, hops and water. Even 477 years after the Bavarian purity law, there is still a lack of attention devoted to yeast as a raw brewing ingredient. Nowadays there is an increasing focus on yeast. MEIER-DÖRNBERG describes yeast as the flavor engine of the brewing industry [2,3]. Yeast metabolism during the fermentation and ripening process gives rise to approximately 80% of all aroma-active compounds in beer, thereby determining its aroma profile [4]. There are more than 300 different volatile and non-volatile fermentation by-products, which vary in their concentration from strain to strain. According to WHITE and ZAINASHEFF, brewer's yeast alone is able to produce about 500 different flavor and aroma compounds [5]. Yeast strains are as diverse as the resulting

flavors, and the choice of yeast strain is therefore directly linked to the individual and special flavors created when developing new beer types and styles.

To date, approximately 1,500 yeast species have been reported [6]. The most important yeast species for fermentation technology belong to the *Saccharomyces* genus and are taxonomically grouped in the *Saccharomyces sensu stricto* complex [7,8]. The *Saccharomyces sensu stricto* complex consists of *Saccharomyces cerevisiae*—the yeast used to produce top-fermented beers (often referred to as "ale"), wine, distillers' mash, sake and many other alcoholic beverages, *Saccharomyces bayanus*—used in wine, cider, cidre and apple wine production, and *Saccharomyces pastorianus*—the starter culture for bottom-fermented beer (lager) and apple wine production, as well as additional species (*S. cariocanus*, *S. jurei*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, *S. arboricolus* and *S. eubayanus*) which are not used industrially [7,9–13]. LIBKIND et al. published that the bottom-fermenting strains of the species *Saccharomyces pastorianus* used in the lager beer production are genetic hybrids of *Saccharomyces cerevisiae* and the Patagonian wild yeast *Saccharomyces eubayanus* [12]. DUNN and SHERLOCK claimed that there were at least two hybridization events and that all *Saccharomyces pastorianus* lager strains consist of at least two types [14]. The *S. pastorianus* strains they studied were divided into the groups Saaz and Frohberg. Some industrial strains exhibiting strong fermentation performance belong to the Frohberg group. PERES-TRAVEZ et al. investigated that *S. bayanus* strains are also hybrids, whereas the parental species are of an European linage of *S. eubayanus*-like strains and *S. bayanus* var. *uvarum* (*S. uvarum*) [15]. Rapid species identification within the *Saccharomyces sensu stricto* group plays a key role in verifying the purity of a species in a beer starter culture, and for detecting cross contamination. Some special beer styles also use non-*Saccharomyces* yeast species as starter cultures. *Schizosaccharomyces pombe* is found in some traditional African beers, and *Dekkera bruxellensis* in Belgian beers and in German Berliner Weiße. *Saccharomycodes ludwigii* is used to produce low-alcohol and non-alcoholic beer styles, while in the production of top-fermented wheat beer, *Torulaspora delbrueckii* can be used as a supplemental yeast strain to create a distinctive fruity aroma [16–18]. Spontaneous beer fermentation may utilize other non-*Saccharomyces* species such as *Debaryomyces* spp., *Meyerozyma guilliermondii*, *Pichia membranifaciens*, *Candida friedrichii*, *Naumovia castellii*, *Dekkera anomala*, and *Priceomyces* spp. in lambic beer [19], and *Cryptococcus keutzingii*, *Rhodotorula mucilaginosa*, *Candida krusei*, *Pichia fermentans*, and *Pichia opuntiae* in American coolship ale [20].

A brewing yeast strain should be taxonomically classified at the species and strain level using molecular biological methods. It should also be characterized in terms of its propagation and fermentation performance as well as its aroma profile. The Frisinga-TUM 34/70® strain is one of the most abundant lager yeast strains in the brewing industry, and is the reference lager strain when comparing the fermentation performance and final pure beer flavor of other lager strains. The genome of Frisinga-TUM 34/70® was also the first of the bottom-fermenting strains to be sequenced and published [21]. A study conducted by MÜLLER-AUFFERMANN used the characteristics of TUM 34/70 as a reference to develop a method to rapidly compare the performance of lager yeast strains [22]. MEIER-DÖRNBERG further optimized small-scale fermentation vessels used to conduct these trials, as well as the fermentation to make it possible to directly compare the investigated yeast strains [23]. These kinds of trials are very useful for breweries wishing to either replace their yeast strain or to introduce a second one to develop specialty beers with specific properties or to modify or improve existing beer styles.

The market share for such beer specialties is steadily increasing. The craft beer movement and the continuing interest in the variety of flavors mean that regional brewers in particular are benefiting from their willingness to experiment with a variety of different yeast strains. While the volume and market share loss of classical beer types such as pils beer and wheat beer has been declining for several years in Germany, the overall beer market remains practically the same [24]. The greater diversity of yeast that can be applied in brewing, along with an improved understanding of yeasts' evolutionary history and biology, is expected have a significant and direct impact on the brewing industry, with potential for improved brewing efficiency, product diversity and, above all, customer

satisfaction [25]. A lot of specialty beers with distinctive flavors are appearing on the market, especially for beers fermented with top-fermenting *Saccharomyces cerevisiae* strains. These strains produce intense flavors, and they are the focus of many craft- and microbreweries. They include Bavarian wheat beer, ales and Belgian specialty beers. The broad biodiversity and availability of different strains of *Saccharomyces cerevisiae* offer brewers a wealth of possibilities to create beers with unique attributes and flavor profiles. Descriptions of these top-fermenting specialty yeast strains are therefore of considerable importance when selecting suitable strains in the development of special products. The Bavarian wheat beer strain *Saccharomyces cerevisiae* LeoBavaricus-TUM 68® is a perfect example of a successful specialist yeast strain. It is phenolic-off-flavor (POF)-positive. Depending on the production process, Bavarian wheat beers can exhibit very strong fruity, clove like, estery flavors or a more neutral, yeasty, top-fermented character with a decent fruity note, or they can fall somewhere in between the two. The finished beer aroma is determined in part by the strain and how it is handled as well as by the process parameters. SCHNEIDERBANGER recently described the impact of the different wheat beer yeast strains on fermentation performance, and their respective aroma profiles [26]. SCHNEIDERBANGER et al. found that the Bavarian wheat beer strain LunaBavaria-TUM 127® used to ferment the first batch does not ferment maltotriose. This produces a different mouthfeel and aroma to wheat beer strains without this maltotriose gap [27]. Very different sensory impressions can be achieved by using other top-fermenting yeasts, as MEIER-DÖRNBERG recently showed. By investigating five different ale yeast isolates under the same fermentation and substrate conditions, entirely different and often surprising flavors could be identified in the finished beers. One yeast strain created citrus and fruity beer notes, whereas a second produced more floral flavors [23]. Describing both existing and new brewing yeast strains will help us understand their characteristics, and will pave the way for innovative brewers around the world to experiment and create novel products for the beer market. There is a more or less infinite potential for increasing biodiversity among brewing yeast strains.

2. Materials and Methods

The following methods were performed according to [23].

2.1. Yeast Isolates and Strains

A total of ten culture yeast strains were obtained in agar slants from the Yeast Center of the Research Center Weihenstephan for Brewing and Food Quality (BLQ) of the Technical University of Munich (TUM). These ten culture yeast strains included two bottom-fermenting *Saccharomyces pastorianus* and eight top-fermenting *Saccharomyces cerevisiae* brewing yeast strains commonly used to produce beer styles dependent on the industrial applications listed in the following Table 1.

Table 1. Technical University of Munich (TUM) culture yeast strains for industrial brewing.

TUM Yeast Strains			
TUM Yeast Strain	**Yeast Species**	**Industrial Application**	**Origin**
Frisinga-TUM 34/70®	*Saccharomyces pastorianus*	lager beer production	Freising-Weihenstephan, Germany
Securitas-TUM 193®	*Saccharomyces pastorianus*	lager beer production	Freising-Weihenstephan, Germany
LeoBavaricus-TUM 68®	*Saccharomyces cerevisiae*	wheat beer production	Freising-Weihenstephan, Germany
LunaBavara-TUM 127®	*Saccharomyces cerevisiae*	wheat beer production	Freising-Weihenstephan, Germany
Colonia-TUM 177®	*Saccharomyces cerevisiae*	kölsch beer production	Krefeld, Germany
Vetus-TUM 184®	*Saccharomyces cerevisiae*	alt beer production	Dusseldorf, Germany
Mel-TUM 211®	*Saccharomyces cerevisiae*	ale and stout beer production	region unknown, Great Britain
Monacus-TUM 381®	*Saccharomyces cerevisiae*	trappist beer production	region unknown, Germany
Tropicus-TUM 506®	*Saccharomyces cerevisiae*	ale beer production	region unknown, Great Britain
Harmonia-TUM 511®	*Saccharomyces cerevisiae*	ale beer production	region unknown, United States of America

2.2. Genetic Identification and Strain Determination

To confirm the genetic distinctiveness of each obtained TUM brewing yeast strain, a real-time polymerase chain reaction (RT-PCR) and a strain typing method based on a PCR-capillary

electrophoresis of partial intergenic spacer 2 (IGS2) fragment (IGS2-314 PCR-capillary electrophoresis) were used according to HUTZLER [28,29]. The RT-PCR was used in each case to identify the species the strain belonged to, and IGS2-314 PCR-capillary electrophoresis was used to confirm that the investigated yeast cultures of the same species represent different strains.

2.2.1. Real-Time Polymerase Chain Reaction

RT-PCR (Light Cycler® 480 II. Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used to taxonomically classify the brewing yeast strains in species level. The primer and TaqMan® probe sequences used are listed in Table 2, and the RT-PCR procedure followed that of HUTZLER [28,29]. All RT-PCR systems listed in Table 2 are compatible and were performed using 10 µL 2x Mastermix (Light Cycler® 480 Probe Master, Roche, Germany), 1.4 µL dd (double distilled) H_2O PCR water, 0.8 µL (400 nM) of each primer (Biomers, Ulm, Germany), 0.4 µL (200 nM) probe (Biomers, Ulm, Germany; MGB probe from ThermoFisher scientific, Applied Biosystems®, USA), 0.5 µL IAC135-f (250 nM), 0.5 µL IAC135-r (250 nM), 0.4 µL IAC135-S (HEX) (200 nM), 0.1 µL IAC135 (dilution $1{:}10^{-13}$), 0.1 µL IAC135 rev (dilution $1{:}10^{-13}$), and 5 µL template DNA, with a total reaction volume of 20 µL, using the same temperature protocol: 95 °C/10 min; 40 cycles of 95 °C/10 s, 60 °C/55 s; 20 °C. IAC135 was developed by RIEDL at the Research Center Weihenstephan for Brewing and Food Quality of the Technical University Munich and is listed in Table 3. IAC (internal amplification control) is a control to confirm that the PCR reaction itself took place. If IAC is negative, the reaction has to be repeated. The yeast strains *S. cerevisiae* (LeoBavaricus-TUM 68®) and *S. pastorianus* (Frisinga-TUM 34/70®) were used as positive and negative controls respectively, according to the RT-PCR system tested.

Table 2. Qualitative real-time PCR systems for brewing yeast species differentiation [30,31].

Real-Time PCR Systems, Primer and Probe Sequences (5′–3′)	System Name	Reference	*S. cer.*	*S. cer.* var. *dia.*	*S. past.*
Sbp-f CTTGCTATTCCAAACAGTGAGACT Sbp-r1 TTGTTACCTCTGGGCGTCGA Sbp-r2 GTTTGTTACCTCTGGGCTCG Sbp ACTTTTGCAACTTTTTCTTTGGGTTTCGAGCA	Sbp	[32,33]	−	−	+
Sc-f CAAACGGTGAGAGATTTCTGTGC Sc-r GATAAAATTGTTTGTGTTTGTTACCTCTG Scer FAM-ACACTGTGGAATTTTCATATCTTTGCAACTT-BHQ1	Sce	[32,33]	+	+	+
Sc-GRC-f CACATCACTACGAGATGCATATGCA Sc-GRC-r GCCAGTATTTTGAATGTTCTCAGTTG Sc-GRC FAM-TCCAGCCCATAGTCTGAACCACACCTTATCT-BHQ1	Sc-GRC3	[30]	+	+	+
TF-f TTCGTTGTAACAGCTGCTGATGT TF-r ACCAGGAGTAGCATCAACTTTAATACC TF-MGB FAM-ATGATTTTGCTATCCCAAGTT-BHQ1 (MGB probe)	TF-COXII	[30]	+	+	−
BF300E CTCCTTGGCTTGTCGAA BF300M GGTTGTTGCTGAAGTTGAGA BF300 FAM-TGCTCCACATTTGATCAGCGCCA-BHQ1	BF-300	[32]	−	−	+
BF-LRE-f ACTCGACATTCAACTACAAGAGTAAAATTT BF-LRE-r TCTCCGGCATATCCTTCATCA BF-LRE FAM-ATCTCTACCGTTTTCGGTCACCGGC-BHQ1	BF-LRE1	[30]	−	−	+
Sd-f TTCCAACTGCACTAGTTCCTAGAGG Sd-r GAGCTGAATGGAGTTGAAGATGG Sdia FAM-CCTCCTCTAGCAACATCACTTCCTCCG-BHQ1	Sdia	[32]	−	+	−

Positive (+), negative (−).

Table 3. Primer, probe and target DNA sequences of the internal amplification control system (IAC135) used for real-time PCR systems.

Real-Time PCR Internal Amplification Control (IAC135)		
System Name	**Primer**	**Primer Sequence (5′–3′)**
IAC135	IAC135-f IAC135-r	TGGATAGATTCGATGACCCTAGAAC TGAGTCCATTTTCGCAGATAACTT
	Probe	**Probe Sequence (5′–3′)**
	IAC135-S	HEX-TGGGAGGATGCATTAGGAGCATTGTAAGAGAG-BHQ1
	Target DNA	**DNA Sequence (5′–3′)**
	IAC135	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGGGAGGATGCATT AGGAGCATTGTAAGAGAGTCGGAAGTTATCTGCGAAAATGGACTCATTCGA GTGGCCTATTGACGGTCGCCCAAGGTGTCGCA
	IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCATTTTCGC AGATAACTTCCGACTCTCTTACAATGCTCCTAATGCATCCTCCCACTAGTTCTA GGGTCATCGAATCTATCCATTCTCTAGCA

2.2.2. DNA Fingerprinting (PCR-Capillary Electrophoresis of the IGS2-314 Fragment)

In order to determine that the TUM brewing yeasts represented different strains, genetic fingerprints were generated using the IGS2-314 method [29]. The IGS2 is a spacer region within the ribosomal cluster. To amplify a partial sequence of the intergenic spacer 2 (IGS2-314) the specific primers IGS2-314f (5′-CGGGTAACCCAGTTCCTCACT-3′) and IGS2-314r (5′-GTAGCATATATTTCTTGTGTGAGAAAGGT-3′) (Biomers GmbH, Ulm, Germany) [34] were used at a concentration of 600 nM as described by HUTZLER [28]. PCR was performed with 22.5 µL RedTaq Mastermix (2x) (Genaxxon, Ulm, Germany) and 2.5 µL template DNA, with a total reaction volume of 25 µL. The Mastermix contained 12.5 µL buffer solution (RedTaq Mastermix), 7.0 µL DNA-free PCR water and 1.5 µL of each primer (Biomers, Munich, Germany). Cycling parameters were: A pre-denaturing step at 95 °C for 300 s, then 35 cycles for denaturing at 95 °C for 30 s, for annealing and elongation at 54 °C for 30 s and 72 °C for 40 s and for final elongation at 72 °C for 300 s. PCR was performed using a SensoQuest LabCycler48s (SensoQuest GmbH, Gottingen, Germany). Amplified fragments were analyzed using a capillary electrophoresis system (Agilent DNA 1000 kit) following the manufacturer's recommendations (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).

2.3. Phenolic Off-Flavor Test

TUM yeast culture strains were taken from wort agar slopes and spread on a yeasts and mold agar plate (YM-agar) containing one of the following precursors: ferulic acid, cinnamic acid and coumaric acid. After three days of incubation at 24 °C, the three single agar plates per yeast isolate were evaluated by sniffing to detect any of the following aromas: ferulic acid becomes 4-vinylguajacol (4-VG, clove-like), cinnamic acid becomes 4-vinylstyrene (4-VS, styrofoam-like) and coumaric acid becomes 4-vinylphenol (4-VP, medicinal-like).

For the YM-agar plates a YM-media was prepared by adding distilled water to 3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone, 11.0 g glucose monohydrate, and 20.0 g agar to 1000 mL, and autoclaved. After autoclaving, an aliquot of the following stock solutions was added to the YM-media at 45–50 °C under sterile conditions. For the stock solution of coumaric acid, 100 mg of the instant were dissolved in 10 mL of 96% (*v/v*) ethanol. The stock solution of ferulic and cinnamic acid was made by dissolving 1 g in 20 mL of 96% (*v/v*) ethanol. 10 mL coumaric acid, 2 mL ferulic acid or 2 mL cinnamic acid stock solution was added for 1000 mL YM-media.

2.4. Brewing Trials

2.4.1. Wort

The wort characteristics used for propagation and the brewing trials are shown in Table 4. The wort was based on hopped barley malt concentrate (N53940; Döhler GmbH, Darmstadt, Germany). To achieve an original gravity of 12.4 °P, wort concentrate was diluted with distilled water and boiled for 5 min to guarantee sterile conditions. The same wort batch preparation was used for the propagation and brewing trials to ensure constant wort composition. Free alpha-amino nitrogen was quantified using the MEBAK II. 2.8.4.1 method. Sugar composition was determined using the HPLC MEBAK II. 3.2.2.1.2 method.

Table 4. Starting wort composition used for propagation and brewing trials (12.4 °P wort).

Wort composition	
Parameter	**Amount**
Original gravity (°P)	12.40
pH	5.19
Spec. weight SL 20/20 °C	1.05
Zinc (mg/L)	0.15
Free amino nitrogen (FAN) (mg/100 mL)	25.00
Total amino acid content (AS) (mg/100 mL)	203.22
Total sugar (g/L)	83.78
EBC-Bittering units	20.20
Glucose (g/L)	11.46
Fructose (g/L)	2.57
Saccharose (g/L)	1.12
Maltose (g/L)	53.65
Maltotriose (g/L)	14.98

2.4.2. Propagation

In order to propagate yeasts, yeast strains were inoculated from agar slants (yeast pure culture) into 60 mL of sterile wort medium in an 100 mL Erlenmeyer flask and incubated for 72 h at ambient temperature (20 °C) and pressure, then agitated at 80 rpm using a WiseShake 207 orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, yeasts were transferred to 4 kg of sterile wort medium and further propagated at the same conditions for an additional 72 hours. After allowing six hours for sedimentation, the supernatant was decanted and 2 kg of sterile wort medium at pitching temperature (20 °C) was added to the yeast sediment in each container. The yeast concentration was determined in terms of cells/g using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area of 0.00025 m^3 per square (Brand GmbH&Co.KG, Wertheim, Germany).

2.4.3. Fermentation

Laboratory-scale brewing trials were performed using stainless steel vessels with dimensions of 10 cm diameter x 33 cm height (2.5 liters) with 20% headspace and clamped down lids, according to MEIER-DÖRNBERG [23]. The vessels were placed in a tempered cooling chamber (2023 Minicoldlab, LKB-Produkter AB, Bromma, Sweden) to guarantee a constant fermentation temperature. To imitate industrial brewery conditions during fermentation, a head pressure of 0.5 bar was applied to simulate a liquid height of 10 m (median hydrostatic pressure). Brewing trials were evaluated by pitching 8.5 L wort per yeast strain. Each batch was then divided into four fermentation vessels. By having four vessels, samples could be taken daily from one of the four vessels to estimate the specific gravity, cells in suspension and pH, while the other three vessels remained undisturbed. Yeast strains were added at an inoculation rate of 15 million cells/g of homogeneous mixed wort medium for the top-fermenting *Saccharomyces cerevisiae* yeast strains (LeoBavaricus-TUM 68®, LunaBavaria-TUM

127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506®, and Harmonia-TUM 511®) and an inoculation rate of 30 million cells/g of homogeneous mixed wort medium for bottom-fermenting *Saccharomyces pastorianus* yeast strains (Frisinga-TUM 34/70® and Securitas-TUM 193®). The wort was not oxygenated. Primary fermentation was maintained at 20 °C for the top-fermenting and 15 °C for the bottom fermenting TUM yeast strains. Fermentation was considered complete once the specific gravity remained constant for two consecutive days. An additional five days for maturation was given following primary fermentation at the same fermentation temperature, and seven days for lagering at 0 °C. The beers were then removed from the fermentation vessels, homogenized, and collected in sterile bottles. The specific gravity and pH of the samples were determined from the filtered fermentation samples using a DMA 35N (Anton-Paar GmbH, Graz, Austria) for specific gravity and a pH3210 (WZW, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) for pH measurement. The samples were filtered using Whatman® folded filter paper with a diameter of 320 mm (GE Healthcare Europe GmbH, Freiburg, Germany).

2.5. Analytical Methods

After lagering, the finished beers were analyzed for physical and chemical attributes, which included the following parameters: ethanol, pH, specific gravity, degree of attenuation, free amino nitrogen, amino acid composition, sugar composition, total SO_2, free and total dimethylsulfide, free vicinal diketones and the concentration of fermentation by-products. Ethanol, pH, specific gravity, and degree of attenuation were measured using an Anton Paar DMA 5000 Density Meter with Alcolyzer Plus measuring module, pH measuring module, and Xsample 122 sample changer (Anton-Paar GmbH, Ostfildern, Germany). Free amino nitrogen and amino acid composition were quantified using the HPLC MEBAK II (2.8.4.1) method. Residual sugar composition was determined using the HPLC MEBAK II (3.2.2.1.2) method. Total SO_2, free and total dimethylsulfide, and free vicinal diketones were quantified using a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham, MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 µm column using a 2.3-hexandione internal standard. The final concentrations of fermentation by-products (e.g., acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols, 4-vinylguajacol, diacetyl, 2.3-pentandione) were measured according to MEBAK II (3.2.21) methods using a gas chromatograph with a headspace unit, and an INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm, 0.5 µm column (Perkin-Elmer, Waltham, MA, USA).

2.5.1. Determining the Cell Count (Cells in Suspension and Total Cell Count)

Cell counts for pitched yeast, cells in suspension until lagering, and total cell count after lagering were determined using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area of 0.00025 m^3 per square (Brand GmbH&Co.KG, Wertheim, Germany). Cells in suspension were analyzed every 24 h up to the start of lagering. To ensure cell count accuracy during fermentation and maturation, 20 mL of green beer was removed from the middle of the fermentation vessel by using a 10 mL volumetric pipette mounted on a stand. Prior to sampling, the head pressure in the vessel was released very slowly so that the cells in suspension were not affected by a pressure surge. The total cell count was determined after the lagering phase. Beers were removed from the fermentation vessels, and the decanted yeast masses were collected by suspending the yeast cells in a total of 50 g distilled water. The yeast cells were washed by centrifugation twice with 50 g distilled H_2O (5 min at 3000 rpm) and resuspended with distilled water up to a total of 100 g. Afterwards, distilled water was added to 1 g of the homogenous yeast suspension to make up to 100 mL. Total cell counts were determined in cells/g using the Thoma cell counting chamber.

2.6. Sensory Evaluation

Three single sensory tests were conducted which included: expected beer type test, DLG-scheme for beer (Deutsche Landwirtschafts-Gesellschaft) and a descriptive sensory evaluation. All beer samples were tasted and evaluated by a sensory panel of seven DLG-certified tasters with long-standing experience in the sensory analysis of beer at the Weihenstephan Research Center for Brewing and Food Quality. Accredited sensory evaluations were performed according to DIN EN 17025. Sensory evaluations were performed in individual walled tasting stations under controlled environmental conditions. Samples were provided in triplicate sets for all beers in dark glasses, each with a three digit code. All samples were served at 12 °C to guarantee optimal conditions to investigate the predominant flavor diversity. At first the panelists associated the beer samples with their expected beer type (e.g., ale, wheat-, Kölsch-, Alt-, stout, Berliner Weisse, porter-, lager-; Bock-; Märzen-, Rauch-, Schwarz-, Dunkles-, malt beer) followed by an examination of the beer samples according to the DLG-scheme. Secondly, a descriptive sensory evaluation was conducted during which trained panelists described specific flavors. Seven main categories were described (e.g., sweet, tropical fruity, fruity (other fruits), citrus, spicy, floral and other flavors). Every category was evaluated from 0, meaning not noticeable, to 5, extremely noticeable.

3. Results

3.1. Genetic Analysis

3.1.1. Real-Time PCR Assays and IGS2-314 PCR-Capillary Electrophoresis

RT-PCR results confirmed the species identity of the investigated TUM yeast strains of the TUM Yeast Center. In addition, the results of the DNA fingerprinting showed unique banding patterns, also confirming that each yeast represents a genetically different strain (Figure 1). Table 5 shows that the tested RT-PCR systems and the obtained results for all strains. All yeast strains were positive for the Sc-GRC3 and Sce loci. The RT-PCR systems Sc-GRC3 and Sce have positive signals when *S. cerevisiae* DNA is measured or the DNA of hybrid strains that contain these DNA loci. The top-fermenting yeast strains LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511® were also positive for the TF-COXII locus, suggesting that they belong to the *S. cerevisiae* strains. The bottom-fermenting yeast strains Frisinga-TUM 34/70® and Securitas-TUM 193® were also positive for loci that correlate with the PCR systems Sbp, BF-LRE1 and BF-300, which detect *S. bayanus*/*S. pastorianus* strains. In addition, all investigated TUM yeast strains were negative for the RT-PCR system Sdia which detects *S. cerevisiae* var. *diastaticus* strains.

Table 5. Qualitative results of the real-time PCR systems used for the investigated yeast strains and the reference strains to differentiate *Saccharomyces sensu stricto* species.

		RT-PCR-System						
Species Confirmation	**TUM Yeast Strain**	**Sc-GRC3**	**Sce**	**TF-COXII**	**Sbp**	**BF-LRE1**	**BF-300**	**Sdia**
S. pastorianus (*S. cerevisiae* hybrid strain)	Frisinga-TUM 34/70®	+	+	−	+	+	+	−
	Securitas-TUM 193®	+	+	−	+	+	+	−
S. cerevisiae	LeoBavaricus-TUM 68®	+	+	+	−	−	−	−
	LunaBavaricus-TUM 127®	+	+	+	−	−	−	−
	Colonia-TUM 177®	+	+	+	−	−	−	−
	Vetus-TUM 184®	+	+	+	−	−	−	−
	Mel-TUM 211®	+	+	+	−	−	−	−
	Monacus-TUM 381®	+	+	+	−	−	−	−
	Tropicus-TUM 506®	+	+	+	−	−	−	−
	Harmonia-TUM 511®	+	+	+	−	−	−	−

Positive (+), negative (−).

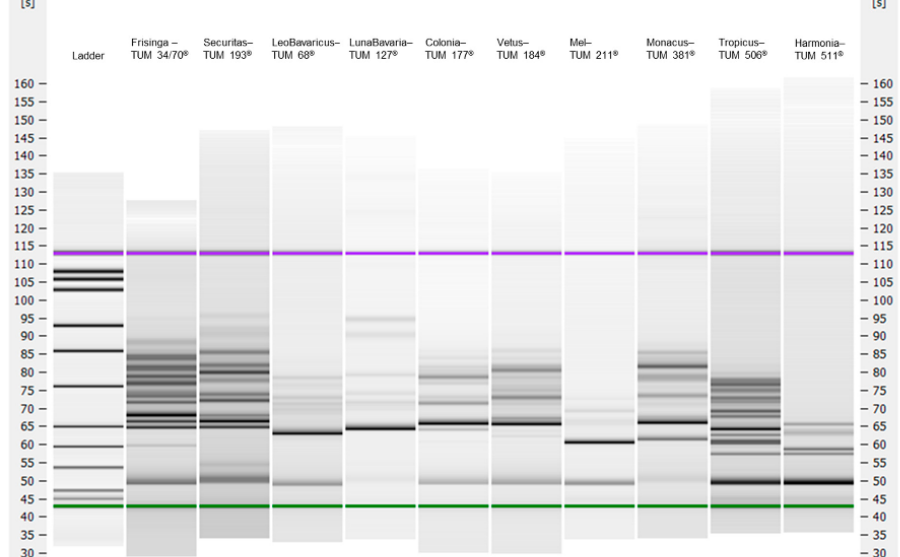


Figure 1. Capillary electrophoresis IGS2-314 ribosomal DNA (rDNA) patterns for the investigated yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; lower and upper internal marker are shown in green and purple.

3.2. Brewing Trials

3.2.1. Sugar Utilization

As Table 6 shows, not all of the strains were able to metabolize all major wort sugars (e.g., glucose, fructose, sucrose, maltose, maltotriose). Maltotriose could not be utilized by all strains. The top-fermenting yeast strain LunaBavaria-TUM 127® could not ferment maltotriose (1.05%), while the culture yeast strains Mel-TUM 211®, Tropicus-TUM 506® and Vetus-TUM 184® only fermented a small amount of maltotriose (26.66%, 59.28% and 60.92%). Variations in the maltotriose utilization for all other strains were above 83%. The results suggested that the utilization degree as well as the ability to utilize maltotriose is strain dependent.

Table 6. Mean percentage of total wort sugar utilization in beer, measured in triplicate after lagering; confidence level 95%.

TUM Yeast Strain	Glucose	Fructose	Sucrose	Maltose	Maltotriose
Frisinga-TUM 34/70®	98.61 ± 0.00	96.15 ± 0.00	100.00 ± 0.00	99.02 ± 0.12	94.06 ± 0.91
Securitas-TUM 193®	98.70 ± 0.14	96.15 ± 0.00	100.00 ± 0.00	96.89 ± 0.12	86.60 ± 0.52
LeoBavaricus-TUM 68®	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.92 ± 0.02	99.65 ± 0.28
LunaBavaria-TUM 127®	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.07 ± 0.10	01.05 ± 2.89
Colonia-TUM 177®	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.85 ± 0.02	94.80 ± 0.78
Vetus-TUM 184®	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	98.00 ± 0.53	60.92 ± 5.87
Mel-TUM 211®	98.55 ± 0.47	98.57 ± 0.21	98.51 ± 0.48	87.23 ± 0.82	26.66 ± 0.26
Monacus-TUM 381®	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.65 ± 0.14	97.75 ± 0.09
Tropicus-TUM 506®	98.23 ± 0.12	98.05 ± 0.36	99.11 ± 0.00	98.48 ± 0.93	59.28 ± 0.81
Harmonia-TUM 511®	99.42 ± 0.12	98.05 ± 0.00	95.54 ± 0.00	99.28 ± 0.09	83.91 ± 0.71

3.2.2. Amino Acid Utilization

The mean amino acid uptake in the finished beers after lagering using the investigated *S. pastorianus* and *S. cerevisiae* yeast strains is shown in Supplementary Material Tables S1 and S2. The commonly accepted amino acid uptake classification is indicated with shading according to JONES and PIERCE [22,23,35,36].

As shown in Tables S1 and S2, the total amino acid utilization followed no defined process and was different for each of the investigated TUM yeast strains. As MEIER-DÖRNBERG previously described using five tested *S. cerevisiae* ale yeast strains, the exact course of absorption and the sequence varies, even if specific amino acids were preferred by the yeast [23]. However, in contrast to the tested top-fermenting *S. cerevisiae* ale yeast strains, the bottom-fermenting *S. pastorianus* strains metabolized the single amino acids in a similar order. This might depend on the same industrial application (lager and export beer production) for which both strains were commonly used. Fermentation conditions are similar between lager and export beer production, and the Frisinga-TUM 34/70® and Securitas-TUM 193® strains may adapt to the same circumstances and react with similar cell metabolisms, even if they are genetically different and show unique sensory profiles. Figures 2 and 3 show the free amino nitrogen (FAN) and the total amino acid (AS) utilization of each yeast strain in comparison with the corresponding residual contents. The utilization rate of FAN and AS was correlated for the same yeast strain, but different across strains.

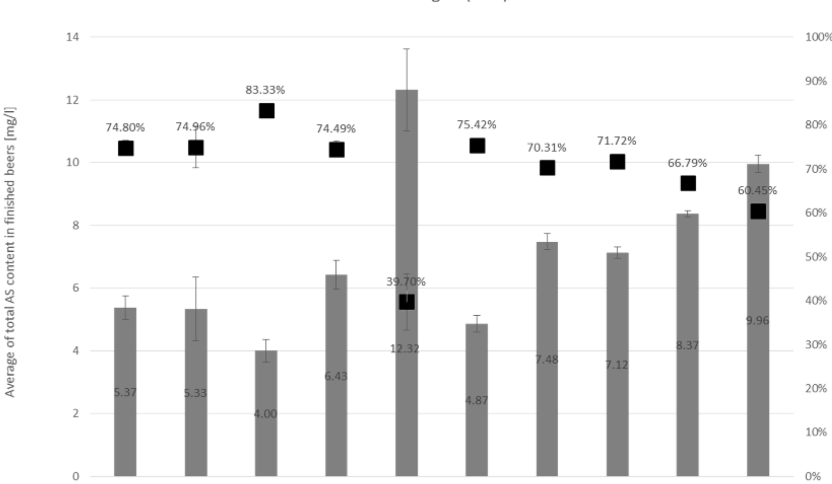


Figure 2. Average of metabolized and free amino nitrogen (FAN) content in finished beers produced with the yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

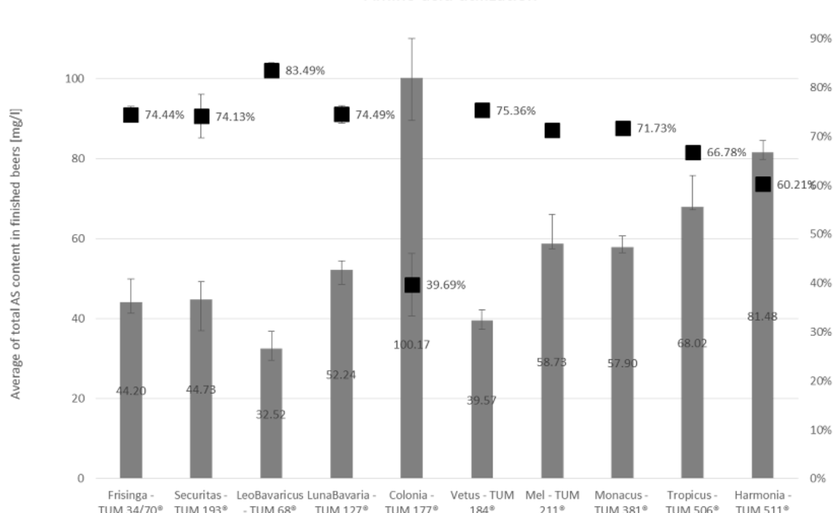
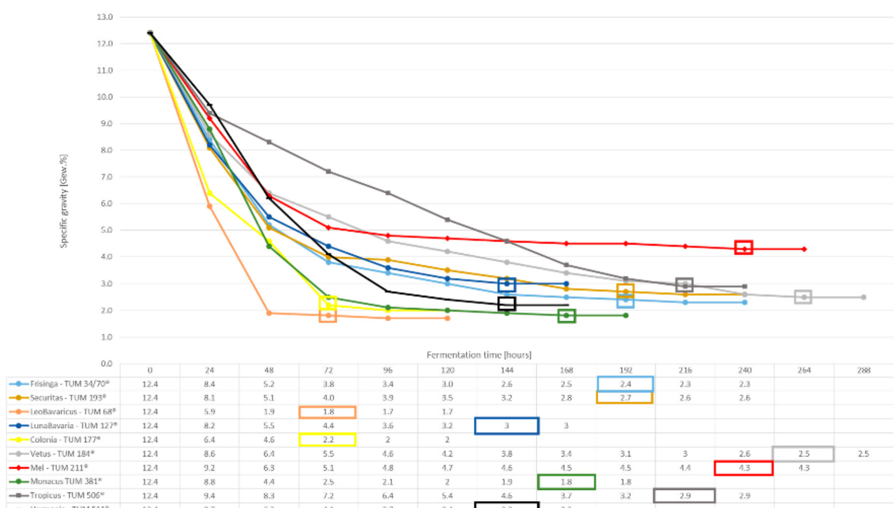


Figure 3. Average of metabolized and total amino acid (AS) content in finished beers produced with yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

3.2.3. Fermentation Kinetics

Figure 4 shows the drop in specific gravity during fermentation by the investigated yeast strains. As shown in Figure 4, LeoBavaricus-TUM 68® has the quickest drop in specific gravity, followed by Colonia-TUM 177®. Both strains reached their final gravity after 96 hours of fermentation. Monacus-TUM 381® needed 72 hours more to reach the final gravity of 1.8 °P after 168 hours. Mel-TUM 211® fermented the wort slower than the other strains but did so continuously until it reached the lowest apparent attenuation of all investigated yeast strains at 66.13% after 216 hours of fermentation.

Table 7 shows the apparent attenuation compared with the fermentation time required by the isolated strains. The different fermentation rates and degrees of apparent attenuation are due to their ability to ferment maltose and maltotriose (see Table 6). LeoBavaricus-TUM 68® and Monacus-TUM 381® reached the attenuation limit of the wort used at an apparent attenuation of 86.17%. The attenuation limit was previously tested according to MEBAK Bd. II and was achieved by using the top-fermenting *Saccharomyces cerevisiae* brewing yeast strain LeoBavaricus-TUM 68® and the same wort used in this trial. The low apparent attenuation of 76.2% by LunaBavaria-TUM 127® was due to the unique strain property of not fermenting one of the major wort sugars, namely maltotriose. The low drop in specific gravity over 144 h was not due to the maltotriose gap in the strain specific sugar metabolism, because maltotriose was taken up by the yeast cell as the last wort sugar, and was therefore not necessary for sufficient cell growth.



	0	24	48	72	96	120	144	168	192	216	240	264	288
Frisinga - TUM 34/70®	12.4	8.4	5.2	3.8	3.4	3.0	2.6	2.5	2.4	2.3	2.3		
Securitas - TUM 193®	12.4	8.1	5.1	4.0	3.9	3.5	3.2	2.8	2.7	2.6	2.6		
LeoBavaricus - TUM 68®	12.4	5.9	1.9	1.8	1.7	1.7							
LunaBavaria - TUM 127®	12.4	8.2	5.5	4.4	3.6	3.2	3	3					
Colonia - TUM 177®	12.4	6.4	4.6	2.2	2	2							
Vetus - TUM 184®	12.4	8.6	6.4	5.5	4.6	4.2	3.8	3.4	3.1	3	2.6	2.5	2.5
Mel - TUM 211®	12.4	9.2	6.3	5.1	4.8	4.7	4.6	4.5	4.5	4.4	4.3	4.3	
Monacus TUM 381®	12.4	8.8	4.4	2.5	2.1	2	1.9	1.8	1.8				
Tropicus - TUM 506®	12.4	9.4	8.3	7.2	6.4	5.4	4.6	3.7	3.2	2.9	2.9		
Harmonia - TUM 511®	12.4	9.7	6.2	4.1	2.7	2.4	2.2	2.2					

Figure 4. Drop in specific gravity measured in a single reference vessel compared with the average in final gravity (marked with box) measured in triplicate in final beers for the tested yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

Table 7. Apparent attenuation (AA%) of the final beer compared with specific time for primary fermentation for the investigated yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

Apparent attenuation (AA %) of the final beer		
TUM Yeast Strain	**AA (%)**	**Fermentation time (hours)**
Frisinga-TUM 34/70®	81.63 ± 0.51	216
Securitas-TUM 193®	79.30 ± 0.51	216
LeoBavaricus-TUM 68®	86.17 ± 0.05	96
LunaBavaria-TUM 127®	76.20 ± 1.76	144
Colonia-TUM 177®	84.93 ± 0.37	96
Vetus-TUM 184®	80.97 ± 3.02	264
Mel-TUM 211®	66.13 ± 0.51	240
Monacus-TUM 381®	86.17 ± 0.11	168
Tropicus-TUM 506®	77.37 ± 1.34	216
Harmonia-TUM 511®	82.70 ± 0.42	144

3.2.4. Flocculation (Cell Count)

The flocculation behavior of a yeast strain is an important selection criterion to ensure reliable product quality in industrial brewing processes. Besides the genetic background of the yeast strain (e.g., variation in FLO genes), the flocculation behavior is affected by the physiological environment (e.g., the pH and availability of metal ions and nutrients of the wort), by the physical environment (e.g., soluble oxygen, hydrodynamic conditions and low agitation), and the fermentation temperature, as well as a sufficient concentration of yeast cells in suspension [37–39]. In this research, all environmental and fermentation conditions were kept constant, in order to investigate and classify the investigated

strains according to their specific flocculation behavior in flocculent and non-flocculent ("powdery") yeast strains (see Table 8). According to ANNEMÜLLER, a flocculent yeast strain accumulates to flocs and settles at the bottom of the fermentation vessel when the nutrients present in brewers wort are largely consumed [40].

As Figure 5 shows, in contrast to the eight top-fermenting TUM yeast strains, the bottom-fermenting yeast strains Frisinga-TUM 34/70® and Securitas-TUM 193® flocculated very rapidly in the first three days of the main fermentation and reached a concentration of cells in suspension below the pitching rate. By reaching their apparent attenuation both strains shows cell concentrations below two million yeast cells/mL (e.g., 1.8 and 1.6 million cells/mL), and could therefore be classified according to their flocculation behavior as flocculent yeast strains (see Table 8). With the exception of the LunaBavaria-TUM 127® and Vetus-TUM 184® yeast strains, the top-fermenting TUM yeast strains LeoBavaricus-TUM 68®, Colonia-TUM 177®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511® were largely dispersed and remained in a suspension that is close to the pitching concentration, even once they have reached their apparent attenuation. According to BÜHLINGEN et al., the strains exhibited a non-flocculent ("powdery") behavior [41]. In contrast, LunaBavaria-TUM 127® and Vetus-TUM 184® showed similar flocculation behavior to the bottom-fermenting yeast strains and flocculated below four million cells/mL (e.g., 3.5 and 0.5 million cells/mL) by reaching their apparent attenuations. LeoBavaricus-TUM 68® also showed noticeable flocculation behavior, but also had the greatest increase in the number of cells, so that, similarly to Colonia-TUM 177®, the concentration of cells in suspension at the time of apparent attenuation was still above the pitching concentration. The flocculation behavior of both strains was therefore classified as powdery, but showed rapid flocculation, with a final concentration of cells in suspension below one million cells/mL at the end of maturation. With the exception of Mel-TUM 211® and Tropicus-TUM 506®, the "powdery" yeast strains fermented beer faster than the "flocculent" yeast strains, but both resulted in similar final attenuations.

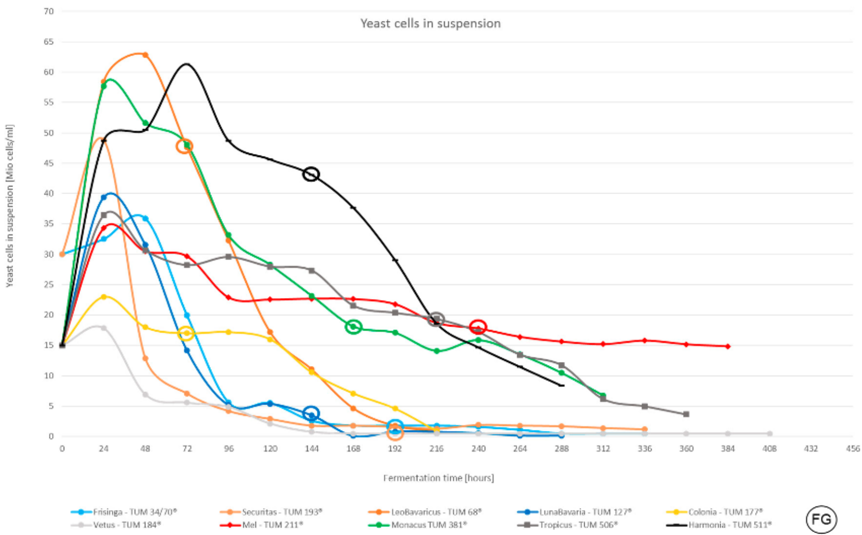


Figure 5. Yeast cells in suspension during the main fermentation and maturing phase. The circle marks the specific final gravity (FG) of the investigated yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®.

Table 8. Difference in maximum yeast cell concentration during primary fermentation and yeast cell concentration by reaching the specific final gravity (FG) and the flocculation behavior of Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®.

Yeast Cell Sedimentation at the end of Primary Fermentation				
TUM Yeast Strain	**Max. Cell Conc.**	**Cell Conc. FG**	**Difference**	**Flocculation Behavior**
Frisinga-TUM 34/70®	35.90	1.80	−34.10	flocculent
Securitas-TUM 193®	48.80	1.30	−47.50	flocculent
LeoBavaricus-TUM 68®	62.81	32.29	−30.52	powdery
LunaBavara-TUM 127®	39.38	7.50	−31.88	flocculent
Colonia-TUM 177®	23.00	17.20	−5.8	powdery
Vetus-TUM 184®	17.83	0.50	−17.33	flocculent
Mel-TUM 211®	34.30	17.80	−16.50	powdery
Monacus-TUM 381®	57.65	18.12	−39.53	powdery
Tropicus-TUM 506®	36.45	19.36	−17.09	powdery
Harmonia-TUM 511®	61.30	43.07	−18.23	powdery

3.2.5. Change in pH Value

Table 9 shows the drop in pH during the first 96 hours of primary fermentation, the pH value after the maturation phase, and the average pH value of the final beer. As shown in Table 9, the drop in pH and the time taken to reach the minimum pH value for primary fermentation is different for all the investigated yeast strains.

Securitas-TUM 193®, Monacus-TUM 381® and Mel-TUM 211® reached their minimum and final beer pH value after 48 hours. Frisinga-TUM 34/70® showed a similar change in pH value to the ale yeast strains Mel-TUM 211®, Tropicus-TUM 506® and Harmonia-TUM 511®, which was already shown by MEIER-DÖRNBERG [23]. These four strains reached their minimum pH value for primary fermentation after 48 hours, and recorded a pH value increase of 0.1 after the maturation and lagering phase. LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177® and Vetus-TUM 184® needed 96 hours to reach their minimum pH value for primary fermentation. Colonia-TUM 177® recorded a pH value increase of 0.1 as measured in triplicate in the final beer, while an increase of 0.2 could be registered in the produced beers with LeoBavaricus-TUM 68® and LunaBavaria-TUM 127®. According to ANNEMÜLLER and MANGER, an increase in the pH value of more than 0.1 could indicate cell autolysis, and might be due to the excretion of yeast metabolites and the uptake and metabolization of pyruvate [42]. Frisinga-TUM 34/70®, LeoBavaricus-TUM 68®, and Harmonia-TUM 511® exhibited the strongest capacity for acidification (ΔpH 0.8) compared with the other yeast strains. Mel-TUM 211® and Tropicus-TUM 506® exhibited the weakest capacity for acidification (ΔpH 0.5), which might be due to cell autolysis caused by low fermentation performance (see Section 3.2.3).

Table 9. Change in pH value during primary fermentation, after the maturation and lagering phase, rounded to two decimal figures, for Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

pH Value Decrease during Primary Fermentation									
TUM Yeast Strain	0 h	24 h	48 h	72 h	96 h	After primary fermentation	After maturation	Final beer (after lagering)	ΔpH
Frisinga-TUM 34/70®	5.2	4.6	4.5	4.5	4.5	4.5	4.5	4.4 ± 0.04	0.8
Securitas-TUM 193®	5.2	4.6	4.5	4.5	4.5	4.5	4.5	4.5 ± 0.01	0.7
LeoBavaricus-TUM 68®	5.2	4.8	4.3	4.3	4.2	4.2	4.2	4.4 ± 0.01	0.8
LunaBavaria-TUM 127®	5.2	4.6	4.5	4.5	4.4	4.4	4.4	4.6 ± 0.01	0.6
Colonia-TUM 177®	5.2	5	4.8	4.8	4.7	4.7	4.7	4.6 ± 0.02	0.6
Vetus-TUM 184®	5.2	4.7	4.6	4.6	4.5	4.5	4.6	4.5 ± 0.04	0.7
Mel-TUM 211®	5.2	4.5	4.4	4.4	4.4	4.4	4.5	4.7 ± 0.01	0.5
Monacus-TUM 381®	5.2	4.6	4.5	4.5	4.5	4.5	4.5	4.5 ± 0.01	0.7
Tropicus-TUM 506®	5.2	4.6	4.5	4.5	4.5	4.5	4.6	4.7 ± 0.01	0.5
Harmonia-TUM 511®	5.2	4.6	4.4	4.4	4.4	4.4	4.4	4.4 ± 0.01	0.8

3.3. Flavor Characterization

3.3.1. Phenolic Off-Flavor

Table 10 shows the results of the POF-tests evaluated by sniffing. As shown in Table 10, not all of the investigated yeast strains were capable of building phenolic flavors. The panelists could only detect aroma active components formed by the top-fermenting yeast strains LeoBavaricus-TUM 68® and LunaBavaria-TUM 127®, commonly used for wheat beer production, and Harmonia-TUM 511® (ale beer production) and for Monacus-TUM 381® (trappist beer production). For these yeasts, all three corresponding POF-flavors were detected by sniffing.

Both bottom-fermenting yeast strains Frisinga-TUM 3470® and Securitas-TUM 193®, as well as the four top-fermenting yeast strains Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, and Tropicus-TUM 506® were POF negative. These strains were normally used for the production of lager beer (Frisinga-TUM 3470®, Securitas-TUM 193®), kölsch (Colonia-TUM 177®), and alt (Vetus-TUM 184®), as well ale beer production (Mel-TUM 211® and Tropicus-TUM 506®). Phenolic off-flavors are typically not desired in these classic beer styles. These three yeast strains cannot decarboxylate any of the precursor acids. Therefore the phenylacrylic acid decarboxylase (*PAD1*) and/or ferulic acid decarboxylase (*FDC1*) gene activity might be inactive or blocked [43–45].

Figure 6 shows the concentrations of 4-vinylguajacol measured in the finished beers after lagering. According to the evaluation by sniffing, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Monacus-TUM 381®, and Harmonia-TUM 511® were POF-positive, with detected concentrations of 4-vinylguajacol above the individual threshold for 4-vinylguajacol of 0.3 mg/L [46].

Table 10. Phenolic off-flavor (POF) test results for the investigated yeast strains.

	POF-Test/Sniffing Perception of:		
TUM Yeast Strain	**4-Vinylguajacol/ Ferulic Acid**	**4-Vinylphenol/ Coumaric Acid**	**4-Vinylstyrene/ Cinnamic Acid**
Frisinga - TUM 3470®	–	–	–
Securitas-TUM 193®	–	–	–
LeoBavaricus-TUM 68®	+	+	+
LunaBavaria-TUM 127®	+	+	+
Colonia-TUM 177®	–	–	–
Vetus-TUM 184®	–	–	–
Mel-TUM 211®	–	–	–
Monacus-TUM 381®	+	+	+
Tropicus-TUM 506®	–	–	–
Harmonia-TUM 511®	+	+	+

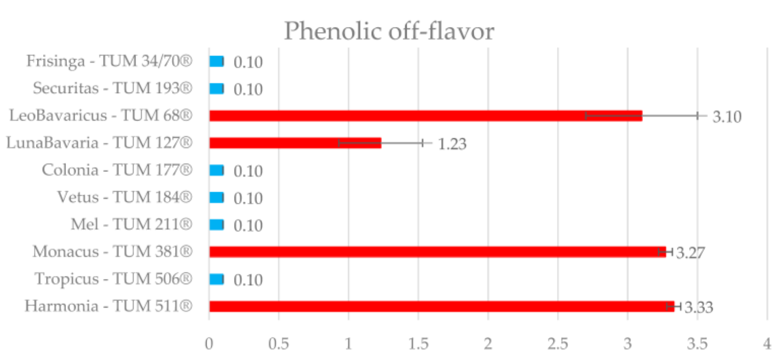


Figure 6. Phenolic off-flavor ability of the investigated yeast strains; confidence level 95%.

3.3.2. Fermentation by-Products

There was a variation in the production of fermentation by-products for all of the yeast strains (Tables 11 and 12). The beers produced with LeoBavaricus-TUM 68®, Luna Bavaria-TUM 127®, Monacus-TUM 381® and Harmonia -TUM 511® had the highest levels of isoamyl acetate and 4-vinylguajacol, with concentrations above 2.9 mg/L for isoamyl acetate (Harmonia-TUM 511®) and above 1.2 mg/L for 4-vinylguajacol. The concentration of these esters specific to the production of wheat beers were within the average reference values for regular wheat beers (2–8 mg/L isoamyl acetate and 1–4 mg/L of 4-vinylguajacol) according to Back [47]. LeoBavaricus-TUM 68® had the highest concentration of higher alcohols (212.07 ± 13.15 mg/L), and the highest level of esters was detected in the beer produced by Harmonia-TUM 511®, with a concentration of 57.33 ± 0.65 mg/L.

Acetaldehyde, 2.3-pentanedione and diacetyl are associated with unmatured beer, and can result in an unpleasant flavor if the concentrations are above their individual thresholds. The concentration of acetaldehyde is below their individual thresholds of 25 mg/L for all strains. Frisinga-TUM 34/70®, LunaBavaria-TUM 127® and Colonia-TUM 177® showed concentrations of diacetyl above the individual threshold of 0.15 mg/L [46]. The production as well as the degradation of diacetyl is strain dependent, and were influenced by fermentation conditions and the yeast management (pitching rate, vitality and viability).

Table 11. Average of important fermentation by-products (FBP) measured in triplicate of the final beers produced with Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127® and Colonia-TUM 177®; confidence level 95%.

FBP	Fermentation by-Products (mg/L)				
	Frisinga-TUM 3470®	**Securitas-TUM 193®**	**LeoBavaricus-TUM 68®**	**LunaBavaria-TUM 127®**	**Colonia-TUM 177®**
Isoamyl acetate	0.63 ± 0.19	1.38 ± 0.05	4.07 ± 0.46	3.97 ± 2.30	2.40 ± 0.16
Ethyl acetate	19.77 ± 2.50	26.07 ± 1.89	32.50 ± 2.97	36.97 ± 2.93	32.87 ± 1.68
∑ Ester (E)	20.40 ± 2.69	27.90 ± 1.94	36.57 ± .3.43	40.93 ± 3.16	35.27 ± 1.83
n-Propanol	11.23 ± 0.77	13.43 ± 0.72	22.77 ± 2.37	15.93 ± 0.70	21.30 ± 1.25
i-Butanol	10.63 ± 0.71	14.27 ± 0.47	62.30 ± 3.51	43.70 ± 3.42	10.53 ± 0.11
Amyl alcohols	60.53 ± 3.31	82.60 ± 2.68	127.00 ± 7.33	91.60 ± 3.34	80.77 ± 1.06
∑ Higher alcohols (HE)	82.40 ± 4.76	110.30 ± 3.86	212.07 ± 13.15	151.23 ± 6.25	112.60 ± 2.32
4-Vinylguajacol	0.10 ± 0.00	0.10 ± 0.00	3.10 ± 0.40	1.23 ± 0.30	0.10 ± 0.00
Diacetyl	0.18 ± 0.07	0.10 ± 0 01	0.11 ± 0.01	0.17 ± 0.03	0.14 ± 0 03
2,3-Pentandione	0.00 ± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
∑ Vicinal diketones	0.18 ± 0.07	0.12 ± 0.02	0.12 ± 0.01	0.19 ± 0.03	0.15 ± 0.04
Acetaldehyde	1.33 ± 0.47	11.17 ± 1.46	7.27 ± 1.80	2.80 ± 0.94	8.23 ± 2.02
Ratio (∑E:∑HE)	4.04	3.95	5.80	3.69	3.19

Table 12. Average of important fermentation by-products (FBP) measured in triplicate of the final beers produced with Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

FBP	Fermentation by-Products (mg/L)				
	Vetus-TUM 184®	**Mel-TUM 211®**	**Monacus-TUM 381®**	**Tropicus-TUM 506®**	**Harmonia-TUM 511®**
Isoamyl acetate	1.80 ± 0.09	2.03 ± 0.05	4.33 ± 0.05	1.53 ± 0.11	2.93 ± 0.05
Ethyl acetate	37.40 ± 0.58	37.93 ± 0.11	50.60 ± 0.85	22.57 ± 1.31	54.40 ± 0.61
∑ Ester (E)	39.20 ± 0.61	39.97 ± 0.14	54.93 ± 0.84	24.10 ± 1.36	57.33 ± 0.65
n-Propanol	15.40 ± 0.46	18.30 ± 0.16	18.30 ± 0.16	20.67 ± 1.01	20.77 ± 0.53
i-Butanol	11.37 ± 0.14	16.20 ± 0.37	24.17 ± 0.66	20.90 ± 1.40	13.13 ± 0.28
Amyl alcohols	67.37 ± 0.91	59.27 ± 1.32	101.00 ± 4.23	88.50 ± 5.90	74.97 ± 1.24
∑ Higher alcohols (HE)	94.13 ± 1.43	93.77 ± 1.83	142.23 ± 5.26	130.07 ± 8.12	108.87 ± 1.23
4-Vinylguajacol	0.10 ± 0.00	0.10 ± 0.00	3.27 ± 0.05	0.10 ± 0.00	3.33 ± 0.05
Diacetyl	0.06 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	0.12 ± 0.01	0.06 ± 0.00
2,3-Pentandione	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
∑ Vicinal diketones	0.06 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	0.14 ± 0.01	0.07 ± 0.00
Acetaldehyde	6.80 ± 0.80	4.60 ± 0.48	6.23 ± 0.77	5.93 ± 0.93	4.03 ± 0.46
Ratio (∑E:∑HE)	2.40	2.35	2.59	5.40	1.90

3.3.3. Sulfur Dioxide

Table 13 shows the SO_2 concentration of the finished beers produced by the investigated TUM yeast strains. As shown in the table, all of the investigated yeast strains form sulfur dioxide (SO_2) during fermentation. The concentration produced varies from strain to strain and differs by up to a about 9 mg/L SO_2 in the finished beers. This can be confirmed by SWIEGERS and ANNEMÜLLER, who reported that differences between 2 and 10 mg/L SO_2 can be detected in the finished beer under identical fermentation conditions [40,48]. In conclusion and also according to the results obtained by MEIER-DÖRNBERG in 2017, the SO_2 formation is mainly influenced by the used yeast strain and is strain dependent [23]. As Table 13 shows, Securitas-TUM 193® produced the highest quantity of SO_2 at a total amount of 9.47 ± 0.68 on average. According to BACK, each additional mg/L SO_2 below 5 mg/L prolongs the flavor stability of beer by about one month (Back Technologisches Seminar Weihenstephan 2015). Therefore TUM yeast strain Securitas-TUM 193® could be very suitable for producing lager beers with a long-term flavor stability. The lowest concentration was produced by yeast strain Monacus-TUM 381® and Harmonia-TUM 511® at a concentration of 0.5 mg/L. The second lowest concentration in the finished beer of 1.63 ± 0.51 mg/L SO_2 was produced by the top-fermenting wheat beer yeast strain LunaBavaria-TUM 127®.

Table 13. SO_2 concentration of the final beers produced using Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Colonia-TUM 177®, Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511®; confidence level 95%.

SO2 Concentration of the Finished Beers	
TUM Yeast Strain	**SO2 (mg/L)**
Frisinga-TUM 34/70®	6.03 ± 0.30
Securitas-TUM 193®	9.47 ± 0.68
LeoBavaricus-TUM 68®	2.87 ± 0.30
LunaBavaria-TUM 127®	1.63 ± 0.51
Colonia-TUM 177®	3.80 ± 0.79
Vetus-TUM 184®	3.10 ± 0.16
Mel-TUM 211®	2.60 ± 0.98
Monacus-TUM 381®	0.50 ± 0.00
Tropicus-TUM 506®	2.23 ± 1.02
Harmonia-TUM 511®	0.50 ± 0.00

3.3.4. Sensory Evaluation

Sensory analysis of the beers was conducted after maturation and lagering. All of the beers produced had no prevailing off-flavors and were rated with a four or a five in every category of the DLG scheme for beer (data not shown). In terms of the descriptive sensory evaluation, the following Figures 7–11 show the aroma profile of each investigated TUM yeast strain. The overall flavor impression is shown in orange, and the most distinct individual flavor attributes are shown in blue. The individual flavor attributes represent the most noted and highest rated flavors by all panelists within the seven main aroma categories. The average values of the single flavor attributes are summarized in main categories and represent the overall flavor impression. As shown in the figures, LeoBavaricus-TUM 68® and LunaBavaria-TUM 127® had a very distinct clove-like aroma. In addition to Monacus-TUM 381® and Harmonia-TUM 511®, all four *Saccharomyces cerevisiae* yeast strains were POF positive, with analytically detected concentrations above the individual threshold of 4-vinylguajacol. A clove-like aroma is the main aroma compound in German wheat beers, and probably the reason for why over 90% of the tasters associated the produced and tasted beers with wheat beer. In contrast to LeoBavaricus-TUM 68®, LunaBavaria-TUM 127® and Monacus-TUM 381®,

the clove-like flavor was not recognized by the panelists for Harmonia-TUM 511® (flavor intensity of 0.14, e.g., Figure 11), even if this strain produced the highest concentration of 4-VG at 3.33 mg/L.

According to MEIER-DÖRNBERG [23], this may be caused and suppressed by synergistic effects and due to the well-balanced flavor profile with citrus and fruity flavors, which were perceived as being slightly more distinct. In conclusion, 28.5% of the panelists could not clearly assign this beer to a wheat nor to an ale style. MEIER-DÖRNBERG suggested that this strain was particularly suitable for brewing a beer with the fruitiness of an ale style brewed beer, underlined by the slightly spicy and yeasty flavors of a wheat beer and proposed calling it "Bavarian Ale". The brewing yeast Mel-TUM 211® and Tropicus-TUM 506® were an ale beer style, and the beers produced were very fruity. Tropicus-TUM 506® has fruity flavors, especially within the tropical fruit category. Vetus-TUM 184® also produces fruity and sweet flavors as well as a flavor reminiscent of wine, which was described as dry, and could be responsible for the drier beer flavor expected in beers of the alt type. Colonia-TUM 177® seemed to be suitable for more than one beer type. The panelists assigned the beer produced using Colonia-TUM 177® to a kölsch and an alt style (27.27% kölsch and alt). The produced beer had a sweet and yeasty flavor with aromas slightly reminiscent of citrus fruits, such as grapefruit. The bottom-fermenting yeast strain Frisinga-TUM 34/70® had a well-balanced aroma profile with a tendency towards floral and fruity flavor impressions. The produced beers were clearly described as being lager beers. In comparison, the beers produced using Securitas-TUM 193® were also assigned to fruity beer types such as ale and kölsch (28.57% Lager, 14.28% Ale and 14.28% kölsch). This could be confirmed by the specific aroma profile. Securitas-TUM 193® was characterized by plenty of fruity flavors, particularly reminiscent of berries, with additional fresh yeasty and sulfuric flavors, typically for lager beers, and a sweet body.

Figure 7. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for the bottom-fermenting *Saccharomyces pastorianus* yeast strains Frisinga-TUM 34/70® and Securitas-TUM 193®.

Figure 8. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for the top-fermenting *Saccharomyces cerevisiae* yeast strains LeoBavaricus-TUM 68® and LunaBavaria-TUM 127®.

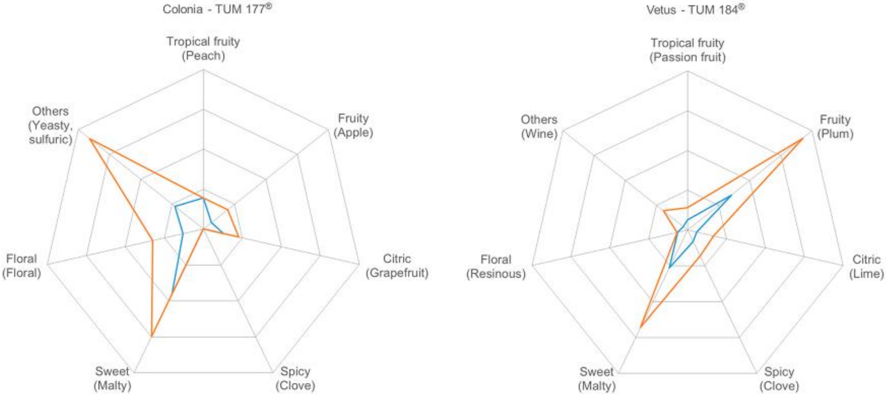


Figure 9. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for top-fermenting *Saccharomyces cerevisiae* yeast strains Colonia-TUM 177® and Vetus-TUM 184®.

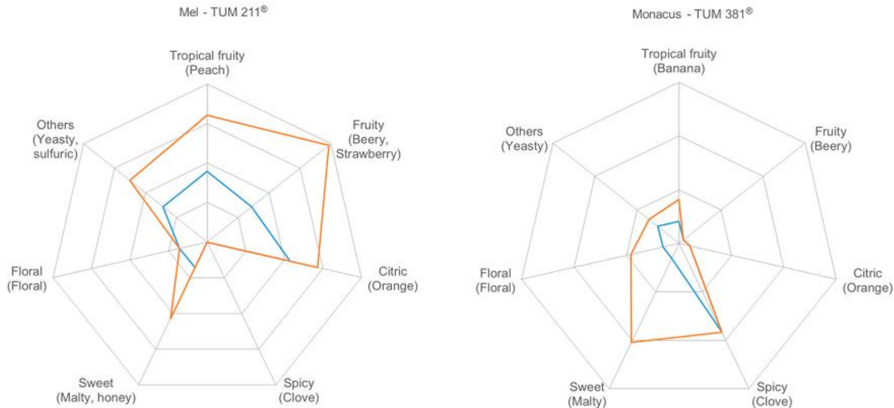


Figure 10. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for top-fermenting *Saccharomyces cerevisiae* yeast strains Mel-TUM 211® and Monacus-TUM 381®.

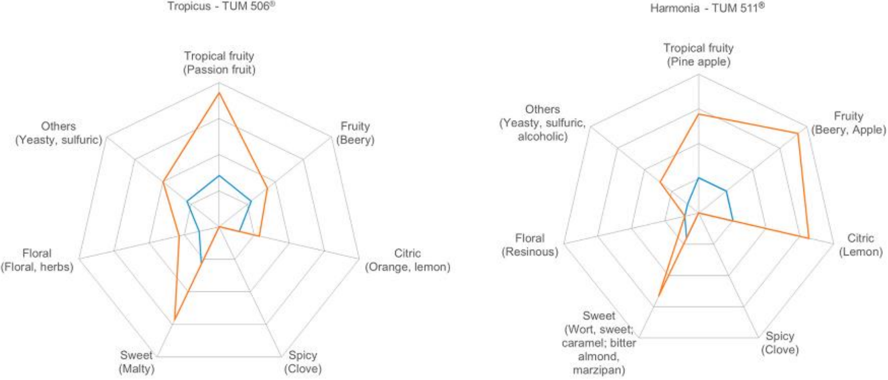


Figure 11. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for top-fermenting *Saccharomyces cerevisiae* yeast strains Tropicus-TUM 506® and Harmonia-TUM 511®.

4. Conclusions

All 10 investigated TUM brewing yeast strains showed different phenotypic characteristics and flavor profiles. The most interesting differences are presented in the following Table 14 and highlighted in red, yellow, or green according to their performance and brewing characteristics. Based on the results of the genetic analysis, the species identity as well as the genetic distinctness of the investigated TUM yeast strains of the TUM Yeast Center could be confirmed. Except Frisinga-TUM 34/70® and Securitas-TUM 193®, which belong to the *Saccharomyces pastorianus* species, all other TUM yeast strains belonged to *Saccharomyces cerevisiae*. All 10 TUM yeast strains showed different fermentation rates and degrees of apparent attenuation and can be explained by their different ability to ferment maltotriose. The top-fermenting yeast strain Mel-TUM 211® only fermented a low level of maltotriose (26.66% ± 0.26%), while the LunaBavaria-TUM 127® yeast strain could not ferment maltotriose at all (01.05% ± 2.89%). In the case of non-fermentation of maltotriose, LunaBavaria-TUM

127® beers reached their apparent attenuation faster than Mel-TUM 211®, and needed less time for fermentation. Except for the strains Mel-TUM 211® and Tropicus-TUM 506®, the pH of the final beer was within the range of 4.4 to 4.6 [49]. Mel-TUM 211® and Tropicus-TUM 506® exhibited the weakest capacity for acidification (ΔpH 0.5) of all the investigated strains, which might be due to cell autolysis caused by the low fermentation performance. The total amino acid utilization was also different for each investigated TUM yeast strain, and no conclusion can be drawn as to cell growth. The cell concentration was measured during the main fermentation phase and maturation phase to classify the investigated strains according to their specific flocculation behavior. Not every top-fermenting yeast strain shows powdery behavior. LunaBavaria-TUM 127® and Vetus-TUM 184® showed a flocculent behavior similar to the bottom-fermenting yeast strains Frisinga-TUM 34/70® and Securitas-TUM 193®. As expected, some of the yeast strains most commonly used in industry Frisinga-TUM 34/70® and LeoBavaricus-TUM 68® showed the best phenotypic characteristics, thereby standing out from the other investigated yeast strains. However, every brewer's ultimate goal is the final desirable taste of the produced beer. In this respect, the main focus in this study was on the individual and main flavor impression of the final beers. Only the top-fermenting yeast strains LeoBavaricus-TUM 68®, LunaBavaria-TUM 127®, Harmonia-TUM 511®, and Monacus-TUM 381® were capable of building phenolic off-flavors, which was confirmed by the concentrations of 4-vinylguajacol in the finished beers, which were above the individual threshold. Except for the Monacus-TUM 381® strain, all panelists recognized the clove like flavor and therefore referred to these beers as wheat style beers. The production of fermentation by-products, as well as the resulting flavor composition in the finished beers was strain-dependent and followed no defined order. The formation of sulfur dioxide (SO_2) during fermentation could be detected in all strains, but concentrations differed from 0.50 ± 0.00 mg/L for Monacus-TUM 381® and Harmonia-TUM 511®, to a considerably higher level on average of 9.47 ± 0.68 mg/L for Securitas-TUM 193®. The bottom-fermenting yeast strain Securitas-TUM 193® could therefore be very suitable for the production of lager beers with high flavor stability. This characterization model for yeast strains allows brewers around the world access to a simplified and targeted selection of brewing yeast strains suitable for their specific purposes. By analyzing and comparing different yeast strains, breweries can be given customized advice when selecting a yeast strain suitable for their brewing process or type of beer, irrespective of whether they want to replace their existing yeast strain to improve the aroma profile of existing beer styles, develop new beer styles, or optimize the fermentation process by selecting a strain with the corresponding fermentation characteristics. Knowledge about the different yeast strain characteristics can, in particular, promote the competitiveness of small and medium-sized breweries and, if necessary, secure their existence by being part of the steadily increasing market for beer specialties [24].

Table 14. Comparison of the investigated 10 TUM yeast strains with the focus on recommended beer style, POF, flocculation behavior, maltotriose utilization, pH drop, SO_2, apparent attenuation and time to reach the final gravity (red=weak, yellow=normal, green=strong).

TUM Yeast Strains									
TUM Yeast Strain	Yeast Species	Recommended Main Beer Style	Phenolic Off-Flavor	Flocculation Behavior	Maltotriose-Utilization (%)	ΔpH	SO_2(mg/L)	AA(%)	Fermentation Time (days)
Frisinga-TUM 34/70®	*S. pastorianus*	lager beer	–	flocculent	94.06 ± 0.91	0.8	6.03 ± 0.30	81.63 ± 0.51	9
Securitas-TUM 193®	*S. pastorianus*	lager bee	–	flocculent	86.60 ± 0.52	0.7	9.47 ± 0.68	79.30 ± 0.51	9
LeoBavaricus-TUM 68®	*S. cerevisiae*	wheat beer	+	powdery	99.65 ± 0.28	0.8	2.87 ± 0.30	86.17 ± 0.05	4
LunaBavaria-TUM 127®	*S. cerevisiae*	wheat beer	+	flocculent	01.05 ± 2.89	0.6	1.63 ± 0.51	76.20 ± 1.76	6
Colonia-TUM 177®	*S. cerevisiae*	kölsch and alt beer	–	powdery	94.80 ± 0.78	0.6	3.80 ± 0.79	84.93 ± 0.37	4
Vetus-TUM 184®	*S. cerevisiae*	alt beer	–	flocculent	60.92 ± 5.87	0.7	3.10 ± 0.16	80.97 ± 3.02	11
Mel-TUM 211®	*S. cerevisiae*	ale beer	–	powdery	26.66 ± 0.26	0.5	2.60 ± 0.98	66.13 ± 0.51	10
Monacus-TUM 381®	*S. cerevisiae*	wheat beer	+	powdery	97.75 ± 0.09	0.7	0.50 ± 0.00	86.17 ± 0.11	7
Tropicus-TUM 506®	*S. cerevisiae*	ale beer	–	powdery	59.28 ± 0.81	0.5	2.23 ± 1.02	77.37 ± 1.34	9
Harmonia-TUM 511®	*S. cerevisiae*	ale and wheat beer	+	powdery	83.91 ± 0.71	0.8	0.50 ± 0.00	82.70 ± 0.42	6

Supplementary Materials: The following Tables are available online at www.mdpi.com/2311-5637/3/3/41. Supplementary Tables: Table S1. Mean percentage of amino acid uptake of the yeast strains Frisinga-TUM 34/70®, Securitas-TUM 193®, LeoBavaricus-TUM 68®, LunaBavaria-TUM 127® and Colonia-TUM 177® after lagering measured in the finished beers (Group A = light gray, Group B = dark gray, Group C = no shading); confidence level 95%; Table S2. Mean percentage of amino acid uptake of the yeast strains Vetus-TUM 184®, Mel-TUM 211®, Monacus-TUM 381®, Tropicus-TUM 506® and Harmonia-TUM 511® after lagering measured in the finished beers (Group A = light gray, Group B = dark gray, Group C = no shading); confidence level 95%.

Acknowledgments: Parts of this work were funded by the German Ministry of Economics and Technology and the Wifö (Wissenschaftsförderung der Deutschen Brauwirtschaft e.V., Berlin, Germany) in project AiF 17698 N.

Author Contributions: Tim Meier-Dörnberg designed and performed the experiments and analyzed the data; Mathias Hutzler contributed reagents/materials/analysis tools; Mathias Hutzler designed primer and probes for Real-Time PCR and DNA based strain differentiation, Tim Meier-Dörnberg and Mathias Hutzler wrote the paper; Frank-Jürgen Methner and Maximilian Michel revised the conception and manuscript, and agreed for submission.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cotterchio, D.; Zarnkow, M.; Jacob, F. Europäische Interpretationen zur Reinheit des Bieres. *Brauwelt* **2016**, *2016*, 50–53.
2. Meier-Dörnberg, T. Genetic and phenotypic characterization of different top-fermenting *Saccharomyces cerevisiae* ale yeast isolates. In Proceedings of the 36th European Brewery Convention, Ljubljana, Slovenia, 14–18 May 2017.
3. Sachon, V.W. The science of brewing in the heart of slovenia: 36th EBC Convention held in Ljubljana. *Brew. Bever. Ind. Int.* **2017**, *102*, 28–31.
4. Hutzler, M.; Meier-Dörnberg, T.; Stretz, D.; Englmann, J.; Zarnkow, M.; Jacob, F. TUM-Hefen—die Geburtsstunde von LeoBavaricus-TUM 68®. *Brauwelt* **2017**, *157*, 266–269.
5. White, C.; Zainasheff, J. *Yeast: The Practical Guide to Beer Fermentation*; Brewers Publications: Boulder, CO, USA, 2010.
6. Gábor, P.; Rosa, C. *Biodiversity and Ecophysiology of Yeasts*; Springer-Verlag: Berlin/Heidelberg, Germany, 2006.
7. Rainieri, S.; Zambonelli, C.; Kaneko, Y. *Saccharomyces sensu stricto*: Systematics, genetic diversity and evolution. *J. Biosci. Bioeng.* **2003**, *96*, 1–9. [CrossRef]
8. Vaughan-Martini, A.; Martini, A. Saccharomyces Meyen ex Reess. In *The yeasts, a taxonomic study*, 5th ed.; Kurtzman, C.P., Fell, J.W., Eds.; Elsevier: Amsterdam, Netherlands, 2011; pp. 733–746.
9. Naseeb, S.; James, S.A.; Alsammar, H.; Michaels, C.J.; Gini, B.; Nueno-Palop, C.; Bond, C.J.; McGhie, H.; Roberts, I.N.; Delneri, D. *Saccharomyces jurei* sp. nov., isolation and genetic identification of a novel yeast species from Quercus robur. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 2046–2052. [CrossRef] [PubMed]
10. Rainieri, S.; Kodama, Y.; Kaneko, Y.; Mikata, K.; Nakao, Y.; Ashikari, T. Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. *Appl. Environ. Microbiol.* **2006**, *72*, 3968–3974. [CrossRef] [PubMed]
11. Bamforth, C.W. *Food, fermentation, and micro-organisms*; Blackwell Science: Oxford, Ames, IA, USA, 2005.
12. Libkind, D.; Hittinger, C.T.; Valério, E.; Gonçalves, C.; Dover, J.; Johnston, M.; Gonçalves, P.; Sampaio, J.P. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 14539–14544. [CrossRef] [PubMed]
13. Wang, S.A.; Bai, F.Y. *Saccharomyces arboricolus* sp. nov., a yeast species from tree bark. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 510–514. [CrossRef] [PubMed]
14. Dunn, B.; Sherlock, G. Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Res.* **2008**, *18*, 1610–1623. [CrossRef] [PubMed]
15. Pérez-Través, L.; Lopes, C.A.; Querol, A.; Barrio, E. On the complexity of the *Saccharomyces bayanus* taxon: Hybridization and potential hybrid speciation. *PLoS ONE* **2014**, *9*, e93729. [CrossRef] [PubMed]
16. Canonico, L.; Agarbati, A.; Comitini, F.; Ciani, M. *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. *Food Microbiol.* **2016**, *56*, 45–51. [CrossRef] [PubMed]

17. Canonico, L.; Comitini, F.; Ciani, M. *Torulaspora delbrueckii* contribution in mixed brewing fermentations with different *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **2017**, *259*, 7–13. [CrossRef] [PubMed]
18. Michel, M.; Meier-Dörnberg, T.; Jacob, F.; Schneiderbanger, H.; Haslbeck, K.; Zarnkow, M.; Hutzler, M. Optimization of Beer Fermentation with a Novel Brewing Strain *Torulaspora delbrueckii* using Response Surface Methodology. *Tech. Q.* **2017**, *54*, 23–33. [CrossRef]
19. Spitaels, F.; Wieme, A.D.; Janssens, M.; Aerts, M.; Daniel, H.-M.; van Landschoot, A.; Vuyst, L.; de Vandamme, P. The microbial diversity of traditional spontaneously fermented lambic beer. *PLoS ONE* **2014**, *9*, e95384. [CrossRef] [PubMed]
20. Bokulich, N.A.; Bamforth, C.W.; Mills, D.A. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. *PLoS ONE* **2012**, *7*, e35507. [CrossRef] [PubMed]
21. Nakao, Y.; Kanamori, T.; Itoh, T.; Kodama, Y.; Rainieri, S.; Nakamura, N.; Shimonaga, T.; Hattori, M.; Ashikari, T. Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Res.* **2009**, *16*, 115–129. [CrossRef] [PubMed]
22. Müller-Auffermann, K.; Caldera, A.; Jacob, F.; Hutzler, M. Characterization of Different Bottom Fermenting *Saccharomyces pastorianus* Brewing Yeast Strains. *BrewingScience* **2015**, *68*, 46–57.
23. Meier-Dörnberg, T.; Michel, M.; Wagner, R.S.; Jacob, F.; Hutzler, M. Genetic and Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale Yeast Isolates. *BrewingScience* **2017**, *70*, 9–25. [CrossRef]
24. Zocher, H. Bierspezialitäten beleben den Biermarkt. *Brauindustrie* **2017**, *102*, 9.
25. Gibson, B.; Geertman, J.-M.A.; Hittinger, C.T.; Krogerus, K.; Libkind, D.; Louis, E.J.; Magalhães, F.; Sampaio, J.P. New yeasts—new brews: Modern approaches to brewing yeast design and development. *FEMS yeast res.* **2017**, *17*. [CrossRef] [PubMed]
26. Schneiderbanger, H.; Koob, J.; Poltinger, S.; Jacob, F.; Hutzler, M. Gene expression in wheat beer yeast strains and the synthesis of acetate esters. *J. Inst. Brew.* **2016**, *122*, 403–411. [CrossRef]
27. Schneiderbanger, H.; Hutzler, M.; Jacob, F. Aromaprofile ausgewählter Weizenbier-Hefestämme. *Brauwelt* **2013**, *153*, 267–270.
28. Hutzler, M.; Geiger, E.; Jacob, F. Use of PCRDHPLC (Polymerase Chain Reaction Denaturing High Performance Liquid Chromatography) for the Rapid Differentiation of Industrial *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* Strains. *J. Inst. Brew.* **2010**, *116*, 464–474. [CrossRef]
29. Hutzler, M. Entwicklung und Optimierung von Methoden zur Identifizierung und Differenzierung von getränkerelevanten Hefen. Ph.D. Thesis, Technische Universität München, Weihenstephan, July 2009. Available online: https://mediatum.ub.tum.de/doc/796815/796815.pdf (accessed on 17 August 2017).
30. Hutzler, M. *Getränkerelevante Hefen—Identifizierung und Differenzierung: Wie Können Hefen Praxisrelevant Unterschieden Werden, und Wie Können Identifizierungsergebnisse Technologisch bewertet Werden?* Südwestdeutscher Verlag für Hochschulschriften: Saarbrücken, Germany, 2009. (In German)
31. Bamforth, C.W.; Bokulich, N.A. *Brewing Microbiology*; Caister Academic Press: Poole, UK, 2017; Available online: https://books.google.com.hk/books/about/Brewing_Microbiology.html?id=LS1FMQAACAAJ&redir_esc=y (accessed on 17 August 2017).
32. Brandl, A. Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien. Ph.D. Thesis, Technische Universität München, Weihenstephan, March 2006. Available online: http://d-nb.info/105814118X/34 (accessed on 17 August 2017).
33. Josepa, S.; Guillamon, J.-M.; Cano, J. PCR differentiation of *Saccharomyces cerevisiae* from *Saccharomyces bayanus*/*Saccharomyces pastorianus* using specific primers. *FEMS Microbiol. Lett.* **2000**, *193*, 255–259. [CrossRef] [PubMed]
34. Büchl, N.-R.; Hutzler, M.; Mietke-Hofmann, H.; Wenning, M.; Scherer, S. Differentiation of probiotic and environmental *Saccharomyces cerevisiae* strains in animal feed. *J. Appl. Microbiol.* **2010**, *109*, 783–791. [CrossRef] [PubMed]
35. Jones, M.; Pierce, J.S. Absorption of amino acids from wort by yeasts. *J. Inst. Brew.* **1964**, *2*, 307–315. [CrossRef]
36. Procopio, S.; Brunner, M.; Becker, T. Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation. *Eur. Food Res. Technol.* **2014**, *239*, 421–439. [CrossRef]

37. Zepf, M. Die Bedeutung des Absetzverhaltens der Hefe: Flokkulationsverhalten. *Der Doemensianer* **2010**, *1*, 51.
38. Vidgren, V. Yeast Flocculation and Sedimentation in Brewing. *J. Inst. Brew.* **2011**, *117*, 475–487. [CrossRef]
39. Soares, E.V. Flocculation in *Saccharomyces cerevisiae*: A review. *J. Appl. Microbiol.* **2011**, *110*, 1–18. [CrossRef] [PubMed]
40. Annemüller, G.; Manger, H.-J.; Lietz, P. *Die Hefe in der Brauerei. Hefemanagement, Kulturhefe-Hefereinzucht, Hefepropagation im Bierherstellungsprozess*, 3rd ed.; VLB: Berlin, Germany, 2013; Available online: https://www.amazon.de/Hefe-Brauerei-Hefemanagement-Hefepropagation-Bierherstellungsprozess/dp/3921690501 (accessed on 17 August 2017).
41. Bühligen, F.; Sträuber, H.; Ondruschka, J.; Harms, H.; Müller, S. Monitoring von Bruch- und Staubhefeanteilen in Brauprozessen: Prozessoptimierung. *BRAUWELT* **2010**, *150*, 18–21.
42. Annemüller, G.; Manger, H.-J. *Gärung und Reifung des Bieres. Grundlagen-Technologie-Anlagentechnik*, 2nd ed.; VLB: Berlin, Germany, 2013; Available online: https://www.vlb-berlin.org/publikationen/fachbuecher/gaerung-und-reifung-des-bieres-0 (accessed on 17 August 2017).
43. European Bioinformatic Institut Cambridge. Available online: http://www.ebi.ac.uk/interpro/IEntry?ac=IPR004507 (accessed on 15 November 2016).
44. Richard, P.; Viljanen, K.; Penttilä, M. Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*. *AMB Express* **2015**, *5*, 2–5. [CrossRef] [PubMed]
45. Mukai, N.; Masaki, K.; Fujii, T.; Lefuji, H. Single nucleotide polymorphisms of PAD1 and FDC1 show a positive relationship with ferulic acid decarboxylation ability among industrial yeasts used in alcoholic beverage production. *J. Biosci. Bioeng.* **2014**, *118*, 50–55. [CrossRef] [PubMed]
46. Meilgaard, M.C. Flavour chemistry of beer. Part 2. Flavour and threshold of 239 aroma volatiles. *Tech. Qual. Master Brew. Assoc. Am.* **1975**, *12*, 151–168.
47. Back, W. *Ausgewählte Kapitel der Brauereitechnologie*; Hans Carl: Nürnberg, 2005; Available online: https://www.amazon.de/Ausgew%C3%A4hlte-Kapitel-Brauereitechnologie-BRAUWELT-Wissen/dp/341800802X (accessed on 17 August 2017).
48. Swiegers, J.H.; Pretorius, I.-S. Modulation of volatile sulfur compounds by wine yeast. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 954–960. [CrossRef] [PubMed]
49. Narziss, L.; Back, W. *Abriss der Bierbrauerei, 7., aktualisierte und erweiterte Aufl*; Wiley-VCH: Weinheim, Germany, 2005; Available online: http://eu.wiley.com/WileyCDA/WileyTitle/productCd-3527310355,subjectCd-HO40.html (accessed on 17 August 2017).

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Part 3 Page 71 - 79	**2.4 Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry – Cases of contamination with *S. cerevisiae* var. *diastaticus* in the period 2008 to 2017**

Saccharomyces cerevisiae var. *diastaticus* is considered to be the most hazardous spoiling yeast in beer and beer-mixed beverages. This yeast is difficult to detect as it can competes directly with the culture strains. In the filled product *S. cerevisiae* var. *diastaticus* causes chemical, physical and sensorial changes due to its ability to ferment residual carbohydrates such as higher dextrin and starch, which are normally not metabolized by brewing culture strains. This super-attenuation leads to an increase in the carbon dioxide concentration, which often results in gushing and bottle bursting. The consequence of this can be product recalls and a loss of benefits and consumer reputation.

This paper presents an overview of the increase in contamination with *S. cerevisiae* var. *diastaticus* and the importance of detection in breweries and the beverage industry in Europe over the past 9.5 years (January 2008 to June 2017). A total of 126 *S. cerevisiae* var. *diastaticus* instances in 52 European companies are evaluated according to their origin (beer, beer-mixed beverages, non-alcoholic beverages, etc.), country, year, and type of contamination. Most of the positive findings occur as secondary contaminations during the filling process in the bottling area or to biofilms in the pipework system of the filler. Only a small number can be traced back to primary contamination in the brewhouse, fermentation cellar and storage cellar.

The evaluation study shows that six positive contaminations with *S. cerevisiae* var. *diastaticus* were detected every year and instances have been steadily increasing since 2015. Most contaminations occur during the third quarter of the year, when beer production and consumption was at the highest level.

<u>Authors/Authorship contribution:</u>

Meier-Dörnberg, T.: Data analysis and interpretation; **Jacob F.**: Supervised the project; **Michel, M.**: Critical review; **Hutzler, M.**: Provided microbiological data of accredited laboratory (TUM RCW BLQ), support in the statistical analysis of data and critical content review

MBAA TQ vol. 54, no. 4 • 2017 • pp. 140–148

Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the Beverage Industry: Cases of Contamination, 2008–2017

Tim Meier-Dörnberg, Fritz Jacob, Maximilian Michel, and Mathias Hutzler
Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany

ABSTRACT

Saccharomyces cerevisiae var. *diastaticus* is an obligatory spoilage microorganism in the beverage industry with high spoilage potential owing to its glucoamylase activity. *S. cerevisiae* var. *diastaticus* yeast strains can lead to an increase of the carbon dioxide concentration in beverages, with gushing and bottle bursting, caused by their superattenuating ability, as possible consequences. Therefore, the determination of beverage contamination by *S. cerevisiae* var. *diastaticus* is of significant interest. This article gives an overview of the incidence of *S. cerevisiae* var. *diastaticus* contamination in Europe over the last 9.5 years (2008–2017). The paper is based on analytical data of about 126 *S. cerevisiae* var. *diastaticus* instances in 52 companies (anonymous) in Europe recorded by the Research Center Weihenstephan for Brewing and Food Quality of the Technical University of Munich, presented according to their origin (beer, beer-mixed beverages, nonalcoholic beverages, etc.), country, year, and type of contamination. The accredited microbiological laboratory of the Research Center Weihenstephan investigates approximately 15,000 microbiological samples of the beverage industry worldwide per year. Real-time polymerase chain reaction analysis for *S. cerevisiae* var. *diastaticus* was conducted and evaluated. About six positive contaminations were detected every year, and 71% of them were caused by contamination events during the filling process of beverages. An increase in contamination incidents and confirmed positive findings can be observed over the last two years. Most problems with *S. cerevisiae* var. *diastaticus* contaminations occurred during the third quarter of the year. This analytical evaluation clearly shows the increase in contamination with *S. cerevisiae* var. *diastaticus* and the importance of detection in the breweries and the beverage industry in general.

Keywords: *Saccharomyces cerevisiae* var. *diastaticus*, Brewing yeasts, Spoilage yeast, Yeast characterization, Superattenuating

Introduction

The aim of every brewer is to manufacture a reproducible and flawless beer. Problems, particularly microbiological issues, can spoil the product. Beer spoilage microorganisms are therefore assessed in terms of their spoilage potential. The most hazardous and widespread of beer spoilage organisms belong to the class of so-called obligate beer spoilage organisms, which are still able to propagate in beer despite the low pH value, bitter substances in the hops, and very low oxygen concentrations. These organisms change the product both in terms of its physical-chemical and sensory properties as a result of metabolization. In addition to beer-spoilage bacteria such as *Lactobacillus brevis*, *Pectinatus*, and *Megasphaera*, so-called beer spoilage yeasts also play an often forgotten role (11).

Beer spoilage yeasts belong to both *Saccharomyces* and non-*Saccharomyces* types. *Saccharomyces* beer-spoiling yeasts are often regarded as being the most hazardous because they are difficult to differentiate from *Saccharomyces* brewing strains and directly compete with the culture strains. In particular, *Saccharomyces cerevisiae* var. *diastaticus* is considered to be an obligatory spoilage microorganism and spoilage yeast (e.g., wild yeast) in beer and beer-mixed beverages (2,7,12). Compared with *Saccharomyces* brewing culture yeasts, *S. cerevisiae* var *diastaticus* yeast strains are able to metabolize residual carbohydrates in naturally conditioned beers such as complex dextrins and starches. This physiological property is considered to be connected to *STA* genes encoding for the enzyme glucoamylase (1,12,14,18). These amylolytic strains of *Saccharomyces* are classified by classical taxonomic criteria to be a separate species from *S. cerevisiae.* However, genetic differences do not make such a clear separation, and genome sequence data unequivocally show that they are strains of the species *S. cerevisiae* (15). Despite multilocus and genome sequencing to solve taxonomic problems, many archaic and misleading synonyms are unfortunately still in use (8). Up to now, there is no correct taxonomic term for superattenuating yeasts of the genus *Saccharomyces*, but the term *S. cerevisiae* var. *diastaticus* is widely used throughout several publications (3,13,16,17). Contamination with *S. cerevisiae* var. *diastaticus* can cause changes in taste, sediment formation, turbidity, gushing, and swelling of the package. Additional fermentation in filled containers (such as bottles, cans, kegs, or disposable drums) can further cause a sharp increase in the carbon dioxide concentration in the product, and bottle bursting can be the consequence (5,21). In Germany, breweries and beverage manufacturers are legally obliged to report positive analysis results and potentially to remove sold products from the market in a public product recall. In cases of recurrent contamination and if entire batches are affected, the contamination source can usually be located and eliminated by microbiological monitoring. Affected batches are not placed on the market. Contamination in the bottling area is generally the greater evil. This type of contamination generally occurs as so-called scatter

Phone: +49-8161-71-3100. E-mail: m.hutzler@tum.de

http://dx.doi.org/10.1094/TQ-54-4-1130-01
© 2017 Master Brewers Association of the Americas

contamination and only affects single or several bottles. Such contamination can occur by aerosol infection owing to hygienic problems of the filler environment or by a so-called wash-out effect of biofilms in the pipework system of the filler. These kinds of trace contaminations are difficult to detect, and this is why finding evidence of *S. cerevisiae* var. *diastaticus* before placing the contaminated product on the market is so difficult. A microbiological contamination with *S. cerevisiae* var. *diastaticus* poses a significant threat to breweries and consumers alike. Product recalls owing to microbiological contaminations with *S. cerevisiae* var. *diastaticus* can cause economic losses and occasionally expose the consumer to risk of injury (4). The purpose of the work presented in this paper is to determine beverage contaminations by *S. cerevisiae* var. *diastaticus* and therefore to show the increase and the importance of detection in breweries and the beverage industry in general. Additional knowledge about the type of contamination and the time of the year *S. cerevisiae* var. *diastaticus* contaminations mainly occur can be helpful for brewers and beverage technologists to avoid microbiological problems. The paper further shows the increasing significance of *S. cerevisiae* var. *diastaticus* as a spoilage yeast.

Materials and Methods

Data Collection and Evaluation

The data collected are based on analysis data at the Technical University of Munich at the Research Center Weihenstephan for Brewing and Food Quality (BLQ). The accredited microbiological laboratory of the BLQ investigates approximately 15,000 microbiological samples of the beverage industry worldwide per year. Instances of *S. cerevisiae* var. *diastaticus* were collected from 2008 to June 2017 since the real-time polymerase chain reaction (PCR) for *S. cerevisiae* var. *diastaticus* was introduced. Instance was defined as being all investigated samples of the same customer/company when contamination with superattenuating yeast was suspected. Therefore, we defined a *S. cerevisiae* var. *diastaticus* instance as when we investigated a suspected *S. cerevisiae* var. *diastaticus* problem in a brewery on site, or when we received multiple samples from one specific brewery within a 3 month period. The result can be positive (positive finding) or negative (negative finding). Positive findings were evaluated as being only those analyses in which real-time PCR provided positive evidence of *S. cerevisiae* var. *diastaticus* (target DNA region *STA1* gene, method described in the next section). Further positive findings for microbiological organisms besides *S. cerevisiae* var. *diastaticus* are not shown and will not be considered in the article. The sample distribution and the number of samples sent to the BLQ depended on the customers/companies. The distribution had a shift depending on the number and structure of customers. Approximately 50% of the customers were German breweries and 35% were from other European countries. As a result, the number of positive findings was always related to the total number of samples sent. The terms primary and secondary contamination identify the type of contamination. Primary contaminations were defined as contamination of the wort, of the yeast (yeast crop and Kräusen addition), of the fermentation, bright beer, and storage tanks, and of the filtration systems used. Secondary contamination was defined as contaminations owing to hygienic problems of the filler environment and the filler hygiene itself. Such contaminations can be traced back to biofilms in the pipework system of the filler or can occur as an aerosolization of contaminating organisms owing to areas of the filler with spoilage organisms, which can become aerosolized by the vortex action of the filler, and dysfunctional drains near the filler that are not draining properly or not maintained with any regularity.

DNA Extraction and Real-Time PCR

DNA isolation and real-time PCR were performed according to the methods of Hutzler and Meier-Dörnberg (9,10,19,20). Specific real-time PCR system patterns can identify *S. cerevisiae* var. *diastaticus*. A specific primer and probe system is situated on the glucoamylase *STA1* gene (1). To amplify the specific rDNA region, the primers Sd-f (5´-TTCCAACTGCA CTAGTTCCTAGAGG-3´) and Sd-r (5´-GAGCTGAATGGAG TTGAAGATGG-3´) and the probe Sdia (5´-FAM-CCTCCTCT AGCAACATCACTTCCTCCG-BHQ1-3´) were used according to the method of Brandl (6). Other systems were also applied to prove the species identity *S. cerevisiae* and the absence of other *Saccharomyces* species. All real-time PCR reactions were performed from single colonies that were obtained as described below. Process and product samples that showed yeast contamination (turbidity, CO_2 development, off-flavor, microscopic picture) were plated on yeast mold (YM) agar, and single colonies were picked for real-time PCR identification. YM agar was produced according to the method of Hutzler (9) (3.0 g of broth malt extract, 3.0 g of yeast extract, 5.0 g

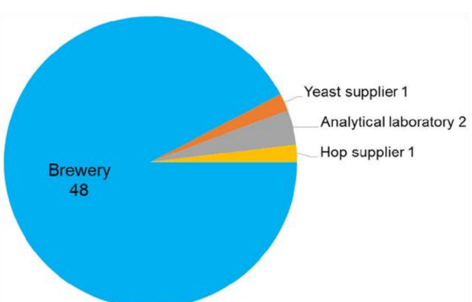


Figure 1. Total number of companies (n = 52) according to type of operation.

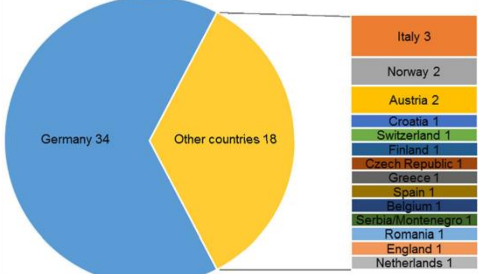


Figure 2. Total number of companies (n = 52) categorized according to operating site (countries) from 2008 to June 2017.

of peptone, 11.0 g of glucose monohydrate, 20.0 g of agar, and 1,000 mL of distilled water, incubated aerobic at 20°C for 5 days). In some cases a liquid preenrichment of yeast was necessary (suspicious samples without obvious spoilage appearance).

- For universal preenrichment, double-concentrated YM broth (without agar) was mixed 1:1 with the liquid sample and incubated at 28°C for 5 days. Sediment was plated on YM agar to obtain single colonies.
- For preenrichment in samples that contained bottom-fermenting brewer's yeast, *S. pastorianus*, double-concentrated YM broth was mixed 1:1 with the liquid sample and incubated at 37°C (selective conditions that suppress *S. pastorianus* growth) for 5 days. Sediment was plated and incubated on YM agar (see above).
- For preenrichment in samples that contained top-fermenting brewer's yeast, *S. cerevisiae*, double-concentrated YM broth containing 400 ppm of $CUSO_4$ was mixed 1:1 with the liquid sample and incubated at 28°C (selective condi-

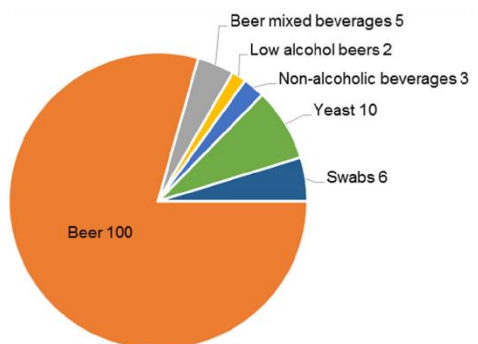


Figure 4. Total number of *S. cerevisiae* var. *diastaticus* instances (n = 126) categorized in a matrix for the period 2008 to June 2017.

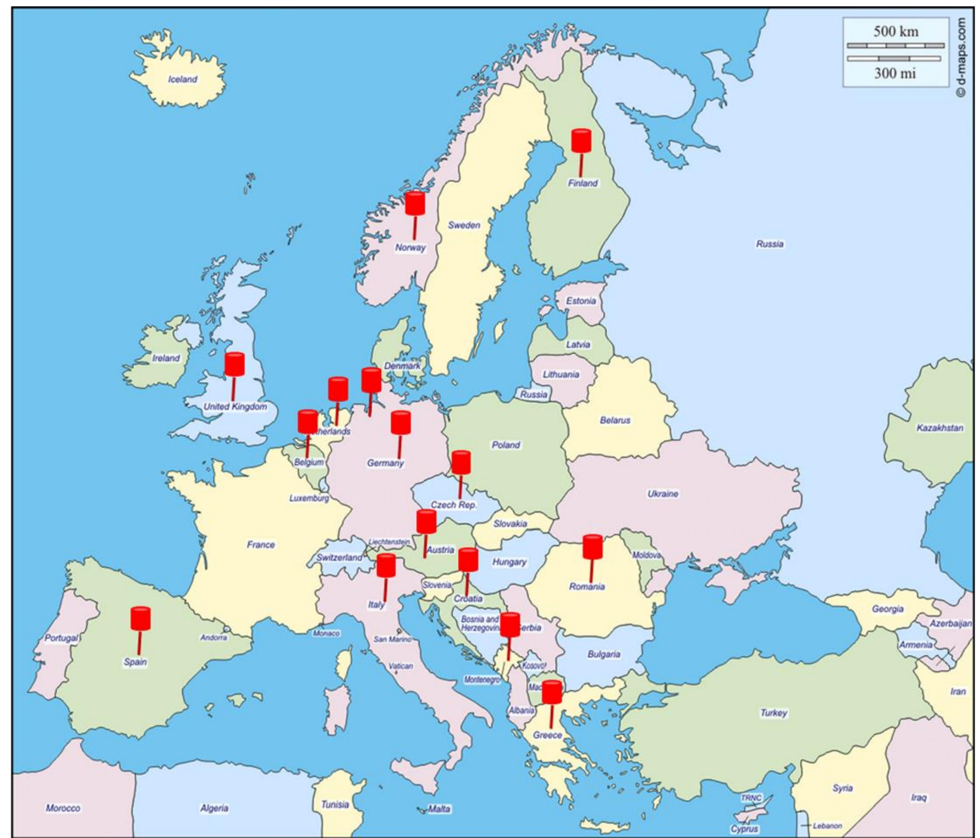


Figure 3. Overview of the investigations for *S. cerevisiae* var. *diastaticus* in Europe (source of the European map: http://d-maps.com/carte.php?num_car=13146&lang=en).

Table 1. Number of negative and positive findings of *S. cerevisiae* var. *diastaticus* per year from January 2008 to June 2017

Year	Negative finding	Positive finding	Total per year
2008	0	1	1
2009	0	1	1
2010	4	4	8
2011	11	4	15
2012	4	5	9
2013	7	4	11
2014	3	3	6
2015	10	17	27
2016	18	19	37
01–06/2017	7	4	11
Total 01/2008 to 06/2017	64	62	126
Mean/month for 114 months	0.56	0.54	1.11

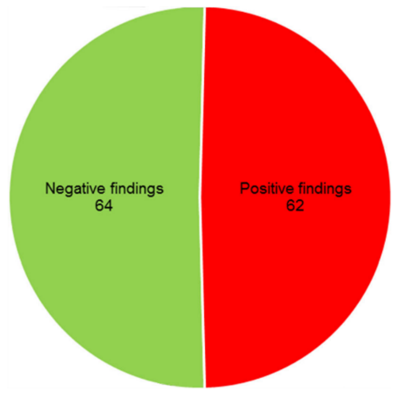


Figure 5. Findings of *S. cerevisiae* var. *diastaticus* (n = 126) for the period 2008 to June 2017.

tions of 200 ppm $CUSO_4$ in final mixed liquid that suppress *S. cerevisiae* growth) for 5 days. Sediment was plated and incubated on YM agar to obtain single colonies (see above).

All real-time PCR results that were performed with the specific *STA1* gene real-time PCR system between 2008 and June 2017 are shown in our study. Species identity was confirmed by other real-time PCR systems as well. Negative results can occur when there are suspicious batches, single samples, or process steps that might be contaminated but *S. cerevisiae* var. *diastaticus* could not be proved in these samples (even with preenrichment steps).

S. cerevisiae var. *diastaticus* Instances from 2008 to June 2017

Total Number of Companies Categorized According to Type of Company and Operating Site (Country)

In the past 9.5 years (2008–2017), samples from a total of 52 different businesses were analyzed for contamination with *S. cerevisiae* var. *diastaticus* (positive and negative findings listed). With the exception of two analytical labs, a hop supplier, and a yeast supplier, these businesses were small-scale and large breweries (Fig. 1).

Of these 52 companies, 65% (i.e., 34 companies) were based in Germany, and the remaining 35% (18 companies) were spread over 14 other European countries. Three customers had their operating site in Italy, two in Austria, and two in Norway. The remaining 11 companies were spread over another 11 European countries. PCR analysis for *S. cerevisiae* var. *diastaticus* was performed for all 52 companies, and therefore the *S. cerevisiae* var. *diastaticus* instances are distributed across 15 countries in Europe (Figs. 2 and 3).

Incidence and Findings in the Beverage Industry

As Figure 4 shows, a total of 126 *S. cerevisiae* var. *diastaticus* instances were investigated within the last 9.5 years (2008

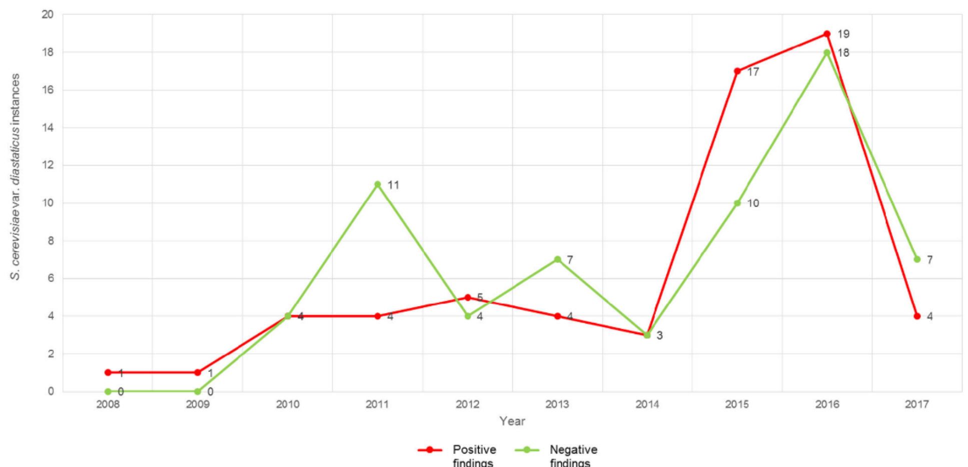


Figure 6. Number of negative and positive findings of *S. cerevisiae* var. *diastaticus* per year from 2008 to June 2017.

to June 2017). This corresponds to 13 instances a year on average or one instance per month (Table 1).

For the 52 companies in this period there was an average of nearly 2.5 incidents and one positive finding per company. It can therefore be assumed that, depending on the type (primary or secondary contamination), contamination with *S. cerevisiae* var. *diastaticus* is difficult to control using conventional brewery countermeasures (cleaning and disinfection) and/or the source of contamination could not be precisely located, which caused further incidents and therefore investigations for each company (Fig. 4).

As Figure 5 shows, 62 positive findings (just under half of the 126 investigated *S. cerevisiae* var. *diastaticus* instances) were established and confirmed.

Figures 6 and 7 show the general results and incidence of *S. cerevisiae* var. *diastaticus* during the period 2008 to June 2017. As shown in the figures, there was a significant increase in investigated instances of *S. cerevisiae* var. *diastaticus* starting in 2015 (27 of 126). In 2016, there were 37 investigated instances. At 19 positive findings, most of the *S. cerevisiae* var. *diastaticus* instances and confirmed contaminations over the last 9.5 years occurred in 2016.

Despite the lower number of investigations and confirmed findings so far in 2017 (first six months), a further increase in investigations is expected compared with 2016, because Figure 7 and Table 2 show that the majority of investigations took place in the late summer months of July to September and therefore in the third quarter of the year.

Type of Contamination in the Positive Findings

Table 3 categorizes the positive findings from 2008 to June 2017 according to their contamination type. In total, 18 of the 62 positive findings were attributed to the brewhouse, fermentation cellar, and storage cellar and were therefore classified as primary contamination.

Most of the confirmed contaminations, with a total of 71%, consisted of secondary contamination: 44 out of the 62 positive findings for *S. cerevisiae* var. *diastaticus* were found in the bottling hall when filling the product in bottles, cans, or kegs. The secondary contamination can be traced back to contaminants in the filler environment (confirmed by swabs) and/or biofilms in the pipework system of the filler. Such contamination can occur by aerosol infection owing to hygienic problems of the filler environment and/or by the so-called wash-out effect of the biofilm in the pipework system of the filler. Aerosol infections usually occur as scattered contamination in single packages and containers (e.g., bottles, cans, and kegs), whereas contaminations owing to biofilms mostly infect the first few packages and containers after starting the filling process.

Incidence and Findings in the Beverage Industry, According to the Matrix

As Figure 8 and Table 4 show, 93.5% of all the 62 proven *S. cerevisiae* var. *diastaticus* findings relate to direct contamination of the product. In total, 56 positive findings were evidenced in beer and two positive findings in beer-mixed beverages.

In addition to these 58 product contaminations (56 beer, two beer-mixed beverages), a further two positive findings were related to the yeast used (pitching yeast and Kräusen addition) and another two positive findings to swab samples taken in the bottling area. No positive result could be detected in the two analyzed low-alcohol beers or the three analyzed nonalcoholic beverages such as lemonade, cola, and so on.

Table 5 shows the number of positive and negative findings per matrix categorized according to the respective country

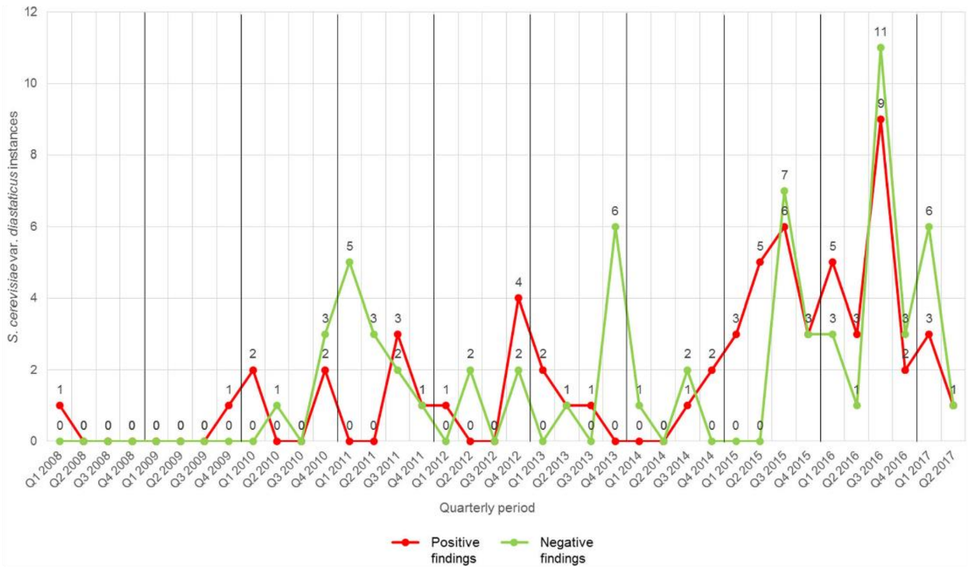


Figure 7. Number of negative and positive findings of *S. cerevisiae* var. *diastaticus* per quarter from 2008 to June 2017.

from which the samples came. As already illustrated in the section on data collection and evaluation, 34 of the 52 clients had their operating site in Germany. As evident from the table, the highest numbers of positive and negative cases were reported in Germany. A total of 60 cases were investigated, and 37 of these cases were analyzed directly in the beer product. Within the categories of beer-mixed beverages, low-alcohol beers, and nonalcoholic beverages, all analyzed cases were reported to be in Germany. With a total of 31 *S. cerevisiae* var. *diastaticus* cases, Norway had the second highest level of investigated beer samples and samples overall.

Positive Findings in Beer According to Country

All the confirmed contaminations (positive findings by real-time PCR) with *S. cerevisiae* var. *diastaticus* in beer are detailed and presented in Figure 9 and Table 6 according to country and year from 2008 to June 2017. The majority of beer contaminations occurred in 2015 and 2016, with 17 positive incidents in each case. In both years, most of the positive findings occurred in Norway.

In Norway, a total of 18 positive instances were recorded, 12 of which occurred in 2015 and six in the following year. Over the entire investigation period from 2008 to June 2017, Germany showed the most product contaminations, with a total of 23 positive findings spread over the years 2010 to June 2017. In England and Greece, beer samples were analyzed on the suspicion of contamination, but these were not confirmed as positive.

Number of Customers per Year Categorized According to Their Type of Business

From 2008 to June 2017, 126 instances of *S. cerevisiae* var. *diastaticus* were reported and then investigated. These cases were spread over a total of 52 different companies, which means that several companies reported multiple cases. Table 7 shows the total number of cases and customers per year categorized according to type of operation. The difference between the total number of 52 companies and the listed total number of 71 customers is owing to the year-dependent evaluation. The same company is evaluated as just one customer, if it has commissioned multiple analyses in the same year.

As already shown in the section on DNA extraction and real-time PCR, the highest number of cases was 37, reported and investigated in 2016. These cases were distributed across a total of 17 different small- and large-scale breweries, which

Table 2. Number of negative and positive findings of *S. cerevisiae* var. *diastaticus* per quarter from 2008 to June 2017

Quarter	Negative finding	Positive finding	Total	Total per year
01–03/2008	0	1	1	1
04–06/2008	0	0	0	
07–09/2008	0	0	0	
10–12/2008	0	0	0	
01–03/2009	0	0	0	1
04–06/2009	0	0	0	
07–09/2009	0	0	0	
10–12/2009	0	1	1	
01–03/2010	0	2	2	8
04–06/2010	1	0	1	
07–09/2010	0	0	0	
10–12/2010	3	2	5	
01–03/2011	5	0	5	15
04–06/2011	3	0	3	
07–09/2011	2	3	5	
10–12/2011	1	1	2	
01–03/2012	0	1	1	9
04–06/2012	2	0	2	
07–09/2012	0	0	0	
10–12/2012	2	4	6	
01–03/2013	0	2	2	11
04–06/2013	1	1	2	
07–09/2013	0	1	1	
10–12/2013	6	0	6	
01–03/2014	1	0	1	6
04–06/2014	0	0	0	
07–09/2014	2	1	3	
10–12/2014	0	2	2	
01–03/2015	0	3	3	27
04–06/2015	0	5	5	
07–09/2015	7	6	13	
10–12/2015	3	3	6	
01–03/2016	3	5	8	37
04–06/2016	1	3	4	
07–09/2016	11	9	20	
10–12/2016	3	2	5	
01–03/2017	6	3	9	11
04–06/2017	1	1	2	
Total	64	62	126	126

Table 3. Number of findings of *S. cerevisiae* var. *diastaticus* and type of contamination per year from 2008 to June 2017

	Finding		Type of contamination and number of positive findings[a]	
Year	Negative	Positive	Primary	Secondary
2008	0	1	0	1
2009	0	1	0	1
2010	4	4	2	2
2011	11	4	1	3
2012	4	5	1	4
2013	7	4	1	3
2014	3	3	0	3
2015	10	17	5	12
2016	18	19	7	12
01–06/2017	7	4	1	3
Total	64	62	18	44
	126		62	

[a] Primary contamination is in the brewhouse, fermentation cellar, and storage cellar, and secondary contamination is in the bottling hall.

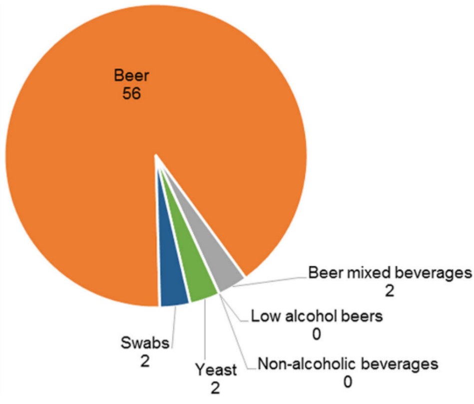


Figure 8. Total number of *S. cerevisiae* var. *diastaticus* positive findings ($n = 62$) characterized by matrix for the period 2008 to June 2017.

Table 4. Number of negative and positive findings of *S. cerevisiae* var. *diastaticus* per matrix per year from January 2008 to June 2017

	Beer		Beer-mixed beverage		Low-alcohol		Nonalcoholic beverage		Yeast		Swab		
Year	–	+	–	+	–	+	–	+	–	+	–	+	**Total**
2008	0	1	0	0	0	0	0	0	0	0	0	0	1
2009	0	0	0	1	0	0	0	0	0	0	0	0	1
2010	3	4	0	0	0	0	0	0	1	0	0	0	4
2011	5	3	2	0	0	0	0	0	4	0	0	1	4
2012	2	5	1	0	0	0	0	0	0	0	1	0	5
2013	6	2	0	1	0	0	0	0	1	1	0	0	4
2014	1	3	0	0	0	0	1	0	1	0	0	0	3
2015	10	17	0	0	0	0	0	0	0	0	0	0	17
2016	11	17	0	0	2	0	2	0	0	1	3	1	19
01–06/2017	6	4	0	0	0	0	0	0	1	0	0	0	4
Total	44	56	3	2	2	0	3	0	8	2	4	2	126
Total	100		5		2		3		10		6		126

Table 5. Number of positive findings of *S. cerevisiae* var. *diastaticus* per country categorized in the matrix from January 2008 to June 2017

	Beer		Beer-mixed beverage		Low-alcohol		Nonalcoholic beverage		Yeast		Swab		
Country	–	+	–	+	–	+	–	+	–	+	–	+	**Total**
Germany	18	19	3	2	2	0	3	0	7	1	3	2	60
Italy	2	4	0	0	0	0	0	0	0	0	0	0	6
Norway	13	18	0	0	0	0	0	0	0	0	0	0	31
Austria	3	2	0	0	0	0	0	0	0	0	0	0	5
Croatia	0	1	0	0	0	0	0	0	0	0	0	0	1
Switzerland	0	1	0	0	0	0	0	0	1	0	0	0	2
Finland	0	1	0	0	0	0	0	0	0	0	0	0	1
Czech Republic	0	1	0	0	0	0	0	0	0	0	0	0	1
Greece	1	0	0	0	0	0	0	0	0	0	0	0	1
Spain	0	1	0	0	0	0	0	0	0	0	0	0	1
Belgium	0	1	0	0	0	0	0	0	0	0	0	0	1
Serbia/Montenegro	0	1	0	0	0	0	0	0	0	0	0	0	1
Romania	1	2	0	0	0	0	0	0	0	0	0	0	3
England	1	0	0	0	0	0	0	0	0	0	0	0	1
Netherlands	5	4	0	0	0	0	0	0	0	1	1	0	11
Total	44	56	3	2	2	0	3	0	8	2	4	2	126
Total	100		5		2		3		10		6		126

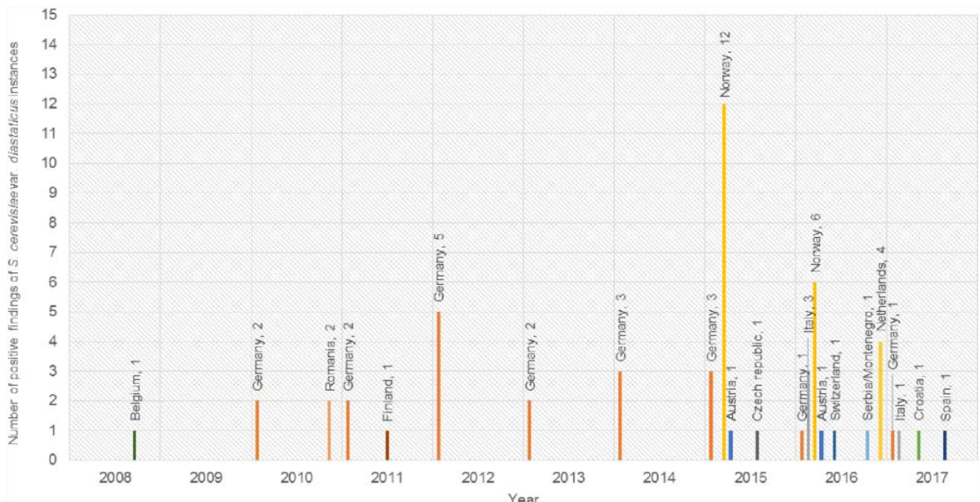


Figure 9. Number of positive findings of *S. cerevisiae* var. *diastaticus* in beer per year and country from 2008 to June 2017.

Table 6. Number of positive findings of *S. cerevisiae* var. *diastaticus* in beer per year and country from January 2008 to June 2017

Year/country	2008	2009	2010	2011	2012	2013	2014	2015	2016	01–06/2017	Total
Germany	0	0	2	2	5	2	3	3	1	1	19
Italy	0	0	0	0	0	0	0	0	3	1	4
Norway	0	0	0	0	0	0	0	12	6	0	18
Austria	0	0	0	0	0	0	0	1	1	0	2
Croatia	0	0	0	0	0	0	0	0	0	1	1
Switzerland	0	0	0	0	0	0	0	0	1	0	1
Finland	0	0	0	1	0	0	0	0	0	0	1
Czech Republic	0	0	0	0	0	0	0	1	0	0	1
Greece	0	0	0	0	0	0	0	0	0	0	0
Spain	0	0	0	0	0	0	0	0	0	1	1
Belgium	1	0	0	0	0	0	0	0	0	0	1
Serbia/Montenegro	0	0	0	0	0	0	0	0	1	0	1
Romania	0	0	2	0	0	0	0	0	0	0	2
England	0	0	0	0	0	0	0	0	0	0	0
Netherlands	0	0	0	0	0	0	0	0	4	0	4
Total	1	0	4	3	5	2	3	17	17	4	56

Table 7. Number of customers per year categorized according to type of company from January 2008 to June 2017

Year	Number of instances	Number of customers	Brewery	Yeast supplier	Analytical lab	Hop supplier
2008	1	1	1	0	0	0
2009	1	1	1	0	0	0
2010	8	3	2	0	1	0
2011	15	11	10	0	1	0
2012	9	6	6	0	0	0
2013	11	9	7	1	1	0
2014	6	6	6	0	0	0
2015	27	8	7	0	1	0
2016	37	17	17	0	0	0
01–06/2017	11	9	8	0	0	1
Total	126	71	65	1	4	1

also represent the main customer base, overall and per year. In 2013, a yeast supplier and an analytical laboratory, in addition to breweries, also sent samples to investigate contamination with *S. cerevisiae* var. *diastaticus*. In the current year, 2017, the customer base also included a hop supplier.

Summary

Overall, 126 cases from a total of 52 companies from 15 countries in Europe were investigated for contamination with *S. cerevisiae* var. *diastaticus* over the past 9.5 years (January 2008 to June 2017), and 62 of these cases were confirmed with a positive result for strains of this yeast. With 71% in total, most confirmed cases (real-time PCR *STA1* gene positive) occurred as secondary contamination in the bottling area (bottling hall) and were attributed to contaminants in the filler environment (confirmed by swabs) and/or to biofilms in the pipework system of the filler. Just 29% of cases related to primary contamination in the brewhouse, fermentation cellar, and storage cellar. Beer, beer-mixed beverages, nonalcoholic beverages, low-alcohol beers, yeast samples, and swabs from breweries, yeast suppliers, hop suppliers, and analytical labs were investigated. Starting in 2015, an increase was recorded in contaminations and confirmed positive results, showing the most cases in the third quarter of each year.

REFERENCES

1. Adam, A. C., Latorre-García, L., and Polaina, J. (2004). Structural analysis of glucoamylase encoded by the *STA1* gene of *Saccharomyces cerevisiae* (var. *diastaticus*). Yeast. 21: 379-388. doi: 10.1002/yea.1102.
2. Andrews, J., and Gilliland, R. B. (1952). Super-attenuation of beer: A study of three organisms capable of causing abnormal attenuations. J. Inst. Brew. 58: 189-196.
3. Bayly, J. C., Douglas, L. M., Pretorius, I. S., Bauer, Florian F., and Dranginis, A. M. (2005). Characteristics of *Flo11*-dependent flocculation in *Saccharomyces cerevisiae*. FEMS Yeast Res. 5: 1151-1156. doi: 10.1016/j.femsyr.2005.05.004.
4. Begrow, W. (2017). Fighting quality threats. Brew. Beverage Ind. Int., 10-13.
5. Boulton, C., and Quain, D. (2009). Brewing Yeast and Fermentation. Blackwell Science, Oxford, U.K.
6. Brandl, A. (2006). Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien. Technische Universität München, Weihenstephan, Germany.
7. Folz, R., Hofmann, R., and Stahl, U. (2011). Impact of permeation of O_2 and CO_2 on the growth behaviour of *Saccharomyces diastaticus* in beer. Brew. Sci. 64: 52-60.
8. Hittinger, C. T. (2013). *Saccharomyces* diversity and evolution: A budding model genus. Trends Genet. 29: 309-317. doi: 10.1016/j.tig.2013.01.002.
9. Hutzler, M. (2009). Entwicklung und Optimierung von Methoden zur Identifizierung und Differenzierung von getränkerelevanten Hefen, Technische Universität München, Weihenstephan, Germany.
10. Hutzler, M., Geiger, E., and Jacob, F. (2010). Use of PCRDHPLC (polymerase chain reaction denaturing high performance liquid chromatography) for the rapid differentiation of industrial *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* strains. J. Inst. Brew. 116: 464-474.
11. Hutzler, M., Koob, J., Riedl, R., Schneiderbanger, H., Müller-Auffermann, K., and Jacob, F. (2015). Yeast identification and characterization. Pages 65-105 in: Brewing Microbiology: Managing Microbes, Ensuring Quality and Valorising Waste. A. E. Hill, ed. Elsevier, Netherlands.

12. Hutzler, M., Riedl, R., Koob, J., and Jacob, F. (2012). Fermentation and spoilage yeasts and their relevance for the beverage industry—A review. Brew. Sci. 65: 33-52.
13. Jespersen, L., van der Aa Kühle, A., and Petersen, K. M. (2000). Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeasts. Int. J. Food Microbiol. 60: 43-53. doi: 10.1016/S0168-1605(00)00326-3.
14. Latorre-García, L., Adam, A. C., Manzanares, P., and Polaina, J. (2005). Improving the amylolytic activity of *Saccharomyces cerevisiae* glucoamylase by the addition of a starch binding domain. J. Biotechnol. 118: 167-176. doi: 10.1016/j.jbiotec.2005.03.019.
15. Liti, G., Carter, D. M., Moses, A. M., Warringer, J., Parts, L., James, S. A., Davey, R. P., Roberts, I. N., Burt, A., Koufopanou, V., Tsai, I. J., Bergman, C. M., Bensasson, D., O'Kelly, M. J. T., van Oudenaarden, A., Barton, D. B. H., Bailes, E., Nguyen, A. N., Jones, M., Quail, M. A., Goodhead, I., Sims, S., Smith, F., Blomberg, A., Durbin, R., and Louis, E. J. (2009). Population genomics of domestic and wild yeasts. Nature 458: 337-341. doi: 10.1038/nature07743.
16. Marín-Navarro, J., Gurgu, L., Alamar, S., and Polaina, J. (2011). Structural and functional analysis of hybrid enzymes generated by domain shuffling between *Saccharomyces cerevisiae* (var. *diastaticus*) *Sta1* glucoamylase and *Saccharomycopsis fibuligera Bgl1* β-glucosidase. Appl. Microbiol. Biotechnol. 89: 121-130. doi: 10.1007/s00253-010-2845-3.
17. Marín-Navarro, J., and Polaina, J. (2011). Glucoamylases: Structural and biotechnological aspects. Appl. Microbiol. Biotechnol. 89: 1267-1273. doi: 10.1007/s00253-010-3034-0.
18. Meaden, P., Ogden, K., Bussey, H., and Tubb, R. S. (1985). A DEX gene conferring production of extracellular amyloglucosidase on yeast. Gene 34: 325-334. doi: 10.1016/0378-1119(85)90141-6.
19. Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.-J., and Jacob, F. (2017). The importance of a comparative characterization of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* strains for brewing. Fermentation 3: 41. doi: 10.3390/fermentation3030041.
20. Meier-Dörnberg, T., Michel, M., Wagner, R.-S., Jacob, F., and Hutzler, M. (2017). Genetic and phenotypic characterization of different top-fermenting *Saccharomyces cerevisiae* ale yeast isolates. Brew. Sci. 70: 9-25. doi: 10.23763/BRSC16-25MEIER-DOERNBERG.
21. Priest, F. G., and Campbell, I. (2003). Rapid identification of microorganisms. Pages 305-328 in: Brewing Microbiology. F. G. Priest and I. Campbell, eds. Springer, Boston, MA.

Part 4
Page 81 -107

2.5 *Saccharomyces cerevisiae* variety *diastaticus* friend or foe? – Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization

Saccharomyces cerevisiae var. *diastaticus* is an obligatory spoilage microorganism in the beverage industry. The amount of contaminations in beer and beer-mixed beverages has increased in the past three years, making it important to detect this microorganism and obtain reliable results on the spoilage potential of *S. cerevisiae* var. *diastaticus* strains.

The following paper investigates the spoilage potential of various *S. cerevisiae* var. *diastaticus* brewery isolates and their application potential for brewing purposes. It could be shown that the spoilage potential, e.g. the super-attenuating power is not directly linked to the presence of *STA1* gene encoding for the enzyme glucoamylase. A developed starch agar test provides a reliable and fast detection of strains with super-attenuating power. A modified Durham test detecting the formation of gas caused by the super-attenuation ability shows clear differences in the spoilage potential and the time needed for noticeable spoilage. Further investigation into the intermediate cell sizes and the sporulation behavior of the strains, and the developed characterization platform for small-scale brewing trials were used to investigate brewing properties and sensorial characteristics (based on publications Part 1 + Part 2). Most of the beers with *S. cerevisiae* var. *diastaticus* are characterized by a dry and winey body with noticeable phenolic off-flavors above the sensory threshold. One *S. cerevisiae* var. *diastaticus* strain did not show any spoilage or super-attenuating ability, even if the *STA1* gene could be verified by RT-PCR. This strain only fermented a low level of maltotriose and resulted in full-bodied beers with lots of fruity aromas and phenotypic brewing properties that were comparable with classical brewing culture strains.

It can be concluded that strains of *S. cerevisiae* var. *diastaticus* may be suitable for brewing applications and may be used in mixed fermentations or secondary bottle fermentation (e.g. post-fermentation) to produce beer specialties with a special flavor profile and/or a low carbohydrate content.

Authors/Authorship contribution:

Meier-Dörnberg, T.: Literature search, writing, data creation and interpretation, study conception and design; **Kory, O.I.**: Data creation, support of method development; **Jacob, F.**: Supervised the project; **Michel, M.**: Support with real-time PCR analytics and interpretation; **Hutzler M.**: Modification of acetate-medium for spore formation, Developed accredited RT-PCR compatible *STA1* detection method, supported the creation of research plan, critical content review.

FEMS Yeast Research, 18, 2018, foy023

doi: 10.1093/femsyr/foy023
Advance Access Publication Date: 6 March 2018
Research Article

RESEARCH ARTICLE

Saccharomyces cerevisiae variety diastaticus friend or foe?—spoilage potential and brewing ability of different *Saccharomyces cerevisiae* variety diastaticus yeast isolates by genetic, phenotypic and physiological characterization

Tim Meier-Dörnberg[1], Oliver Ingo Kory[2], Fritz Jacob[1], Maximilian Michel[1] and Mathias Hutzler[1,*]

[1]Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality, Alte Akademie 3, 85354 Freising, Germany and [2]Miho Inspektionssysteme GmbH, Obervellmarsche Str. 12, 334292 Ahnatal, Germany

*Corresponding author: Technical University of Munich, Research Center Weihenstephan for Brewing and Food Quality, Alte Akademie 3, 85354 Freising, Germany. Tel: +49(0)8161713100; Fax: +49(0)8161714181; E-mail: m.hutzler@tum.de

One sentence summary: This paper presents novel findings on the spoilage potential and the use of *S. cerevisiae* var. *diastaticus* yeast strains as beer fermentation starter cultures.
Editor: Isak Pretorius

ABSTRACT

Saccharomyces cerevisiae variety *diastaticus* is generally considered to be an obligatory spoilage microorganism and spoilage yeast in beer and beer-mixed beverages. Their super-attenuating ability causes increased carbon dioxide concentrations, beer gushing and potential bottle explosion along with changes in flavor, sedimentation and increased turbidity. This research shows clear differences in the super-attenuating properties of *S. cerevisiae* var. *diastaticus* yeast strains and their potential for industrial brewing applications. Nineteen unknown spoilage yeast cultures were obtained as isolates and characterized using a broad spectrum of genetic and phenotypic methods. Results indicated that all isolates represent genetically different *S. cerevisiae* var. *diastaticus* strains except for strain TUM PI BA 124. Yeast strains were screened for their super-attenuating ability and sporulation. Even if the *STA1* gene responsible for super-attenuation by encoding for the enzyme glucoamylase could be verified by real-time polymerase chain reaction, no correlation to the spoilage potential could be demonstrated. Seven strains were further characterized focusing on brewing and sensory properties according to the yeast characterization platform developed by Meier-Dörnberg. Yeast strain TUM 3-H-2 cannot metabolize dextrin and soluble starch and showed no spoilage potential or super-attenuating ability even when the strain belongs to the species *S. cerevisiae* var. *diastaticus*. Overall, the beer produced with *S. cerevisiae* var. *diastaticus* has a dry and winey body with noticeable phenolic off-flavors desirable in German wheat beers.

Keywords: *Saccharomyces cerevisiae* variety diastaticus; brewing; spoilage yeast; yeast characterization; super-attenuation; fermentation

Received: 12 January 2018; **Accepted:** 5 March 2018
© FEMS 2018. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

INTRODUCTION

Saccharomyces cerevisiae var. *diastaticus* is generally considered to be an obligatory spoilage microorganism and spoilage yeast (i.e. wild yeast) in beer and beer-mixed beverages (Andrews and Gilliland 1952; Folz, Hofmann and Stahl 2011; Hutzler *et al.* 2012). As a widespread and abundant spoilage microorganism in bottled beverages, this yeast can cause changes in flavor, sedimentation or increased turbidity. *Saccharomyces cerevisiae* var. *diastaticus* is described as a super-attenuating yeast due to its ability to ferment residual carbohydrates in beer (dextrins and soluble starch), which are not normally metabolized by pure culture yeast strains. This so-called super attenuation leads to an increase in carbon dioxide (CO_2) caused by secondary fermentation and consequences include gushing of beer and bottle bursting (Priest and Campbell 2003; Boulton and Quain 2009). Even draft beer foams up and stops the tapping process working.

A microbiological contamination with *S. cerevisiae* var. *diastaticus* can cause economic losses and occasionally expose the consumer to risk of injury. In the event of a contamination, German breweries and food manufacturers are legally obliged to report this to the authorities to avoid administrative offence. Products that have already been sold are publicly recalled. In the event of a contamination, breweries have to recall their cases of beer resulting in a loss of profits and potentially harm their reputation with the consumer (Rees 2014; The Denver Post 2016). In 2014, the 10 Barell Brewing Co. recalled bottles of their sour beer named ‘Swill’. The recall was prompted by reports of beer gushing out of the bottles and one report of a glass bottle breaking as a result of contamination with *Saccharomyces cerevisiae* var. *diastaticus* (Rees 2014). In 2016, the Left Hand Brewing Company of Longmont, Colorado, USA, which the US Brewers Association ranks at 44 on the list of the largest craft breweries in the USA, recalled at least 20 000 cases of their craft beer called ‘Nitro Milk Stout’. The beer was gushing as a result of secondary fermentation and increased CO_2. It appears that the house ale strain was contaminated with the source of the contamination allegedly coming from White Labs stock culture (The Denver Post 2016; Begrow 2017). The company has now filed a lawsuit against the yeast supplier White Labs, which is accused of supplying yeast contaminated with *S. cerevisiae* var. *diastaticus* (The Denver Post 2017). All in all, product defects due to *S. cerevisiae* var. *diastaticus* may often be incorrectly categorized as production issues. Along with public recall processes, it is almost unheard of for a brewery to publicize the contamination and subsequently destroy a batch of beer (Begrow 2017).

Yeast strains of *S. cerevisiae* var. *diastaticus* occur as primary contaminants in the yeast/fermentation cellar and as secondary contaminants in the filling process. A primary contamination can lead to a competition with culture yeast during main fermentation and to a strong increase in the diastaticus cells in the fermentation substrate. Usually occurring as secondary contaminants derived from residues in bottles or in the formation of biofilms, this super-attenuating yeast can contaminate the finished product directly via contact with the product through beer lines, by air circulation in the area of the filling machine and the capper, by carryovers in the brewery or by insufficient heat treatment (flash- or tunnel pasteurizer). Even subsequent pasteurization of the product cannot always prevent the effects and harmful consequences of a previous contamination. Associated with a release of carbonic acid, pressure surges or the loss of pressure by the used booster pump can lead to cell growth in the product, caused by the ability to form ascispores in a single yeast cell. Contamination of the filled product is mostly a result of a random presence. This so-called scatter contamination is therefore almost impossible for breweries to detect. Subsequent detection of yeast cells in the bottled product is practically no longer possible. Most *S. cerevisiae* var. *diastaticus* contaminations are secondary contaminants and originate from poor hygienic conditions in the filler environment and/or from biofilms in the pipework system of the filler (Meier-Dörnberg *et al.* 2017b). Depending on the *S. diastaticus* yeast strain and the corresponding spoilage/damage potential, cell growth and resulting overproduction of CO_2, it takes longer for contamination to be detected visually or by tasting the product. A common brewery detection method uses fermented beer medium according to the Mitteleuropäischen Brautechnischen Analysenkommission e.V. (MEBAK). The first indication of an infection can be established by measuring the residual extract present in the beer.

The finding of a yeast which secreted diastase was firstly reported in 1943 and appears to be the first reported observation of a yeast of the *diastaticus* type (Gilliand 1966). In 1952, Andrews and Gilliland isolated yeast strains on several separate occasions from bottled beer from various breweries that were able to produce abnormal attenuation by fermenting soluble starch and dextrin in naturally conditioned beers. They referred to these yeast strains as super-attenuating yeasts and proposed the name *S. diastaticus* (Andrews and Gilliland 1952). In contrast to the classification system for yeast according to the fermentation of glucose, galactose, sucrose, raffinose, maltose and lactose as well as the size and shape of the cells, Andrews and Gilliand distinguished the yeast as a separate species as a result of the ability to ferment dextrin or starch (Andrews and Gilliland 1952). In a paper published by Gilliland in 1966, he justified the separation of starch-fermenting *Saccharomyces* as a separate species named *Saccharomyces diastaticus* (Gilliand 1966). To date, there is no correct taxonomic term for super-attenuating yeasts of the genus *Saccharomyces*. Adam described the taxonomic classification as follows: ‘Amylolytic strains of *Saccharomyces* are classified by classical taxonomic criteria as *S. diastaticus*, and they are considered to be a separate species from *S. cerevisiae* (Adam, Latorre-García and Polaina 2004). However, genetic differences do not make clear such a separation’. Liti also reported that genome sequence data unequivocally show that they are strains of *S. cerevisiae* (Liti *et al.* 2009). Despite multilocus and genome sequencing to solve taxonomic problems, many archaic and misleading synonyms are unfortunately still in use (Hittinger 2013). According to Vaughan-Martini, the yeast *S. cerevisiae* has more than 80 synonyms including *S. boulardii* and *S. diastaticus* (Vaughan-Martini and Martini 2011). The use of synonyms still results in misleading taxonomic terms. It is correct that *S. cerevisiae* var. *diastaticus* is not a correct taxonomic term, but it is used widely throughout several publications for super-attenuating/highly fermentative yeast strains belonging to the species *S. cerevisiae* (Jespersen, van der Aa Kühle and Petersen 2000; Bayly *et al.* 2005; Marín-Navarro and Polaina 2011; Marín-Navarro *et al.* 2011). This physiological property is described to be connected to *STA* genes encoding for the enzyme glucoamylase (Adam, Latorre-García and Polaina 2004; Hutzler *et al.* 2012). The *STA* genes are not present in normal *S. cerevisiae* strains and can therefore be used for species-specific identification of *S. cerevisiae* var. *diastaticus* yeast strains (Balogh and Maráz 1996). However, *S. cerevisiae* yeast strains are also able to build the enzyme glucoamylase. In the case of sporulation, they build an intracellular form of glucoamylase which were encoded by the sporulation-specific glucoamylase gene *SGA1*. Even if *S. cerevisiae* as well as *S. cerevisiae* var. *diastaticus* strains are

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

able to build the enzyme glucoamylase, only *S. cerevisiae* var. *diastaticus* strains show super-attenuation as a result of extracellular glucoamylase which will be secreted into the fermentation substrate during normal vegetative cell growth (Meaden *et al.* 1985; Latorre-García *et al.* 2005). The enzyme degrades starch and higher dextrins in the fermentation substrate into glucose units which can then be metabolized by the *S. cerevisiae* var. *diastaticus* yeast cells, whereas the natural substrate of the glucoamylase of *S. cerevisiae* strains is intracellular glycogen which were used at the beginning of fermentation or as a reserve carbohydrate while yeast storage (Adam, Latorre-García and Polaina 2004; Kunze 2011). We therefore consider it very important to differentiate and emphasize this special property in an industrial environment by using the terminology *S. cerevisiae* var. *diastaticus*.

Yeast strains of *S. cerevisiae* var. *diastaticus* are subject to current research projects for an alternative and direct, one-step process of starch fermentation. Commercial enzymes in particular, which are commonly used in the production process of industrial and fuel ethanol from starchy biomass, could be further replaced by the yeast (Laluce and Mattoon 1984). *Saccharomyces cerevisiae* var. *diastaticus* carrying at least one *STA* (*STA1*, *STA2* and *STA3*) or *DEX* gene produce extracellular glycoamylases for starch degradation (Laluce and Mattoon 1984; Yamashita, Hatano and Fukui 1984; Meaden *et al.* 1985). In conclusion, many researchers try to clone and transfer this enzyme into yeast cells by using sexual hybridization or induced protoplast fusion to increase the productivity of such targeted yeast strains for industrial purposes (Yamashita and Fukui 1983; Janderová *et al.* 1986; Erratt 1987; Latorre-García, Adam and Polaina 2008; Favaro, Basaglia and Casella 2012).

Saccharomyces cerevisiae var. *diastaticus* can lead to changes in flavor but does not give an unpleasant taste to beer (Andrews and Gilliland 1952). With better knowledge of phenotypic and physiological brewing properties, yeast strains with super-attenuating ability could be further used to produce carbohydrate-reduced and calorie-reduced dealcolyzed beers and beverages. Back in 1986, Janderová reported low-dextrin beers produced by adding fungal amyloglucosidase to the fermenter (Janderová *et al.* 1986). *Saccharomyces cerevisiae* var. *diastaticus* can be used in secondary or mixed fermentations to produce beers with low-carbohydrate content (Janderová *et al.* 1986; Vanderhaegen *et al.* 2002). Its use in high-gravity brewing can also increase profitability. Economy efficiency goes hand in hand with the availability and cost of raw materials and the technology for their conversion into a liquid fermentation substrate (Amin *et al.* 1985). Depending on the local conditions, this cost factor amounts to 50%–70% of the total production cost of ethanol (Faust, Präve and Schlingmann 1983).

In this case, the following research shows the potential of different *S. cerevisiae* var. *diastaticus* yeast strains as beer fermentation starter cultures and their suitability and potential to produce beers with low-carbohydrate content along with their resulting sensory profile. Additional testing into the sporulation behavior and the ability to build phenolic off-flavors will help to give strain-specific information. Furthermore, a detection method for the super-attenuating ability and the spoilage potential of *S. cerevisiae* var. *diastaticus* yeast strains was carried out to differentiate spoilage *S. cerevisiae* var. *diastaticus* yeast strains and culture strains in brewing practice. To do so, the presence of the glucoamylase gene *STA1* was investigated using a specific real-time polymerase chain reaction (RT-PCR) system to evaluate if it is directly linked to the super-attenuating ability.

MATERIALS AND METHODS

The methods in sections 'Genetic isolate identification and strain differentiation', 'Analytical methods' and 'Sensory evaluation' as well as the methods in 'Phenolic off-flavor test', Propagation and Fermentation were performed according to Meier-Dörnberg (Meier-Dörnberg *et al.* 2017a).

Yeast isolates and strains

A total of 32 yeast cultures were obtained in agar slants from the Yeast Center of the Research Center Weihenstephan for Brewing and Food Quality (BLQ) including 13 *Saccharomyces* brewing culture yeast strains (two bottom-fermenting *S. pastorianus* and 11 top-fermenting *S. cerevisiae* brewing yeast strains) and 19 spoilage yeast cultures. All spoilage yeasts were obtained as isolates and were given a TUM identifier (Table 1) except spoilage yeast DSM 70487, which was obtained in agar slant from the German Collection of Microorganisms and Cell Cultures (DSMZ). The brewing culture yeast strains were commonly used to produce beer styles dependent on the industrial applications and the recommended beer style according to the results obtained by Meier-Dörnberg listed in the following Table 1. Within the 18 unknown spoilage yeast isolates, eight were isolated from beverages of various breweries, which had attained a very low specific gravity. Six spoilage yeasts were isolated from beer, one was isolated from a beer-mixed beverage and one spoilage yeast was isolated from lemonade. We refer to these initial cultures as isolates until species confirmation and confirmation that they represent different strains. We define a strain as being genetically distinct and/or physiologically distinct.

Genetic isolate identification and strain differentiation

The genetic distinctiveness of each TUM yeast isolate was determined by RT-PCR (see section 'Real-time polymerase chain reaction'), ITS1-5.8S-ITS2 and D1/D2 26S ribosomal rRNA gene PCR sequencing (see section 'PCR sequencing of the D1/D2 domain of the 26S rRNA gene and the ITS1-5.8S-ITS2'), and a strain typing method based on a PCR-capillary electrophoresis of partial intergenic spacer 2 (IGS2) fragment (IGS2-314 PCR-capillary electrophoresis see section 'DNA fingerprinting (PCR-capillary electrophoresis of the IGS2-314 fragment)'). The RT-PCR and sequencing methods were used to identify if the isolate belonged to *S. cerevisiae* var. *diastaticus*.

DNA extraction

To isolate the DNA from each investigated yeast isolate, cultures were taken from wort agar slants using an inoculation loop, transferred to a 1.5-mL tube, and mixed with an aliquot of 200 μL InstaGene Matrix solution (Biorad, Munich, Germany). Each tube was vortexed for 10 s and incubated at 56°C for 30 min, followed by another 10 s of vortexing and incubation at 96°C for 8 min. The incubation steps occurred in a Thermomix 5436 (Eppendorf, Hamburg, Germany). After incubation, the tubes were centrifuged at 13 000× *g* for 2 min, and then a 100-μL aliquot of the DNA-containing supernatant was transferred to a new 1.5-mL tube (Hutzler 2009). The DNA concentration was adjusted to 25 ng μL^{-1} after being measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Real-time polymerase chain reaction

RT-PCR (Light Cycler® 480 II, Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used to taxonomically

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 1. *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates with TUM identifier and the as reference strains used brewing culture yeast strains.

TUM yeast isolates/strains			
TUM yeast isolate/strain	Yeast species	Industrial application	Obtained from
DSM 70487	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast (control strain)	Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures GmbH
TUM PI BA 31	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM PI BA 45	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM PI BA 109	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM PI BA 124	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM PI BB 105	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from beer-mixed beverage
TUM PI BB 121	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM PI BB 124	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM PI BB 125	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM PI BB 133	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM PI BB 159	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM 1-B-8	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM 3-D-2	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM PI BB 182 (17-E-7)	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from lemonade
TUM 2-F-1	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM 1-G-7	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM 1-H-7	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
TUM 3-H-2	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	Isolated from brewery
TUM 71	*Saccharomyces cerevisiae* var. *diastaticus*	spoilage yeast	BLQ
LeoBavaricus—TUM 68®	*Saccharomyces cerevisiae*	wheat beer production	BLQ commercial yeast culture
LunaBavaria—TUM 127®	*Saccharomyces cerevisiae*	wheat beer production	BLQ commercial yeast culture
Colonia—TUM 177®	*Saccharomyces cerevisiae*	kölsch and alt beer production	BLQ commercial yeast culture
Vetus—TUM 184®	*Saccharomyces cerevisiae*	alt beer production	BLQ commercial yeast culture
Pensum—TUM 210®	*Saccharomyces cerevisiae*	ale beer production	BLQ commercial yeast culture
Mel—TUM 211®	*Saccharomyces cerevisiae*	ale beer production	BLQ commercial yeast culture
TUM 213	*Saccharomyces cerevisiae*	wheat beer production	BLQ commercial yeast culture
Monacus—TUM 381®	*Saccharomyces cerevisiae*	wheat beer production	BLQ commercial yeast culture
TUM 503	*Saccharomyces cerevisiae*	ale beer production	BLQ commercial yeast culture
Tropicus—TUM 506®	*Saccharomyces cerevisiae*	ale beer production	BLQ commercial yeast culture
Harmonia—TUM 511®	*Saccharomyces cerevisiae*	ale and wheat beer production	BLQ commercial yeast culture
Frisinga—TUM 34/70®	*Saccharomyces pastorianus*	lager beer production	BLQ commercial yeast culture
Securitas—TUM 193®	*Saccharomyces pastorianus*	lager beer production	BLQ commercial yeast culture

classify the isolates. The primer and TaqMan® probe sequences used are listed in Table 2 and the RT-PCR procedure followed that of Hutzler (Hutzler 2009; Hutzler, Geiger and Jacob 2010). All RT-PCR systems listed in Table 2 are compatible and were performed with 10 μL 2× Mastermix (Light Cycler® 480 Probe Master, Roche, Germany), 1.4 μL ddH$_2$O PCR water, 0.8 μL (400 nM) of each primer (Biomers, Ulm, Germany), 0.4 μL (200 nM) probe (Biomers, Ulm, Germany; MGB probe from ThermoFisher scientific, Applied Biosystems®, USA), 0.5 μL IAC135-f (250 nM), 0.5 μL IAC135-r (250 nM), 0.4 μL IAC135-S (HEX) (200 nM), 0.1 μL IAC135 (dilution 1:10^{-13}), 0.1 μL IAC135 rev (dilution 1: 10^{-13}) and 5 μL template DNA with a total reaction volume of 20 μL, using the same temperature protocol: 95°C/10 min; 40 cycles of 95°C/10 s, 60°C/55 s; 20°C. IAC135 was developed by Riedl at the Research Center Weihenstephan for Brewing and Food Quality of the Technical University Munich (see Table 3). IAC (internal amplification control) is a control to confirm that the PCR reaction itself took place. If IAC is negative, the reaction has to be repeated. The yeast strains *S. cerevisiae* (LeoBavaricus—TUM 68®) and *S. pastorianus* (Frisinga—TUM 34/70®) were used as a positive and negative control according to the RT-PCR system tested.

PCR sequencing of the D1/D2 domain of the 26S rRNA gene and the ITS1-5.8S-ITS2

To amplify the D1/D2 domain of the 26S rRNA gene, the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used according to Kurtzman (Kurtzman and Robnett 2003). PCR was performed with 25 μL RedTaq Mastermix 2× (Genaxxon bioscience GmbH, Ulm, Germany), 16 μL ddH$_2$O PCR water, 2 μL of each primer having a concentration of 400 nM (Biomers, Munich, Germany) and 5 μL template DNA with a total reaction volume of 50 μL, using the temperature protocol according to Hutzler (2009): 95°C/5 min; 35 cycles of 95°C/30 s, 52°C/60 s; 72°C/60 s; 72°C/10 min.

To amplify the ITS1-5.8S-ITS2, the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used according to White (Innis 1990). PCR was performed with 25 μL RedTaq Mastermix 2× (Genaxxon bioscience GmbH, Ulm, Germany), 15 μL ddH$_2$O PCR water, 2.5 μL of each primer having a concentration of 500 nM (Biomers, Munich, Germany) and 5 μL template DNA with a total reaction volume of 50 μL, using the temperature protocol according to Hutzler (2009) 95°C/5 min; 40 cycles of 95°C/30 s, 55.5°C/60 s; 72°C/60 s; 72°C/10 min.

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 2. Qualitative RT-PCR systems for brewing yeast species differentiation (Hutzler 2010; Bamforth and Bokulich 2017).

RT-PCR systems, primer and probe sequences (5′→3′)	System name	Reference	*S. cer.*	*S. cer. var. dia.*	*S. past.*
Sbp-f CTTGCTATTCCAAACAGTGAGACT Sbp-r1 TTGTTACCTCTGGGCGTCGA Sbp-r2 GTTTGTTACCTCTGGGCTCG Sbp ACTTTTGCAACTTTTTCTTTGGGTTTCGAGCA	Sbp	(Brandl 2006); (Josepa, Guillamon and Cano 2000)	–	–	+
Sc-f CAAACGGTGAGAGATTTCTGTGC Sc-r GATAAAATTGTTTGTGTTTGTTACCTCTG Scer FAM-ACACTGTGGAATTTTCATATCTTTGCAACTT-BHQ1	Sce	(Brandl 2006); (Josepa, Guillamon and Cano 2000)	+	+	+
Sc-GRC-f CACATCACTACGAGATGCATATGCA Sc-GRC-r GCCAGTATTTTGAATGTTCTCAGTTG Sc-GRC FAM-TCCAGCCCATAGTCTGAACCACACCTTATCT-BHQ1	Sc-GRC3	(Hutzler 2010)	+	+	+
TF-f TTCGTTGTAACAGCTGCTGATGT TF-r ACCAGGAGTAGCATCAACTTTAATACC TF-MGB FAM-ATGATTTTGCTATCCCAAGTT-BHQ1 (MGB probe)	TF-COXII	(Hutzler 2010)	+	+	–
BF300E CTCCTTGGCTTGTCGAA BF300M GGTTGTTGCTGAAGTTGAGA BF300 FAM-TGCTCCACATTTGATCAGCGCCA-BHQ1	BF-300	(Brandl 2006)	–	–	+
BF-LRE-f ACTCGACATTCAACTACAAGAGTAAAATTT BF-LRE-r TCTCCGGCATATCCTTCATCA BF-LRE FAM-ATCTCTACCGTTTTCGGTCACCGGC-BHQ1	BF-LRE1	(Hutzler 2010)	–	–	+
Sd-f TTCCAACTGCACTAGTTCCTAGAGG			–	+	–
Sd-r GAGCTGAATGGAGTTGAAGATGG Sdia FAM-CCTCCTCTAGCAACATCACTTCCTCCG-BHQ1	Sdia	(Brandl 2006)			

Table 3. Primer, probe and target DNA sequences of the internal amplification control system (IAC135) used for real-time PCR systems.

Real-time PCR internal amplification control (IAC135)		
System name	Primer	Primer sequence (5′-3′)
IAC135	IAC135-f	TGGATAGATTCGATGACCCTAGAAC
	IAC135-r	TGAGTCCATTTTCGCAGATAACTT
	Probe	Probe sequence (5′-3′)
	IAC135-S	HEX-TGGGAGGATGCATTAGGAGCATTGTAAGAGAG-BHQ1
	Target DNA	DNA sequence (5′-3′)
	IAC135	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGGGAGGATGCATTAGGAGCATTGTAAGAGAGTC GGAAGTTATCTGCGAAAATGGACTCATTCGAGTGGCCTATTGACGGTCGCCCAAGGTGTCGCA
	IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCATTTTCGCAGATAACTTCCGACTCTCTT ACAATGCTCCTAATGCATCCTCCCACTAGTTCTAGGGTCATCGAATCTATCCATTCTCTAGCA

Amplified fragments were purified using a QIAquick® Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's recommendations. The quality of amplicons was subsequently checked by capillary gel electrophoresis (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA). The DNA concentration of the purified amplicons was adjusted to 25 ng μL^{-1} after being measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The sequencing reaction was assigned to GATC Biotech AG (Konstanz, Germany). For this reason, Sanger sequencing for PCR amplificons was chosen.

Each sequence was subsequently trimmed and analyzed with MEGA6 (Molecular Evolutionary Genetics Analysis Software). The D1/D2 26S rDNA as well as the ITS1-5.8S-ITS2 rDNA nucleotide sequences were identified for each yeast isolate separately using the GenBank Basic Local Alignment Search Tool (BLAST) of the NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike, Bethesda MD, USA) (Altschul *et al.* 1997). Afterwards, sequences were compared with the sequences of the reference strains (Frisinga—TUM 34/70®, LeoBavaricus—TUM 68®) and the type strain *S. cerevisiae* CBS 1171 via ClustalW alignment using MEGA6.

The analysis involved 10 nucleotide sequences of D1/D2 26S rDNA (TUM 3-D-2, Frisinga—TUM 34/70®, LeoBavaricus—TUM 68®, TUM 71, TUM 3-H-2, TUM PI BA 124, TUM PI BB 121, DSM 70487, TUM 1-H-7 and CBS 1171 GenBank accession nos AF528077; AY046146) and except DSM 70487 and TUM 3-H-2, eight nucleotide sequences of the ITS1-5.8s-ITS2 rDNA. Nucleotide sequence polymorphism was shown for the D1/D2 26S rDNA as well as the ITS1-5.8S-ITS2 rDNA compared with the *S. cerevisiae* CBS 1171 yeast strain (CBS 1171 GenBank accession nos AF528077/AY046146).

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

DNA fingerprinting (PCR-capillary electrophoresis of the IGS2-314 fragment)

In order to determine if isolates represented different or identical strains, genetic fingerprints were generated using the IGS2-314 method (Hutzler 2009). The IGS2 is a spacer region within the ribosomal cluster. To a partial sequence of the intergenic spacer 2 (IGS2-314), the specific primers IGS2-314f (5′-CGGGTAACCCAGTTCCTCACT-3′) and IGS2-314r (5′-TAGCATATATTTCTTGTGTGAGAAAGGT-3′) (Biomers GmbH, Ulm, Germany) (Büchl *et al.* 2010) were used at a concentration of 600 nM as described by Hutzler, Geiger and Jacob (2010). PCR was performed with 22.5 μL RedTaq Mastermix (2×) (Genaxxon, Ulm, Germany) and 2.5 μL template DNA with a total reaction volume of 25 μL. The Mastermix contained 12.5 μL buffer solution (Red-Taq Mastermix), 7.0 μL DNA-free PCR water and 1.5 μL of each primer (Biomers, Munich, Germany). Cycling parameters were as follows: a pre-denaturing step at 95°C for 300 s, then 35 cycles for denaturing at 95°C for 30 s, for annealing and elongation at 54°C for 30 s and 72°C for 40 s and for final elongation at 72°C for 300 s. PCR was performed using a SensoQuest LabCycler48s (SensoQuest GmbH, Gottingen, Germany). Amplified fragments were analyzed using a capillary electrophoresis system (Agilent DNA 1000 kit) following the manufacturer‘s recommendations (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).

Phylogenetic analysis of the IGS2-314 fingerprint patterns using Bionumerics Software 7.6

Based on the specific capillary electrophoresis IGS2-314 rDNA patterns, a dendrogram was built using the Bionumerics program 7.6 (Applied Maths, Belgium) to show the relationship between the investigated yeast isolates and reference strains. To create the dendrogram, a curve-based cluster was analyzed using a Pearson correlation with an optimization degree of 0.5% and a band-based cluster was analyzed using a Jaccard correlation with an optimization of 0.5% and a tolerance set of 1%.

Morphological and cultural characters

Microscope images and determination of intermediate cell sizes

Single yeast cultures were taken from wort agar slants and diluted in distilled water at room temperature. Microscope images were conducted by phase microscopy using an oil immersion lens of 100× magnification and a Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany). Cell sizes were measured using a Nikon Eclipse Ti microscope of 60× magnification and the associated analysis software (Nikon GmbH, Düsseldorf, Germany).

Sodium acetate agar test for sporulation behavior

The agar plate test was conducted with 0.2 mL of the propagated yeast suspension (50 mL Erlenmeyer flask). Therefore, yeasts were propagated in 10 mL yeast extract malt extract (YM) broth at 28°C for 72 h (e.g 3 days). The yeast sediment was taken out and spread on sodium acetate agar plate containing 5.0 g L^{-1} sodium acetate and 2.0 g L^{-1} agar using a sterilized spreader rod. The agar plates were incubated anaerobically at 28°C for 192 h (i.e. 8 days). To calculate the sporulation efficiency, the number of asci spores present was estimated after 120 and 192 h (i.e. 5 and 8 days) by counting at least 600 single yeast cells using a Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany). To do so, smears were scraped off the agar plates using a sterilized inoculation loop and transferred into 0.01 mL distilled water on a microscope slide. Sporulated cultures were examined by phase microscopy using an oil immersion lens with a focus of 100×.

Physiological (pre)-screening

Phenolic off-flavor test

TUM yeast culture isolates were taken from wort agar slopes and spread on a YM agar plate containing one of the precursors: ferulic acid, cinnamic acid and coumaric acid. After 3 days of incubation at 24°C, the three single agar plates per yeast isolate were evaluated by sniffing to detect any of the following aromas: ferulic acid becomes 4-vinylguajacol (4-VG, clove-like), cinnamic acid becomes 4-vinylstyrene (4-VS, styrofoam-like) and coumaric acid becomes 4-vinylphenol (4-VP, medicinal-like). *Saccharomyces cerevisiae* LeoBavaricus—TUM 68® and *S. pastorianus* Frisinga—TUM 34/70® were used as a positive and a negative control, respectively (Hutzler, Geiger and Jacob 2010). For the YM-agar plates, a YM media was made by adding distilled water to 3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone, 11.0 g glucose monohydrate and 20.0 g agar to 1000 mL and autoclaved. After autoclaving, an aliquot of the following sterile stock solutions was added to the YM media at 45°C–50°C under sterile conditions. For the stock solution of coumaric acid, 100 mg of the instant was dissolved in 10 mL of 96% [v/v] ethanol. The stock solution of ferulic and cinnamic acid was made by dissolving 1 g in 20 mL of 96% [v/v] ethanol. Ten milliliter coumaric acid, 2 mL ferulic acid or 2 mL cinnamic acid stock solution was added for 1000 mL YM media.

Modified Durham tube test with fermented beer medium — gas-forming potential

To pre-screen for their super-attenuating ability, 19 *S. cerevisiae* var. *diastaticus* yeast isolates were tested for their gas-forming potential in two separate trials at a cell concentration of 3 and 5 × 10^6 yeast cells per mL. In order to propagate yeasts, isolates were inoculated from agar slants into 60 mL of sterile wort medium in an 100 mL Erlenmeyer flask and incubated for 48 h at ambient temperature (20°C) and pressure, and agitated at 80 rpm using a WiseShake 207 orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, 5 mL of each yeast suspension was transferred into 15 mL Sarstedt tubes (Sarstedt AG & Co., Nümbrecht, Germany) and resuspended with 5 mL of distilled water by vortexing for 5 s. After homogenization, the resuspended yeast cells were washed by centrifugation for 5 min at 3000 rpm. The supernatant was removed from the tube, and the decanted yeast cell pellet was vortexed and washed again by centrifugation thrice with 10 mL distilled water. To pitch with a yeast cell concentration of 3 and 5 × 10^6 yeast cells per mL, cell concentrations of 30 and 50 × 10^6 yeast cells per mL were determined using the Thoma cell counting chamber and adjusted with distilled water in sterile Sarstedt tubes. After homogenization by vortexing for 5 s, 1 mL of the adjusted yeast suspension was further inoculated in pasteurized fermented beer medium under sterile conditions (86 AA % using Frisinga—TUM 34/70) according to MEBAK Bd. 3 (10.4.2). For this purpose, 9 mL of the fermented beer medium was pipetted into sterile plugged Durham tubes and autoclaved at 121°C for 5 min. Tubes were incubated at 28°C for 480 h (i.e. 20 days) and observed for the accumulation of gas in the inserts over a period of 20 days (e.g. day 2, 4, 5, 6, 7, 9, 10, 12, 14 and 20). To evaluate the build-up of CO_2, the used Durham tubes (1.40 mL volume, 39.95 mm in length) are divided into four parts by volume described in intervals from >0.00 mL to 0.35 mL as G1/4, from 0.35 mL to

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 4. Agar plate composition to detect super-attenuating yeasts.

Agar plate for detecting super-attenuating yeasts		
Parameter	Dextrin agar	Starch agar
Agar ($g L^{-1}$)	15	15
Carbohydrate source ($g L^{-1}$)	15 (Dextrin)	15 (potato starch)
Yeast nitrogen base ($g L^{-1}$)	6.78	6.78
pH (0.1 M HCl/NaOH)	5.2	5.2
Bidistilled water (L)	1	1
Bromophenol blue ($mg L^{-1}$) (Supplement in trial 3)	0	40

Table 5. Starting wort composition used for propagation and brewing trials (12.4 °P wort).

Wort composition	
Parameter	Amount
Original gravity (°P)	12.40
pH	5.19
Spec. weight SL 20/20 °C	1.05
Zinc ($mg L^{-1}$)	0.15
FAN ($mg 100 mL^{-1}$)	25.00
Total AS ($mg 100 mL^{-1}$)	203.22
Total sugar ($g L^{-1}$)	83.78
EBC-Bittering units (EBC)	20.20
Glucose ($g L^{-1}$)	11.46
Fructose ($g L^{-1}$)	2.57
Saccharose ($g L^{-1}$)	1.12
Maltose ($g L^{-1}$)	53.65
Maltotriose ($g L^{-1}$)	14.98

0.70 mL as G2/4, from 0.70 mL to 1.05 mL as G3/4, from 1.05 mL to 1.40 mL as G4/4 and without gas formation as G-.

Starch- and dextrin-agar plate test

The agar plate test was conducted in two main test trials. In the first trial, 10 mL of the propagated yeast suspension (50 mL Erlenmeyer flask) was centrifuged at 3000 rpm for 5 min. The supernatant was decanted, and the yeast sediment was resuspended with 10 mL sterile physiological saline solution. The yeast cells were washed by centrifugation twice with 10 mL sterile saline solution (5 min at 3000 rpm), resuspended with 5 mL sterile saline solution and stored to starve for 24 h. For the second trial, yeast cells were used without the additional washing step. Yeast cells were adjusted to a concentration of 5×10^6 yeast cells mL^{-1} and spread on an agar plate containing 15 $g L^{-1}$ dextrin or 15 $g L^{-1}$ starch (see Table 4) using a sterilized spreader rod. The agar plates were incubated aerobically and anaerobically at 25°C over a period of 888 h. In a third trial, 40 $mg L^{-1}$ bromophenol blue was added to the starch agar plates, to make it quicker and easier to detect cell growth. If the pH drops from 5.2 to between 4.6 and 3.0, the color of the agar plate changes from blue/violet to yellow as a result of cell metabolites.

Brewing trials

Wort

The wort characteristics used for propagation and the brewing trials are shown in Table 5. The wort was based on hopped barley malt concentrate (N53940; Döhler GmbH, Darmstadt, Germany). To achieve an original gravity of 12.4 °P, wort concentrate was diluted with distilled water and boiled for 5 min to sterilize. The same wort batch preparation was used for the propagation and brewing trials to ensure constant wort composition. Free alpha-amino nitrogen was quantified using the MEBAK WBBM (2.6.4.1.2) method (Jacob 2012). Sugar composition was determined using the HPLC MEBAK WBBM (2.7) method (Jacob 2012).

Propagation

In order to propagate yeasts, isolates were inoculated from agar slants (yeast pure culture) into 60 mL of sterile wort medium in an 100 mL Erlenmeyer flask and incubated for 72 h at ambient temperature (20°C) and pressure, and agitated at 80 rpm using a WiseShake 207 orbital shaker (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, yeasts were transferred to 4 kg of sterile wort medium and further propagated at the same conditions for an additional 72 h. After allowing 6 h for sedimentation, the supernatant was decanted and 2 kg of sterile wort medium at pitching temperature (20°C) was added to the yeast sediment in each container. Yeast concentration was determined in cells/g using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area per square of 0.00025 m^3 (Brand GmbH&Co.KG, Wertheim, Germany).

Fermentation

Laboratory-scale brewing trials were performed using stainless steel vessels with dimensions of 10 cm diameter × 33 cm height (2.5 L) with 20% headspace and clamped-down lids according to Meier-Dörnberg (Meier-Dörnberg et al. 2017a,c). The vessels were placed in a tempered cooling chamber (2023 Minicoldlab, LKB-Produkter AB, Bromma, Sweden) to guarantee a constant fermentation temperature. To imitate industrial brewery conditions during fermentation, a head pressure of 0.5 bar was applied to simulate a liquid height of 10 m (median hydrostatic pressure).

Brewing trials were evaluated by pitching 8.5 L wort per yeast isolate/strain. Each batch was then divided into four fermentation vessels. By having four vessels, samples could be taken daily from one of the four vessels to estimate the specific gravity, cells in suspension and pH, while the other three vessels remained undisturbed. Yeast isolates were added at an inoculation rate of 15 million cells g^{-1} of homogeneous mixed wort medium. The wort was not oxygenated. Primary fermentation was maintained at 20°C and considered complete after the specific gravity remained constant for 2 consecutive days. An additional 5 days for maturation was given following primary fermentation at same temperature of 20°C, and 7 days for lagering at 0°C. The beers were then removed from the fermentation vessels, homogenized and collected in sterile bottles. The specific gravity and pH of samples were determined from the filtered fermentation samples using a DMA 35N (Anton-Paar GmbH, Graz, Austria) for specific gravity and a pH3210 (WZW, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) for pH measurement. The samples were filtered using Whatman® folded filter paper with a diameter of 320 mm (GE Healthcare Europe GmbH, Freiburg, Germany).

Analytical methods

After lagering, the finished beers were analyzed for physical and chemical attributes, which included the following parameters: ethanol, pH, specific gravity, degree of attenuation, free amino nitrogen, amino acid composition, sugar composition, total SO_2,

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

free and total dimethylsulfide, free vicinal diketones and the concentration of fermentation by-products. Analysis was performed according to MEBAK WBBM methods (Jacob 2012). The method number is listed in brackets next to the respective analysis.

Ethanol, pH, specific gravity and degree of attenuation were measured using an Anton Paar DMA 5000 Density Meter with Alcolyzer Plus measuring module, pH measuring module and Xsample 122 sample changer (Anton-Paar GmbH, Graz, Austria) (2.9.6.3). Free amino nitrogen, the total amino acid composition as well as the residual sugar composition were determined using the HPLC method (2.6.4.1.2 and 2.7).Total SO_2 (2.21.8.2), free and total dimethylsulfide (2.23.1) and free vicinal diketones (2.21.5.1) were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, USA) with a headspace unit and Elite 5 60 m 1.5DF column using a 2.3-hexandione internal standard. The final concentrations of fermentation by-products (2.21.1) (e.g. acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols) and 4-VG (2.21.3.3) were quantified using a gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 μm column (Perkin-Elmer, USA).

Determining the cell count (cells in suspension and total cell count)

Cell counts for pitched yeast, cells in suspension until lagering and total cell count after lagering were determined using a Thoma cell counting chamber with a chamber depth of 0.1 mm and an area per square of 0.00025 m^3 (Brand GmbH&Co.KG, Wertheim, Germany).

Cells in suspension were analyzed every 24 h up to the start of lagering. To ensure cell count accuracy during fermentation and maturation, 20 mL of green beer was removed from the middle of the fermentation vessel by using a 10-mL volumetric pipette mounted on a stand. Prior to sampling, the head pressure in the vessel was released very slowly so that the cells in suspension were not affected by a pressure surge.

The total cell count was determined after the lagering phase. Beers were removed from the fermentation vessels and the decanted yeast masses were collected by suspending the yeast cells in a total of 50 g distilled water. The yeast cells were washed by centrifugation twice with 50 g distilled H_2O (5 min at 3000 rpm) and resuspended with distilled water up to a total of 100 g. Afterwards, distilled water was added to 1 g of the homogenous yeast suspension to make up to 100 mL. Total cell counts were determined in cells g^{-1} using the Thoma cell counting chamber.

Sensory evaluation

Sensory analysis of the beers was conducted after maturation and lagering. Three single sensory tests were conducted which included: expected beer type test, DLG (Deutsche Landwirtschafts-Gesellschaft) scheme for beer and a descriptive sensory evaluation. All beer samples were tasted and evaluated by a sensory panel of seven DLG-certified tasters with long-standing experience in the sensory analysis of beer at the Weihenstephan Research Center for Brewing and Food Quality. Sensory evaluations were performed in individual walled tasting stations under controlled environmental conditions. Samples were provided in triplicate sets for all beers in dark glasses, each with a three-digit code. All samples were served at 12°C to guarantee optimal conditions to investigate the predominant flavor diversity. At first, the panelists associated the beer samples with their expected beer type (e.g. ale, wheat-, Kölsch-, Alt-, stout, Berliner Weisse, porter-, lager-; Bock-; Märzen-, Rauch-, Schwarz-, Dunkles-, malt beer) followed by an examination of the beer samples according to the DLG scheme for beer. Finally, a descriptive sensory evaluation was conducted during which trained panelists described specific flavors. Seven main categories were described (e.g. sweet, tropical fruity, fruity (other fruits), citrus, spicy, floral and other flavors). Every category was evaluated from 0, meaning not noticeable, to 5, extremely noticeable.

RESULTS AND DISCUSSION

Genetic (pre)-screening

RT-PCR assays

Table 6 shows the tested RT-PCR systems and the obtained results for all yeast isolates. Based on the RT-PCR results, all of the selected TUM yeast isolates were positive for the Sc-*GRC3* and Sce loci. The RT-PCR systems Sce (PCR system located on ITS1-5.8S-ITS2 rDNA) and Sc-*GRC3* have positive signals when *S. cerevisiae* DNA is measured or DNA of hybrid strains that contain these DNA loci. Except for TUM PI BA 124, all selected TUM yeast isolates were positive for the TF-*COXII* locus, suggesting that they belong to *S. cerevisiae* and negative for loci that correlate with the RT-PCR systems Sbp, BF-*LRE1* and BF-300, which detect *S. bayanus/S. pastorianus* strains. In addition, these yeast isolates were positive for the Sdia loci, determining the yeast isolates as *diastaticus* variety of *S. cerevisiae* (i.e. *S. cerevisiae* var. *diastaticus*). The specific primer and probe system for Sdia is situated on the glucoamylase *STA1* gene. In contrast, TUM yeast isolate TUM PI BA 124 was negative for the PCR systems TF-*COXII* and Sdia, but positive for loci that correlate with the PCR systems Sbp, BF-*LRE1* and BF-300. The results obtained by RT-PCR indicate that TUM yeast isolate TUM PI BA 124 belongs to the yeast species *S. bayanus/S. pastorianus*. Reference strain patterns of Frisinga—TUM 34/70® and LeoBavaricus—TUM 68® corresponded to the proposed patterns.

Physiological (pre)-screening

Phenolic off-flavor potential

Table 7 shows the results of the phenolic-off flavor potential of the tested *S. cerevisiae* var. *diastaticus* yeast strains. Each strain was spread on three YM-agar plates containing one phenolic off-flavor precursor. After time for incubation, the ability to build phenolic off-flavors was detected by sniffing the corresponding aroma-active flavors. Except for the yeast strain *S. pastorianus* TUM PI BA 124 and the bottom-fermenting reference strain Frisinga—TUM 34/70®, all strains were able to produce phenolic off-flavors. For strains TUM PI BA 31, TUM PI BB 105, TUM 1-B-8, TUM 3-D-2, TUM 2-F-1, TUM 1-G-7 and TUM 3-H-2, only two of three aroma-active compounds can be detected by sniffing. The most dominating and most important phenolic off-flavor (POF), namely 4-VG, could be detected in all *S. cerevisiae* var. *diastaticus* strains.

Modified Durham tube test with fermented beer medium — gas-forming potential

The modified Durham tube test was performed with cell concentrations of 3 and 5 × 10^6 yeast cells per mL. The previous propagation was used to obtain vital yeast cells in order to minimize the adaptation phase to the substrate and thus to obtain short-term and fast results. Prior to inoculating the Durham tubes, the yeast suspension was washed with distilled water. As preliminary experiments showed (data not shown), the experiments

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 6. Qualitative results of the RT-PCR systems used for the 18 investigated *S. cerevisiae* var. *diastaticus* yeast isolates and the reference strains to differentiate *Saccharomyces sensu stricto* species; positive (+), negative (–).

		RT-PCR system						
Species	Yeast isolates/reference strains	Sc-GRC3	Sce	TF-*COXII*	Sbp	BF-*LRE1*	BF-300	Sdia
S. cerevisiae var. *diastaticus*	DSM 70487	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BA 31	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BA 45	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BA 109	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 105	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 121	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 124	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 125	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 133	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM PI BB 159	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 1-B-8	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 3-D-2	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 17-E-7	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 2-F-1	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 1-G-7	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 1-H-7	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 3-H-2	+	+	+	–	–	–	+
S. cerevisiae var. *diastaticus*	TUM 71	+	+	+	–	–	–	+
S. cerevisiae	LeoBavaricus—TUM 68®	+	+	+	–	–	–	–
S. pastorianus	Frisinga—TUM 34/70®	+	+	–	+	+	+	–
S. pastorianus	TUM PI BA 124	+	+	–	+	+	+	–

Table 7. POF potential of the investigated *Saccharomyces cerevisiae* var. *diastaticus* and the reference strains Frisinga—TUM 34/70® and LeoBavaricus—TUM 68®.

	POF test/sniffing perception of		
	Product/precursor		
Yeast strain (TUM identifier)	4-VG/ ferulic acid	4-VP/ coumaric acid	4-VS/ cinnamic acid
DSM 70487	+	+	+
TUM PI BA 31	+	+	–
TUM PI BA 45	+	+	+
TUM PI BA 109	+	+	+
TUM PI BB 105	+	–	+
TUM PI BB 121	+	+	+
TUM PI BB 124	+	+	+
TUM PI BB 125	+	+	+
TUM PI BB 133	+	+	+
TUM PI BB 159	+	+	+
TUM 1-B-8	+	–	+
TUM 3-D-2	+	–	+
TUM 17-E-7	+	+	+
TUM 2-F-1	+	–	+
TUM 1-G-7	+	–	+
TUM 1-H-7	+	+	+
TUM 3-H-2	+	–	+
TUM 71	+	+	+
LeoBavaricus—TUM 68®	+	+	+
Frisinga—TUM 34/70®	–	–	–
TUM PI BA 124	–	–	–

led to false positive results when the yeast cells were not first washed with the result that *S. pastorianus* and *S. cerevisiae* also grew in fermented beer medium and produced carbon dioxide. This is presumably due to low molecular weight carbohydrates of the wort used for propagation which still adheres to the yeast and these can probably be used as energy sources to grow in the fermented beer medium.

Table 8 shows the gas formation of the investigated yeast strains within the experimental period of 20 days for an inoculation rate of 5 × 10^6 yeast cells mL^{-1}. With the exception of strains TUM 3-H-2, TUM PI BA 124 and Frisinga—TUM 34/70®, all investigated yeast strains were capable of metabolizing higher dextrins (e.g. starch) present in the fermented beer medium. The bottom-fermenting *S. pastorianus* reference yeast strain Frisinga—TUM 34/70® as well as the *S. pastorianus* strain TUM PI BA 124 did not develop carbon dioxide as expected. Even if strain TUM 3-H-2 was primarily identified as belonging to the species *S. cerevisiae* var. *diastaticus*, no carbon dioxide formation and therefore no super-attenuating ability could be detected. The *S. cerevisiae* var. *diastaticus* yeast strains TUM PI BA 109, TUM PI BB 133 and TUM 1-B-8 had the highest gas-forming potential and ended with a completely filled gas tube (G4/4) after 2 days of inoculation. The *S. cerevisiae* var. *diastaticus* control strain DSM 70487 also developed carbon dioxide very rapidly and reached the same gas amount of G4/4 within 3 days of incubation. At the end of each test series, yeast samples were removed from the respective test tubes and checked by RT-PCR systems to confirm that the grown yeast belonged to the pre-identified yeast species. This was confirmed with each sample taken during the trials. As the results show, the investigated super-attenuating yeasts have a distinct spoilage/harmful potential. The metabolism of dextrins and starch is strain dependent and differs for identical experimental conditions. An increase in the pitching cell concentration from 3 to 5 × 10^6 yeast cells mL^{-1} leads to the gas formation being detected twice as fast (data not shown for cell concentration of 3 × 10^6 yeast cells mL^{-1}). Depending on the strains examined, a further increase in the pitching concentration to accelerate the experimental results is probably only possible to a limited extent in order to

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 8. Gas-forming potential of the investigated yeast strains using a modified Durham tube test with a pitching cell concentration of 5×10^6 yeast cells per mL; Durham tube volume described in intervals from >0.00 to 0.35 mL as G1/4, from 0.35 to 0.70 mL as G2/4, from 0.70 to 1.05 mL as G3/4, from 1.05 to 1.40 mL as G4/4 (highlighted in bold) and without gas formation as G-.

		Gas formation and days needed										
Yeast species	Yeast strain (TUM identifier)	Day 2	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 11	Day 12	Day 14	Day 20
S. cerevisiae var. *diastaticus*	DSM 70487	G -	G 3/4	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BA 31	G -	G -	G -	G -	G -	G -	G -	G 1/4	G 1/4	G 2/4	G 3/4
S. cerevisiae var. *diastaticus*	TUM PI BA 45	G -	G -	G 1/4	G 2/4	G 3/4	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BA 109	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BB 105	G -	G -	G -	G -	G -	G -	G -	G -	G -	G 2/4	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM P IBB 121	G -	G -	G 1/4	G 2/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4
S. cerevisiae var. *diastaticus*	TUM PI BB 124	G 2/4	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BB 125	G 1/4	G 2/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BB 133	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM PI BB 159	G 1/4	G 1/4	G 1/4	G 1/4	G 1/4	G 1/4	G 2/4	G 2/4	G 2/4	G 2/4	G 2/4
S. cerevisiae var. *diastaticus*	TUM 1-B-8	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM 3-D-2	G 3/4	G 3/4	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM 17-E-7	G 1/4	G 2/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4
S. cerevisiae var. *diastaticus*	TUM 2-F-1	G -	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**	**G 4/4**
S. cerevisiae var. *diastaticus*	TUM 1-G-7	G -	G 1/4	G 1/4	G 1/4	G 1/4	G 1/4	G 2/4	G 2/4	G 2/4	G 2/4	G 2/4
S. cerevisiae var. *diastaticus*	TUM 1-H-7	G -	G -	G -	G -	G -	G -	G -	G -	G 1/4	G 1/4	G 2/4
S. cerevisiae var. *diastaticus*	TUM 3-H-2	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -
S. cerevisiae var. *diastaticus*	TUM 71	G -	G -	G 1/4	G 2/4	G 2/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4	G 3/4
S. cerevisiae	LeoBavaricus- TUM 68®	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -
S .pastorianus	Frisinga- TUM 34/70®	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -
S .pastorianus	TUM PI BA 124	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -	G -

Table 9. Percentage of *S. cerevisiae* var. *diastaticus* yeast strains with or without gas production subdivided according to evaluation period.

	Physiological gas test of all 18 investigated *S. cerevisiae* var. *diastaticus* yeast strains										
n strains	Day 2	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 11	Day 12	Day 14	Day 20
% Gas production	8	11	14	14	14	14	14	15	16	17	17
percentage	44.44	61.11	77.77	77.77	77.77	77.77	77.77	83.33	88.88	94.44	94.44
% No gas production	10	7	4	4	4	4	4	3	2	1	1
Percentage	55.55	38.88	22.22	22.22	22.22	22.22	22.22	16.66	11.11	05.55	05.55

continue to ensure the harmful potential or the speed at which the strains are investigated. As Table 9 shows, a period of 14 days is needed to make a reliable statement on the super-attenuating ability of the *S. cerevisiae* var. *diastaticus* yeast strains investigated in this test. Investigated strains (77.77%) showed carbon dioxide formation after 5 days of inoculation. The last strain to build carbon dioxide could be detected after 14 days of inoculation (TUM PI BB 105). Between day 5 and 20, the percentage of yeast strains with gas production rose from 77.77% to 94.44%.

Physiological (pre)-screening summary

The following Table 10 shows the results of the tested super-attenuating ability of the investigated yeast strains as well as their potential to build phenolic off-flavor. According to the days needed for full gas production in the modified Durham tube test (e.g G4/4), the strains were listed according to their super-attenuating power. In addition, Table 11 shows the percentage of all 18 investigated *S. cerevisiae* var. *diastaticus* yeast strains with full gas formation (G4/4) over the experimental period.

As can be seen from the tables, only 16.66% of all investigated 18 *S. cerevisiae* var. *diastaticus* yeast strains ended with a completely filled gas tube (G4/4) after 2 days of inoculation (e.g TUM PI BA 109, TUM PI BB 133 and TUM 1-B-8). TUM PI BB 124 and TUM 2-F-1 needed 4 days for full gas production (27.77%) and TUM 3-D-2 and control strain DSM 70487 needed an additional day to completely fill the gas tube G4/4 (38.88%). After 8 days of inoculation, also TUM PI BA 145 showed the full super-attenuation of the fermented beer medium (44.44%). As can be seen in the tables, after day 8 of the modified Durham tube test with fermented beer medium, no further full gas production of a strain could be observed until day 20. TUM PI 105 and TUM PI BB 125 needed 20 days for full gas production, while 44.44% of the investigated *S. cerevisiae* var. *diastaticus* yeast strains could not ferment the complete amount of higher dextrins and starch in the observed period of 20 days.

Yeast strain selection for further physiological screening and brewing trials

According to the results obtained by the above-specified pre-screening and genetic (qPCR) tests, seven *Saccharomyces* yeast strains (Table 12) were selected for further genetic tests (D1/D2 26S rRNA gene and ITS gene sequencing, ITS1-5.8S-ITS2 and IGS2-314 PCR-capillary electrophoresis), the starch and dextrin agar plate test and brewing trials. Strain DSM 70487 was selected as a positive control strain and TUM PI BA 124 as a

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 10. Physiological (pre)-screening summary (gas production, qualitative super-attenuating ability and phenolic off-flavor) of the all investigated *Saccharomyces cerevisiae* var. *diastaticus* yeast strains and the brewing yeast strains Frisinga—TUM 34/70® and LeoBavaricus—TUM 68® used as references.

		Gas formation (Durham test)		
Species identification	TUM identifier	Day for G4/4	Super-attenuation (qualitative)	Phenolic off-flavor
S. cerevisiae var. *diastaticus*	TUM PI BA 109	2	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 133	2	+	+
S. cerevisiae var. *diastaticus*	TUM 1-B-8	2	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 124	4	+	+
S. cerevisiae var. *diastaticus*	TUM 2-F-1	4	+	+
S. cerevisiae var. *diastaticus*	DSM 70487	5	+	+
S. cerevisiae var. *diastaticus*	TUM 3-D-2	5	+	+
S. cerevisiae var. *diastaticus*	TUM PI BA 45	8	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 105	20	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 125	20	+	+
S. cerevisiae var. *diastaticus*	TUM PI BA 31	>20	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 121	>20	+	+
S. cerevisiae var. *diastaticus*	TUM PI BB 159	>20	+	+
S. cerevisiae var. *diastaticus*	TUM 17-E-7	>20	+	+
S. cerevisiae var. *diastaticus*	TUM 1-G-7	>20	+	+
S. cerevisiae var. *diastaticus*	TUM 1-H-7	>20	+	+
S. cerevisiae var. *diastaticus*	TUM 71	>20	+	+
S. cerevisiae var. *diastaticus*	TUM 3-H-2	0	–	+
S. cerevisiae	LeoBavaricus—TUM 68®	0	–	+
S. pastorianus	Frisinga—TUM 34/70®	0	–	–
S. pastorianus	TUM PI BA 124	0	–	–

Table 11. Percentage of *S. cerevisiae* var. *diastaticus* yeast strains with full gas production G4/4 and <G4/4 subdivided according to evaluation period.

	Physiological gas test of all 18 investigated *S. cerevisiae* var. *diastaticus* yeast strains										
n strains	Day 2	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 11	Day 12	Day 14	Day 20
% Gas production category G4/4	3	5	7	7	7	8	8	8	8	8	10
percentage	16.66	27.77	38.88	38.88	38.88	44.44	44.44	44.44	44.44	44.44	55.55
% Gas production category < G4/4	15	13	11	11	11	10	10	10	10	10	8
Percentage	83.33	72.22	61.11	61.11	61.11	55.55	55.55	55.55	55.55	55.55	44.44

Table 12. Yeast strains selected for species confirmation, strain determination, further physiological screening and brewing trials.

Species identification by qPCR	TUM identifier	Industrial application	Selection criterion
S. cerevisiae var. *diastaticus*	DSM 70487	Spoilage yeast	Control strain
S. cerevisiae var. *diastaticus*	TUM PI BB 121	Spoilage yeast	Weak super-attenuation
S. cerevisiae var. *diastaticus*	TUM 3-D-2	Spoilage yeast	High super-attenuation
S. cerevisiae var. *diastaticus*	TUM 1-H-7	Spoilage yeast	Weak super-attenuation
S. cerevisiae var. *diastaticus*	TUM 71	Spoilage yeast	Weak super-attenuation
S. cerevisiae var. *diastaticus*	TUM 3-H-2	Spoilage yeast	No super-attenuation power
S. pastorianus	TUM PI BA 124	Unknown	Outlier

negative control strain belonging to the species *S. pastorianus* with no super-attenuation power. TUM 3-D-2 was selected due to its high super-attenaution, whereas TUM 3-H-2 showed no super-attenuation even if the presence of *STA1* genes could be detected. Strains TUM PI BB 121, TUM 1-H-7 and TUM 71 were selected as a result of their weak super-attenuation (see gas formation Duham tube test) to ensure better application of the performed starch and dextrin agar plate test. This would then provide a reliable statement on the time required to detect *S. cerevisiae* var. *diastaticus* yeast strains with low super-attenuation power.

Genetic analysis

PCR-DNA sequencing (D1/D2 26S rRNA gene and ITS)

The results obtained by RT-PCR were confirmed by sequence analysis of the D1/D2 26S and ITS1-5.8S-ITS2 ribosomal DNA. Sequence analyses were conducted in MEGA6 (Tamura *et al.* 2013).

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 13. ITS1-5.8S-ITS2 sequence polymorphisms of the investigated yeast isolates compared with *S. cerevisiae* CBS 1171 Accession no. AY046146 by sequence alignment (MEGA6 ClustalW-Alignment).

ITS1-5.8s-ITS2 rDNA sequence polymorphism of the investigated TUM yeast isolates compared to *S. cerevisiae* CBS 1171 Access. No. AY046146								
TUM yeast isolate sequence	ITS1-5.8s-ITS2 rDNA polymorphism							
S. cerevisiae var. *diastaticus* TUM 71	C	C	T	A	A	–	–	T
S. cerevisiae var. *diastaticus* TUM PI BB 121	G	C	–	A	A	–	–	T
S. cerevisiae var. *diastaticus* TUM PI BA 124	G	C	T	A	A	T	T	–
S. cerevisiae var. *diastaticus* TUM 1-H-7	G	C	–	A	A	–	–	T
S. cerevisiae var. *diastaticus* TUM 3-D-2	G	C	–	A	A	T	–	–
*S. cerevisiae*LeoBavaricus—TUM 68®	G	T	T	A	C	T	T	–
S. pastorianus Frisinga—TUM 34/70®	G	C	T	C	C	T	T	–
S. cerevisiae CBS 1171	G	C	–	A	A	–	–	–
Number of base pairs *S. cerevisiae* CBS 1171	64	271	280	493	532	606	607	691

Nucleotide sequence polymorphism was evaluated for the D1/D2 26S rRNA gene as well as the ITS1-5.8S-ITS2 region in contrast to the *S. cerevisiae* type strain (CBS 1171 GenBank accession nos. AF528077/AY046146). The D1/D2 26S rDNA nucleotide sequences of the nine investigated yeasts (TUM 3-D-2, Frisinga—TUM 34/70®, LeoBavaricus—TUM 68®, TUM 71, TUM 3-H-2, TUM PI BA 124, TUM PI BB 121, DSM 70487, TUM 1-H-7) are exactly the same as the D1/D2 26S rDNA sequence of *S. cerevisiae* type strain CBS 1171 (GenBank accession No. AF528077). In conclusion, no polymorphisms could be detected. Table 13 shows the ITS1-5.8S-ITS2 sequence polymorphism compared with *S. cerevisiae* CBS 1171 except for DSM 70487 and TUM 3-H-2. Sanger sequencing for PCR amplificons of the DSM 70487 and TUM 3-H-2 ITS1-5.8S-ITS2 region delivered short nucleotide sequences which could not be used for reliable genetic analysis. The ITS1-5.8S-ITS2 sequences of all the investigated yeast isolates and strains are different to the ITS1-5.8S-ITS2 sequence of *S. cerevisiae* type strain CBS 1171 (GenBank accession No. AY046146). Yeast isolates TUM PI BB 121, TUM 1-H-7 and TUM 3-D-2 have a total of one, TUM 71 and TUM PI BA 124 a total of three and LeoBavaricus—TUM 68® as well as Frisinga—TUM 3470® have a total of five sequence polymorphisms compared with *S. cerevisiae* type strain CBS 1171.

IGS2-314 PCR-capillary electrophoresis

The PCR of the IGS2-314 locus was used to investigate if different isolates represented different strains by amplifying amplicon fragments of different sizes. Each isolate was compared with two reference strains: the yeast strains *Saccharomyces cerevisiae* LeoBavaricus—TUM 68®, a top-fermenting strain, and *S. pastorianus* Frisinga—TUM 34/70®, a bottom-fermenting strain. The results showed unique banding patterns suggesting that each isolate represents a genetically different strain (Fig. 1).

Phylogenetic analysis of the IGS2-314 patterns using Bionumerics Software 7.6

Based on the specific capillary electrophoresis IGS2-314 patterns, a band-based (Fig. 2) and a curve-based (Fig. 3) cluster analysis were performed using the Bionumerics program 7.6 (Applied Maths, Ghent, Belgium). Dendrograms were built to visualize the genetic relationship between the investigated yeast isolates and reference strains. Figures 2 and 3 show that all the investigated yeast isolates are genetically different. Within the band-based cluster analysis shown in Fig. 2, the banding patterns show a similarity of 85.7% between TUM 3-D-2 and DSM 70487. With a similarity of 97.7% for curve-based cluster analysis shown in Fig. 3, these two yeast isolates seem to be genetically closely related. Within the curve-based cluster analysis, the banding patterns of the yeast isolate TUM PI BB 121, TUM 1-H-7 have the highest similarity of all isolates with a similarity of 99.0%. DSM 70487 has a similarity of 98.3% to TUM PI BB 121 and TUM 1-H-7. A significant genetic relationship to the top-fermenting or to the bottom-fermenting reference strain LeoBavaricus—TUM 68® and Frisinga—TUM 34/70® could not be determined for the investigated yeast isolates either by means of curve-based or band-based cluster analysis of the IGS2-314 patterns.

Starch and dextrin agar plate test

In addition to the modified Durham tube test with fermented beer medium (see section 'Modified Durham tube test with fermented beer medium — gas-forming potential' in 'Results and Discussion'), a starch agar plate test and dextrin agar plate test were conducted to achieve faster and reliable results which can be easily and fastly done in brewing practice. Therefore, the previously selected six *S. cerevisiae var. diastaticus* yeast strains and *S. pastorianus* yeast strain TUM PI BA 124 were compared with 12 top-fermenting *S. cerevisiae* and one bottom-fermenting *S. pastorianus* TUM brewing culture yeast strain listed in section 'Yeast isolates and strains' (Table 1). The brewing culture strains were used as control strains to make the test applicable to breweries that use different culture strains. Based on the fact that the super-attenuating ability of *S. cerevisiae* var. *diastaticus* yeast strains means it is possible to metabolize higher dextrins as well as starch, one agar plate test was conducted with dextrin and a second agar plate test with starch as the only carbohydrate source. As the results show, only starch agar plates under anaerobic incubation conditions can be used as a detection method for super-attenuating *S. cerevisiae* var. *diastaticus* yeast strains.

Preliminary tests using an undefined yeast cell concentration extracted from green beer did not give reliable results (data not shown). In fact most of the used *S. cerevisiae* and *S. pastorianus* brewing yeast strains showed visible cell growth on both agar plate types whether they were incubated aerobically or anaerobically at 25°C for 888 h. These results lead to the same presumption already obtained in the Durham tube test, that a further washing step of the cells is necessary to remove still-adherent wort carbohydrates. Even if the yeast cells were washed and starved to remove all possible carbohydrates from the cell, dextrin was not useful for detecting super-attenuating yeasts. As can be seen in Table 14, almost all *Saccharomyces* yeast strains

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

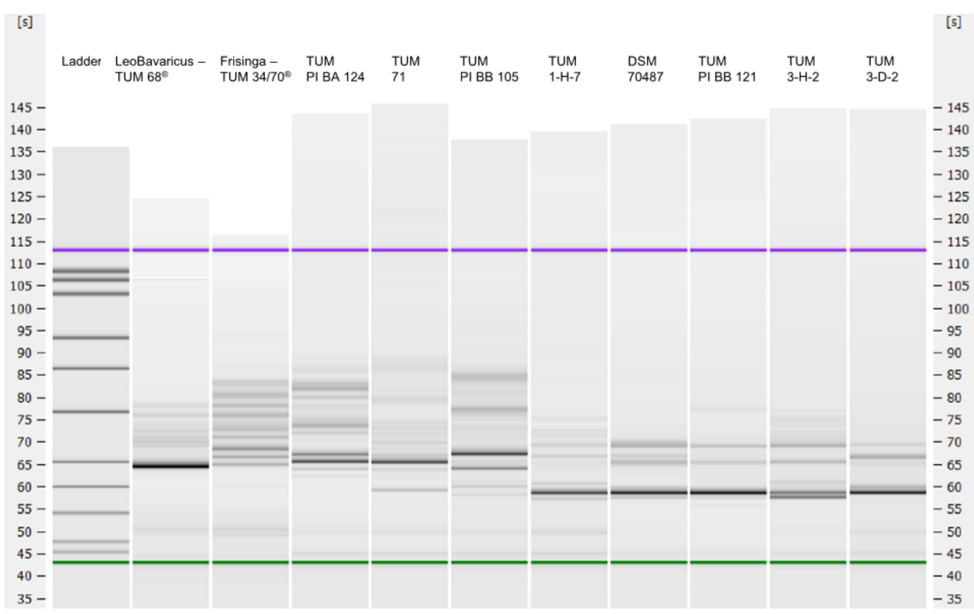


Figure 1. Capillary electrophoresis IGS2-314 rDNA patterns of all yeast isolates (LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2).

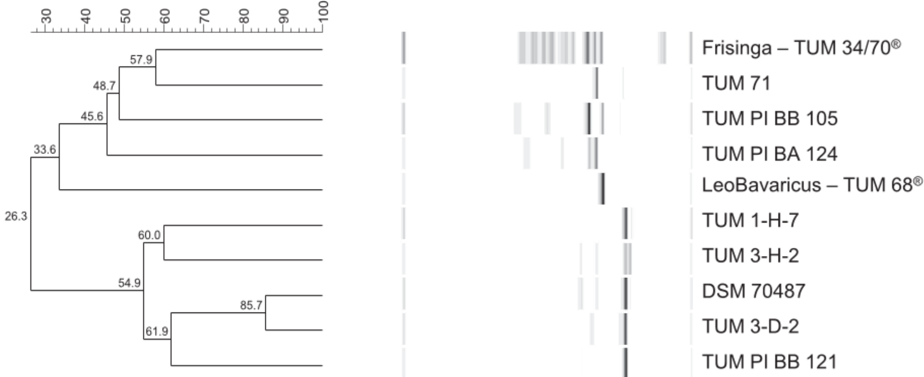


Figure 2. IGS2-314 rDNA band-based genetic relationship in percentage with capillary electrophoresis patterns between LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2 (dendrogram built with Bionumerics 7.6).

were able to grow on the dextrin agar plates. One possible explanation for the growth of the reference yeasts is that the dextrin used was not pure dextrin and may have contained other fermentable sugars. Since dextrin is a superordinate term for a degradation product of the starch, the molecular size is defined only as a region between starch and oligosaccharides. Thus, at least a partial degradation of dextrin by non-super-attenuating yeast strains is conceivable. The saccharification of dextrin and starch is generally due to the enzyme glucoamylase, which hydrolyzes successive glucose units from the non-reducing ends of starch chains, where it hydrolyzes the starch molecules. Erratt and Stewart tested the activity of the enzyme glucoamylase against a number of substrates (Erratt and Stewart 2013). The results showed that the initial rate of hydrolysis is faster with large molecular weight substrates, e.g. dextrin, freeze-dried beer or soluble potato starch, than for the disaccharide maltose. In

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

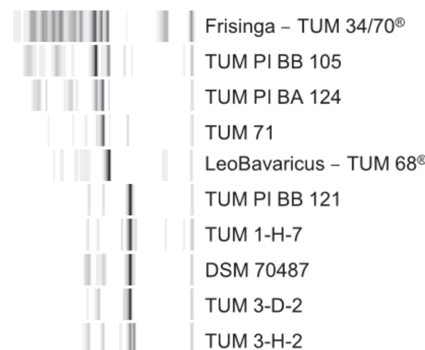


Figure 3. IGS2-314 rDNA curve-based genetic relationship in percentage with capillary electrophoresis patterns between LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2 (dendrogram built with Bionumerics 7.6).

Table 14. Yeast cell growth on dextrin agar plates inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated aerobically at 25°C evaluated after 888 h.

Yeast cell growth on dextrin agar plates inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated anaerobically at 25°C evaluated after 888 h		
Yeast isolate/strain	Species	Anaerobic
DSM 70487	*S. cerevisiae* var. *diastaticus*	strong
TUM PI BB 121	*S. cerevisiae* var. *diastaticus*	strong
TUM 3-D-2	*S. cerevisiae* var. *diastaticus*	strong
TUM 1-H-7	*S. cerevisiae* var. *diastaticus*	medium
TUM 71	*S. cerevisiae* var. *diastaticus*	strong
TUM 3-H-2	*S. cerevisiae* var. *diastaticus*	strong
LeoBavaricus—TUM 68®	*S. cerevisiae*	medium
LunaBavaria—TUM 127®	*S. cerevisiae*	medium
Colonia—TUM 177®	*S. cerevisiae*	low
Vetus—TUM 184®	*S. cerevisiae*	low
Pensum—TUM 210®	*S. cerevisiae*	strong
Mel—TUM 211®	*S. cerevisiae*	strong
TUM 213	*S. cerevisiae*	negative
Tropicus—TUM 506®	*S. cerevisiae*	medium
TUM 503	*S. cerevisiae*	negative
Harmonia—TUM 511®	*S. cerevisiae*	medium
Monacus—TUM 381®	*S. cerevisiae*	weak
Frisinga—TUM 34/70®	*S. pastorianus*	medium
Securitas -TUM 193®	*S. pastorianus*	low
TUM PI BA 124	*S. pastorianus*	strong

addition to that, the nature (i.e. type) of the starch or dextrin used and the fermentation medium pH had substantial effects on the rate and extent of growth of the *S. cerevisiae* var. *diastaticus* yeast cells. Commercial dextrin was not as good a substrate as dextrins prepared by digesting starch with alpha-amylase (Laluce and Mattoon 1984).

Table 15 shows the cell growth on starch agar plates inoculated with washed yeast cells at a concentration of 5 million cells mL^{-1} incubated at 25°C for 888 h. Except for TUM 3-H-2, all investigated *S. cerevisiae* var. *diastaticus* yeast strains showed visible cell growth on starch agar plates. The investigated *S. cerevisiae* and *S. pastorianus* brewing yeast strains did not grow under the appropriate conditions. Even if strain TUM 3-H-2 was identified by RT-PCR as *S. cerevisiae* var. *diastaticus*, this strain shows no super-attenuating ability. This result could be confirmed by the modified Durham tube test. The presence of oxygen did not seem to affect the growth conditions of the washed yeast cells on starch agar. In order to facilitate the classification of cell growth, bromophenol blue was added to the starch agar as an indicator dye. Under anaerobic incubation conditions, the same and consistent results could be achieved after 144 h (see Table 16). By using aerobic conditions almost all the investigated yeast strains showed cell growth. Bromophenol blue was dissolved in ethanol before it was added to the agar. Under aerobic conditions ethanol may be used by yeast strains as an energy source for cell growth. In conclusion, the starch and dextrin agar plate test confirmed that the use of starch agar plates with bromophenol blue as an indicator dye can be used to reliably and rapidly detect super-attenuating *S. cerevisiae* var. *diastaticus* yeast strains. In comparison with the commonly used Durham tube test with fermented beer medium according to MEBAK, the performed agar plate test is cheaper, faster and easier to use in common brewing labs. Amin showed that the conversion efficiency and ethanol production from *S. cerevisiae* var. *diastaticus* yeast strains depends on the initial concentrations of higher dextrins and soluble starch (Amin *et al.* 1985). The highest conversion efficiency was achieved with a dextrin concentration of 200 g L^{-1}. In contrast to the fermentation rate, the conversion efficiency decreased with increasing dextrin concentration up to 400 g L^{-1}. The amylase activity is affected by increasing the temperature until amylase production is completely inhibited, and fermentation occurred at 42°C (Amin *et al.* 1985). According to this finding, the starch and dextrin agar plate test should be performed in future analysis with similar initial dextrin or starch concentrations to potentially evaluate the results more quickly.

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 15. Yeast cell growth on starch agar plates inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated aerobically and anaerobically at 25°C and evaluated after 888 h.

Yeast cell growth on starch agar plates inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated at 25°C evaluated after 888 h			
Yeast isolate/strain	Species	Aerobic	Anaerobic
DSM 70487	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM PI BB 121	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 3-D-2	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 1-H-7	*S. cerevisiae* var. *diastaticus*	weak	weak
TUM 71	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 3-H-2	*S. cerevisiae* var. *diastaticus*	negative	negative
LeoBavaricus—TUM 68®	*S. cerevisiae*	negative	negative
LunaBavaria—TUM 127®	*S. cerevisiae*	negative	negative
Colonia—TUM 177®	*S. cerevisiae*	negative	negative
Vetus—TUM 184®	*S. cerevisiae*	negative	negative
Pensum—TUM 210®	*S. cerevisiae*	negative	negative
Mel—TUM 211®	*S. cerevisiae*	negative	negative
TUM 213	*S. cerevisiae*	negative	negative
Tropicus—TUM 506®	*S. cerevisiae*	negative	negative
TUM 503	*S. cerevisiae*	negative	negative
Harmonia—TUM 511®	*S. cerevisiae*	negative	negative
Monacus—TUM 381®	*S. cerevisiae*	negative	negative
Frisinga—TUM 34/70®	*S. pastorianus*	negative	negative
Securitas -TUM 193®	*S. pastorianus*	negative	negative
TUM PI BA 124	*S. pastorianus*	negative	negative

Table 16. Yeast cell growth on starch agar plates with bromophenol blue inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated aerobically and anaerobically at 25°C evaluated after 144 h.

Yeast cell growth on starch agar plates with bromophenol blue inoculated with washed yeast cells at a concentration of 5 million cells per mL incubated at 25°C and evaluated after 144 h			
Yeast isolate/strain	Species	Aerobic	Anaerobic
DSM 70487	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM PI BB 121	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 3-D-2	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 1-H-7	*S. cerevisiae* var. *diastaticus*	weak	weak
TUM 71	*S. cerevisiae* var. *diastaticus*	strong	strong
TUM 3-H-2	*S. cerevisiae* var. *diastaticus*	negative	negative
LeoBavaricus—TUM 68®	*S. cerevisiae*	weak	negative
LunaBavaria—TUM 127®	*S. cerevisiae*	negative	negative
Colonia—TUM 177®	*S. cerevisiae*	weak	negative
Vetus—TUM 184®	*S. cerevisiae*	weak	negative
Pensum—TUM 210®	*S. cerevisiae*	weak	negative
Mel—TUM 211®	*S. cerevisiae*	weak	negative
TUM 213	*S. cerevisiae*	strong	negative
Tropicus—TUM 506®	*S. cerevisiae*	weak	negative
TUM 503	*S. cerevisiae*	negative	negative
Harmonia—TUM 511®	*S. cerevisiae*	strong	negative
Monacus—TUM 381®	*S. cerevisiae*	weak	negative
Frisinga—TUM 34/70®	*S. pastorianus*	weak	negative
Securitas -TUM 193®	*S. pastorianus*	weak	negative
TUM PI BA 124	*S. pastorianus*	negative	negative

Morphological and cultural characters

Microscope images

The typical cells for *S. cerevisiae* var. *diastaticus* yeast strains are shown in Fig. 4 using the example of the *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487 and TUM 1-H-7. To show morphological differences in the size and shape of the yeast cells, both *S. cerevisiae* var. *diastaticus* yeast strains were compared to the bottom- and top-fermenting reference strains *S. pastorianus* Frisinga—TUM 34/70® and *S. cerevisiae* LeoBavaricus—TUM 68® used in common brewing practice. Figure 4 shows the microscopic images of (a) *S. pastorianus* Frisinga—TUM 34/70® (b) *S. cerevisiae* LeoBavaricus—TUM 68® (c) *S.*

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Figure 4. Types of cell morphology associated with *Saccharomyces* yeast species at 100× magnification. Nikon Eclipse Ti microscope images using DIC 100× of **(a)** *S. pastorianus* Frisinga—TUM 34/70® **(b)** *S. cerevisiae* LeoBavaricus—TUM 68® **(c)** *S. cerevisiae* var. *diastaticus* DSM 70487 **(d)** *S. cerevisiae* var. *diastaticus* TUM 1-H-7.

cerevisiae var. *diastaticus* DSM 70487 (d) *S. cerevisiae* var. *diastaticus* TUM 1-H-7.

On wort agar slants at room temperature, the cells of all four *Saccharomyces* yeast strains show a uniform egg-shaped (oval), elongated and spherical morphology. According to Chant, the cell form or shape is often linked closely to budding patterns. Chant reported that cells that show an oval cell form usually exhibit either an axial or a bipolar budding pattern, while cells that are elongated tend to produce buds in an almost exclusively bipolar fashion (Chant 1995). As can be seen in Fig. 4, the bottom-fermenting yeast strain *S. pastorianus* Frisinga—TUM 34/70® shows (a) oval cells and *S. cerevisiae* var. *diastaticus* TUM 1-H-7 and (d) more spherical cells with an axial budding pattern. *Saccharomyces cerevisiae* LeoBavaricus—TUM 68® shows (b) a uniform oval cell morphology and *S. cerevisiae* var. *diastaticus* DSM 70487 and (c) elongated cells with a bipolar budding pattern.

The cells varied in size from 3.99 μm (DSM 70487) in diameter to 6.01 μm (Frisinga—TUM 34/70®) with all intermediate sizes. With the exception of the top-fermenting culture yeast *S. cerevisiae* LeoBavaricus—TUM 68®, the cells appeared singly or in pairs from one to four cells. *Saccharomyces cerevisiae* LeoBavaricus—TUM 68® appeared in chains or clumps with cell sizes of 5.48 μm in diameter and 22.65 μm in area (n = 156) on average. Culture brewing yeast typically comprises a population of uniform cells between 6 and 10 μm in diameter. The bottom-fermenting *S. pastorianus* yeast strain Frisinga—TUM 34/70® shows cell sizes of 6.01 μm in diameter and 29.77 μm in area (n = 116). Within their population, brewing yeasts normally show a high degree of morphological homogeneity (Powell and Kerruish 2017). In contrast, beer-spoiling yeast can show a wide variety of cell shapes and sizes (Powell and Kerruish 2017). *Saccharomyces cerevisiae* var. *diastaticus* yeast strain TUM 1-H-7 shows intermediate cell sizes with a diameter of 4.08 μm and an area of 14.08 μm (n = 120) and strain DSM 70487 is 3.99 μm in diameter and an area of 13.01 μm (n = 149). As already reported by Powell and Kerruish (2017), the results show that the *S. cerevisiae* var. *diastaticus* yeast strains exhibit a smaller cell size in contrast to the lager and ale brewing yeast strains.

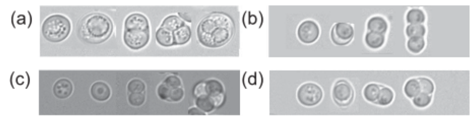


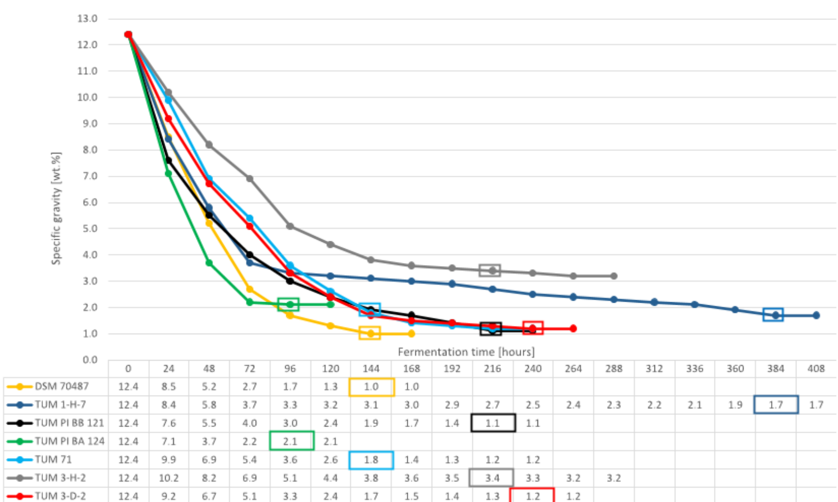
Figure 5. Ascus with ascospores of the *Saccharomyces* yeast strains **(a)** *S. pastorianus* Frisinga—TUM 34/70®, **(b)** *S. cerevisiae* var. *diastaticus* TUM 3-D-2, **(c)** *S. cerevisiae* var. *diastaticus* DSM 70487 and **(d)** *S. cerevisiae* var. *diastaticus* TUM 1-H-7 at 100× magnification using Nikon Eclipse Ti microscope images with DIC 100×.

Sodium acetate agar test for sporulation behavior

Figure 6 shows the asci with spores of (a) *S. pastorianus* Frisinga—TUM 34/70®, (b) *S. cerevisiae* var. *diastaticus* TUM 3-D-2 (c) *S. cerevisiae* var. *diastaticus* DSM 70487 and (d) *S. cerevisiae* var. *diastaticus* TUM 1-H-7. Microscopic evaluation of sporulated cells of yeast strains of the species *S. cerevisiae* has been examined by a number of investigators (Lomander and Gundersen 1963; Merritt and Hurley 1972; Rousseau *et al.* 1972; Davidow, Goetsch and Byers 1980). Due to the environmental conditions, yeast cells proliferate vegetatively or asexually. The majority of brewing yeasts reproduce predominantly via mitosis, which results in theoretically identical new cells and cell populations barring random mutation events. In most cases, vegetative growth occurs through budding, employing multilateral or bipolar division patterns. According to Powell, beer-spoiling yeasts also have the ability to reproduce sexually by cell fusion to form a zygote (karyogamy) and subsequently meiosis, which results in the formation of spores contained within an ascus. This phenomenon can be induced under certain conditions such as sudden changes in environmental conditions or nutrient deficiency. The shape of the ascus as well as spore formation is highly variable and dependent on the yeast genus and species (Powell and Kerruish 2017). *Saccharomyces* yeasts tend to produce one to four spores typically enclosed within an ellipsoidal or tetrahedral-shaped ascus, which can also be seen in Fig. 5: (a) Frisinga—TUM 34/70®, (b) TUM 3-D-2 and (d) TUM 1-H-7. Strain DSM 70487 also shows linear shaped asci including three spores (c).

As can be seen in Tables 17 and 18, the number of ascispores remained approximately at the same level after 5 and 8 days on sodium acetate agar at 28°C. However, the brewing culture yeast strains Frisinga—TUM 34/70® and LeoBavaricus—TUM 68® showed less sporulation behavior than the investigated *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487, TUM 71, TUM PI BB 121, TUM PI BA 124, TUM 1-H-7, TUM 3-H-2 and TUM 3-D-2. The top-fermenting *S. cerevisiae* yeast strain LeoBavaricus—TUM 68® had the highest percentage of unsporulated yeast cells after 5 and 8 days (e.g. 98.65% and 96.91%) followed by the bottom-fermenting *S. pastorianus* yeast strains TUM PI BA 124 (88.28% and 86.67%) and Frisinga—TUM 34/70® (87.69% and 83.31%). Both *S. pastorianus* strains showed similar sporulation behavior with similar spore formation of mostly one to two spores contained within an ascus. It can be concluded that the cells of the investigated *S. cerevisiae* var. *diastaticus* yeast strains show faster and higher spore formation with a higher percentage of more than one spore contained within an ascus. Strain DSM 70487 showed the lowest number of sporulated cells of 73 sporulated cells in total, whereas strain TUM 3-H-7 showed the highest number with 266 followed by TUM 1-H-7 with 243 sporulated yeast cells in total under identical experimental conditions. All investigated *S. cerevisiae* var. *diastaticus* yeast strains showed a spore formation of mostly one to three spores contained within an ascus, except for TUM 3-H-2 and TUM 1-H-7, which showed main spore formation of two spores within an ascus.

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018



	0	24	48	72	96	120	144	168	192	216	240	264	288	312	336	360	384	408
DSM 70487	12.4	8.5	5.2	2.7	1.7	1.3	1.0	1.0										
TUM 1-H-7	12.4	8.4	5.8	3.7	3.3	3.2	3.1	3.0	2.9	2.7	2.5	2.4	2.3	2.2	2.1	1.9	1.7	1.7
TUM PI BB 121	12.4	7.6	5.5	4.0	3.0	2.4	1.9	1.7	1.4	1.1	1.1							
TUM PI BA 124	12.4	7.1	3.7	2.2	2.1	2.1												
TUM 71	12.4	9.9	6.9	5.4	3.6	2.6	1.8	1.4	1.3	1.2	1.2							
TUM 3-H-2	12.4	10.2	8.2	6.9	5.1	4.4	3.8	3.6	3.5	3.4	3.3	3.2	3.2					
TUM 3-D-2	12.4	9.2	6.7	5.1	3.3	2.4	1.7	1.5	1.4	1.3	1.2	1.2						

Figure 6. Drop in specific gravity measured in a single reference vessel compared with the average in final gravity (marked with box) measured in triplicate for the tested yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

Table 17. Number of ascispores in % after 5 days on sodium acetate agar at 28°C (total counted number).

Number of ascispores in % after 5 days on sodium acetate agar at 28°C; (total counted number)							
Identifier	Unsporulated	1-spored	2-spored	3-spored	4-spored	Total sporulated cells	Total number counted
DSM 70487	87.50 (525)	02.50 (15)	09.33 (58)	00.33 (2)	00.00 (0)	12.50 (75)	600
TUM PI BB 121	76.24 (459)	14.12 (85)	08.47 (51)	01.16 (7)	00.00 (0)	23.76 (143)	602
TUM 3-D-2	72.37 (440)	15.95 (97)	08.39 (51)	02.30 (14)	00.00 (0)	27.63 (162)	608
TUM 1-H-7	62.32 (402)	05.27 (34)	26.36 (170)	05.74 (37)	00.31 (2)	37.68 (243)	645
TUM 71	73.28 (469)	16.25 (104)	07.81 (50)	02.66 (17)	00.00 (0)	26.72 (171)	640
TUM 3-H-2	57.57 (361)	03.35 (21)	31.10 (195)	06.54 (41)	01.43 (9)	42.43 (266)	627
LeoBavaricus—TUM 68®	98.65 (659)	00.90 (6)	00.45 (3)	00.00 (0)	00.00 (0)	01.35 (9)	668
Frisinga—TUM 34/70®	87.69 (527)	04.49 (27)	07.15 (43)	00.66 (4)	00.00 (0)	12.31 (74)	601
TUM PI BA 124	88.28 (595)	08.01 (54)	03.71 (25)	00.00 (0)	00.00 (0)	11.72 (79)	674

Brewing trials

Fermentation performance

Figure 6 shows the drop in specific gravity during main fermentation by the investigated yeast strains. As shown in Fig. 6, the *S. pastorianus* yeast strain TUM PI BA 124 has the quickest drop in specific gravity, followed by the *S. cerevisiae* control strain DSM 70487. TUM PI BA 124 reached final gravity after 96 h of fermentation. TUM 71 needed 144 h to reach the final gravity of 1.8 °P measured in the finished beers, but needed an additional 48 h (total 216) to reach the final gravity of 1.2 °P measured in the single reference vessel. Therefore, TUM 71 seems to ferment the wort slower than the other strains but did so continuously until an apparent attenuation similar to the other investigated *S. cerevisiae* var. *diastaticus* yeast strains could be achieved. Strain TUM 1-H-7 shows the lowest drop in specific gravity and reached a final apparent attenuation of 87.47% after 384 h of fermentation.

Table 19 shows the apparent attenuation compared with the fermentation time required by the isolated strains. The different fermentation rates and degrees of apparent attenuation are due to their ability to ferment maltose and maltotriose (see TUM 3-H-2) as well as starch and higher dextrins normally not fermented by yeast strains without super-attenuating ability, like common brewing yeasts. According to Andrews and Gilliland, super-attenuation is due to the conversion of dextrin into fermentable sugars. As a result of their super-attenuating power, the investigated *S. cerevisiae* var. *diastaticus* yeast strains reached apparent attenuations above the attenuation limit of approximately 86% for commercial brewing strains, which was previously shown for the *S. cerevisiae* yeast strain LeoBavaricus—TUM 68® by Meier-Dörnberg (Meier-Dörnberg *et al.* 2017a). Erratt defined the attenuation limit as the lowest specific gravity that can normally be reached by the brewing yeast *S. cerevisiae* (Erratt 1987) and is dependent on the wort and the yeast used. The *S. diastaticus* yeast strains DSM 70487, TUM PI BB 121, TUM 3-D-2 and TUM 1-H-7 reached apparent attenuations from 87.47% to 92.77%. In this case, these *S. cerevisiae* var. *diastaticus* yeast strains show super-attenuating ability. According to Erratt, these

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 18. Number of ascispores in % after 8 days on sodium acetate agar at 28°C (total counted number).

	Number of ascispores in % after 8 days on sodium acetate agar at 28°C (total counted number)						
Identifier	Unsporulated	1-spored	2-spored	3-spored	4-spored	Total sporulated cells	Total number counted
DSM 70487	83.06 (510)	02.60 (16)	12.54 (77)	01.79 (11)	00.00 (0)	16.94 (104)	614
TUM PI BB 121	78.75 (630)	10.75 (86)	09.25 (74)	01.25 (10)	00.00 (0)	21.25 (170)	800
TUM 3-D-2	87.37 (609)	03.73 (26)	06.17 (43)	02.44 (17)	00.29 (2)	12.63 (88)	697
TUM 1-H-7	62.91 (419)	04.65 (31)	28.98 (193)	03.45 (23)	00.00 (0)	37.09 (247)	666
TUM 71	70.13 (446)	11.00 (70)	15.10 (96)	03.30 (21)	00.47 (3)	29.87 (190)	636
TUM 3-H-2	60.95 (412)	05.62 (38)	27.66 (187)	05.62 (38)	00.15 (1)	39.05 (264)	676
LeoBavaricus—TUM 68®	96.91 (597)	02.92 (18)	00.00 (0)	00.16 (1)	00.00 (0)	03.09 (19)	616
Frisinga—TUM 34/70®	83.31 (514)	07.45 (46)	07.94 (49)	01.30 (8)	00.00 (0)	16.69 (103)	617
TUM PI BA 124	86.67 (520)	09.50 (57)	03.83 (23)	00.00 (0)	00.00 (0)	13.33 (59)	600

Table 19. Apparent attenuation (AA %) of the final beer compared with the specific time for primary fermentation measured in a single reference vessel for the investigated yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

	Apparent attenuation (AA %) of the final beer	
TUM yeast strain	AA (%)	Fermentation time (h)
DSM 70487	92.77 ± 0.32	144
TUM 1-H-7	87.47 ± 1.83	384
TUM PI BB 121	91.73 ± 0.14	216
TUM PI BA 124	84.47 ± 0.14	96
TUM 71	86.47 ± 1.39	216
TUM 3-H-2	73.80 ± 9.31	264
TUM 3-D-2	90.67 ± 0.14	240

S. cerevisiae var. *diastaticus* yeast strains show super-attenuations due to the specific gravity of the wort which falls below the attenuation limit. The low apparent attenuation of 73.80% by TUM 3-H-2 was due to the unique strain property of not fermenting one of the major wort sugars, namely maltotriose. In conclusion, commonly used yeast strains used in brewing practice show a final gravity of nearly 1.8 °P, whereas beers produced under the same fermentation conditions show a final gravity up to 0.9 °P. The apparent attenuation increases from approximately 83%–87% to 87%–90%.

Sugar utilization

As Table 20 shows, all of the strains were able to metabolize the major wort sugars (e.g. glucose, fructose, sucrose, maltose, maltotriose). Besides TUM 3-H-2, all yeast strains fermented almost all wort sugars to the full extent. Variation in glucose utilization was above 98%, fructose and sucrose were utilized completely. TUM 3-H-2 had the lowest utilization rate for maltose (80.58%) and maltotriose (45.14%), while all other strains were above 92%. The results suggested that the *S. cerevisiae* var. *diastaticus* yeast strain TUM 3-H-2 does not utilize maltose and maltotriose completely.

Flocculation (cell count)

Yeast flocculation is an important and natural way for brewers to clarify beer, and it provides a cost-effective means of collecting yeast for repitching. Flocculation is based on the non-sexual aggregation of yeast cells. In *Saccharomyces* yeasts, flocculation is governed by the genetic background as well as the environmental and fermentation conditions (Zepf 2010; Soares 2011; Vidgren 2011; Meier-Dörnberg *et al.* 2017a). It would be expected that beer-spoilage yeasts lead to a reduction in flocculation potential compared with classical brewing strains which are highly flocculent at the basic level (Powell and Kerruish 2017). However, in previous investigations by Meier-Dörnberg, it was shown that the flocculation potential in yeast differs from strain to strain and seems to be due to the physiological properties of each strain (Meier-Dörnberg *et al.* 2017a,c).

As Fig. 7 shows, the *S. pastorianus* yeast strain TUM PI BA 124 as well as the *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487, TUM 3-H-2 and TUM 3-D-2 flocculated continuously to cell concentrations below the pitching rate after reaching their apparent attenuation. With a concentration below 1 million yeast cells mL^{-1}, TUM 3-H-2 shows the lowest concentration of cells in suspension by reaching their apparent attenuation (00.14 million yeast cells mL^{-1}). Compared to yeast strains TUM 3-H-2 and TUM 3-D-2, TUM PI BA 124 and DSM 70487 show concentrations of cells in suspension close to the pitching rate (12.66 and 12.81 million yeast cells mL^{-1}), which is caused by a higher concentration of cells during the main fermentation. According to their flocculation behavior, these strains can be classified as flocculent yeast strains (Table 21). In contrast to these flocculent yeast strains, strains TUM 1-H-7, TUM PI BB 121 and TUM 71 remained in a suspension that is close to the pitching concentration, even once they reached their apparent attenuation. TUM 1-H-7, TUM PI BB 121 and TUM 71 can therefore be classified as powdery yeast strains (Table 21). Across all the investigated yeast strains, TUM PI BB 121 reached the highest concentration of cells in suspension. After 72 h of fermentation, TUM PI BB 121 had a maximum concentration of cells in suspension of 186.61 million yeast cells mL^{-1}. At reaching their apparent attenuation of 91.73% after 216 h of fermentation, strain TUM PI BB still had a concentration of cells in suspension of 68.28 million yeast cells mL^{-1} which is, with the exception of strain TUM 1-H-7, higher than the overall maximum concentration of all the compared strains.

Change in pH value

Table 22 shows the drop in pH during the first 96 h of primary fermentation, the pH value after maturation phase and the average pH value of the final beer. As shown in Table 22, time to reach the minimum pH value for primary fermentation differs between the investigated yeast strains. Strains TUM PI BB 121 and TUM PI BA 124 show the quickest drop in pH value and reached their minimum pH value for primary fermentation after 24 h. Strains DSM 70487 and TUM 1-H-7 needed 48 h and

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 20. Mean percentage of total wort sugar utilization in beer, measured in triplicate after lagering; confidence level 95%.

	Total sugar utilization in beer after lagering (%)				
TUM yeast strain	Glucose	Fructose	Sucrose	Maltose	Maltotriose
DSM 70487	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.50 ± 0.07	98.29 ± 0.40
TUM 71	98.34 ± 0.14	100.00 ± 0.00	100.00 ± 0.00	97.39 ± 0.25	98.90 ± 0.32
TUM PI BB 121	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.09 ± 0.07	96.22 ± 0.28
TUM PI BA 124	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	99.14 ± 0.10	96.03 ± 0.37
TUM 1-H-7	99.10 ± 0.10	100.00 ± 0.00	100.00 ± 0.00	99.12 ± 0.12	92.81 ± 4.27
TUM 3-H-2	98.70 ± 0.22	100.00 ± 0.00	100.00 ± 0.00	80.58 ± 2.29	45.14 ± 9.05
TUM 3-D-2	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	98.94 ± 0.06	98.21 ± 0.15

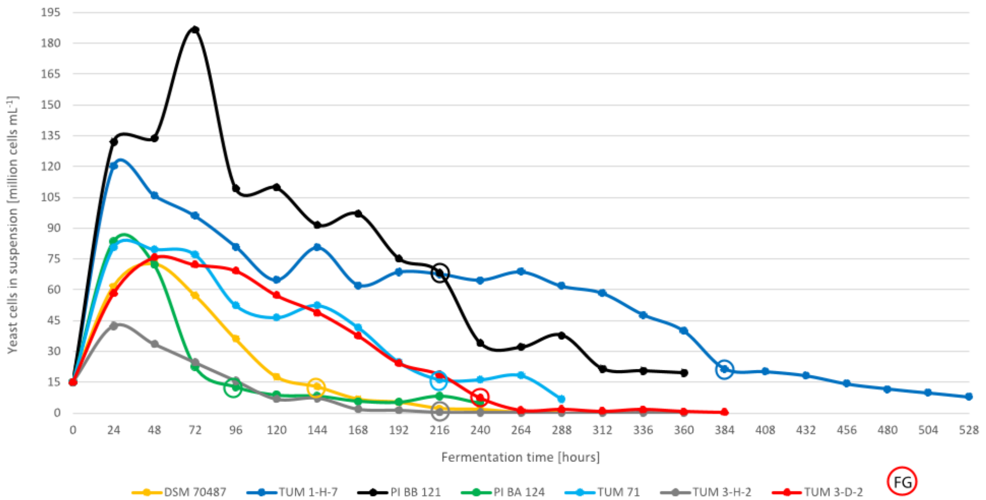


Figure 7. Yeast cells in suspension during the main fermentation and maturing phase. The circle marks the specific final gravity of the investigated yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2.

Table 21. Difference in maximum yeast cell concentration during primary fermentation and yeast cell concentration by reaching the specific final gravity (FG) and the flocculation behavior of DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2.

	Yeast cell sedimentation at the end of primary fermentation (Hz mL^{-1})			
Yeast strain	Max. cell conc.	Cell conc. FG	Difference	Flocculation behavior
DSM 70847	72.84	12.81	–60.03	flocculent
TUM 1-H-7	120.20	21.35	–98.85	powdery
TUM PI BB 121	186.61	68.28	–118.33	powdery
TUM PI BA 124	83.40	12.66	–70.74	flocculent
TUM 71	80.52	16.20	–64.32	powdery
TUM 3-H-2	42.24	00.14	–42.10	flocculent
TUM 3-D-2	75.76	07.33	–68.43	flocculent

TUM 71, TUM 3-H-2 and TUM 3-D-2 needed 72 h to reach their minimum pH value for primary fermentation. With the exception of TUM PI BB 121, the used yeast strains recorded a pH value increase after the maturation and lagering phase. This was already shown by Meier-Dörnberg in 2017 and might be due to the excretion of yeast metabolites and the uptake and metabolization of pyruvate (Meier-Dörnberg *et al.* 2017a,c). Due to the super-attenuating ability of these yeast strains and the related higher amount of yeast cells after fermentation compared with common brewing yeast strains (Meier-Dörnberg *et al.* 2017a), a pH value increase of up to 0.2 (TUM PI BA 124) can be observed.

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 22. Change in pH value during primary fermentation, after the maturation and lagering phase, rounded to two decimal figures, confidence level 95%.

	pH value decrease during primary fermentation								
TUM yeast strain	0 h	24 h	48 h	72 h	96 h	After primary fermentation	After maturation	Final beer (after lagering)	ΔpH
DSM 70487	5.2	4.4	4.2	4.2	4.2	4.3	4.4	4.4 ± 0.06	0.8
TUM 1-H-7	5.2	4.5	4.3	4.3	4.3	4.4	4.5	4.5 ± 0.01	0.7
TUM PI BB 121	5.2	4.4	4.4	4.4	4.4	4.4	4.5	4.4 ± 0.01	0.8
TUM PI BA 124	5.2	4.4	4.4	4.4	4.4	4.4	4.5	4.6 ± 0.01	0.6
TUM 71	5.2	4.6	4.4	4.3	4.3	4.3	4.4	4.4 ± 0.01	0.8
TUM 3-H–2	5.2	4.6	4.5	4.4	4.4	4.3	4.4	4.4 ± 0.09	0.8
TUM 3-D-2	5.2	4.4	4.3	4.2	4.2	4.2	4.3	4.3 ± 0.01	0.9

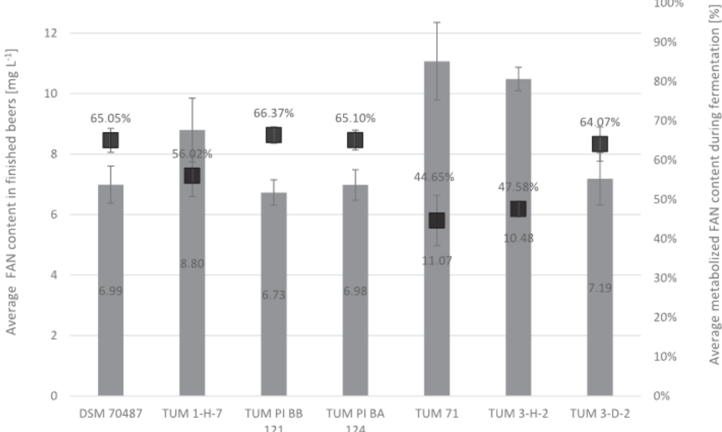


Figure 8. Average metabolized and FAN content in finished beers produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

Amino acid utilization

The mean amino acid uptake in the finished beers after lagering by the investigated *S. cerevisiae* var. *diastaticus* yeast strains is shown in Tables S1 and S2, Supporting Information. The commonly accepted amino acid uptake classification is indicated with shading according to Jones and Pierce (Jones and Pierce 1964; Procopio, Brunner and Becker 2014; Müller-Auffermann *et al.* 2015; Meier-Dörnberg *et al.* 2017a,c). As Meier-Dörnberg described in former research characterizing 15 *Saccharomyces* brewing yeast strains, the total amino acid utilization followed no defined process and was different for each investigated yeast strain (Meier-Dörnberg *et al.* 2017a,c).

In contrast to the characterized *Saccharomyces* brewing strains, all 13 *S. cerevisiae* var. *diastaticus* strains as well as the *S. pastorianus* strain TUM PI BA 124 metabolized a lower free amino nitrogen (FAN) and total AS amount on average. The brewing strains show a metabolization rate of over 70%, whereas the *S. diastaticus* strains metabolized 44.65% to 66.37% of the wort amino acids. The single FAN and total amino acid (AS) utilization of each investigated strain in comparison with the corresponding residual contents can be seen in Figs 8 and 9. The utilization rate of FAN and AS is correlated for the same yeast strain but is different across strains.

Flavor characterization

Phenolic off-flavor

Table 23 shows the results of the POF tests evaluated by sniffing. As shown in Table 23, all yeast strains that belong to *S. cerevisiae* var. *diastaticus* can build phenolic flavors. Yeast strain TUM PI BA 124, genetically classified as *S. pastorianus*, is POF negative and no corresponding POF could be detected by sniffing. According to the tested *S. pastorianus* lager beer strains by Meier-Dörnberg, TUM PI BA 124 cannot decarboxylate any of the precursor acids (Meier-Dörnberg *et al.* 2017a). Therefore, the phenylacrylic acid decarboxylase (*PAD1*) and/or ferulic acid decarboxylase (*FDC1*) gene activity might be inactive or blocked (European Bioinformatic Institut Cambridge 2010; Mukai *et al.* 2014; Richard, Viljanen and Penttilä 2015).

Figure 10 shows the concentrations of 4-VG measured in the finished beers after lagering. According to the evaluation by sniffing, DSM 70847, TUM 1-H-7, TUM PI BB 121, TUM 71, TUM 3-H-2 and TUM 3-D-2 were POF positive, with detected concentrations of 4-VG above the individual threshold for 4-VG of 0.3 mg L^{-1} measured for lager beers (Meilgaard 1975). The concentrations of 4-VG measured in the finished beers produced with the *S. cerevisiae* var. *diastaticus* yeast strains are also above

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

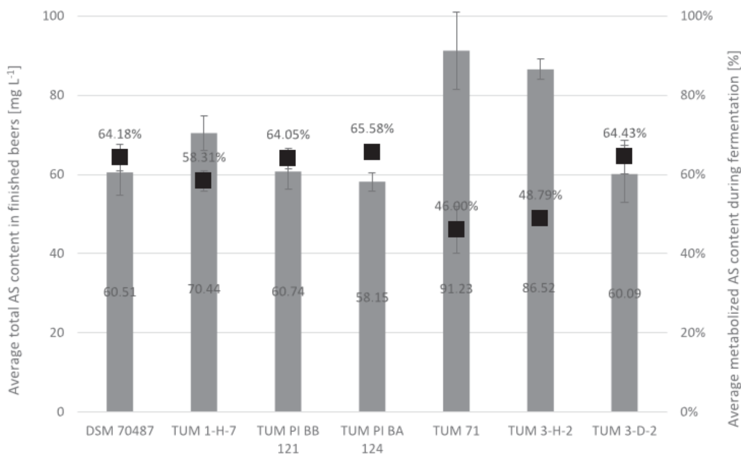


Figure 9. Average metabolized and total amino acid (AS) content in finished beers produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

Table 23. POF results of the investigated yeast strains.

	POF test/sniffing perception of		
TUM yeast strain	4-VG/ Ferulic acid	4-VP/ Coumaric acid	4-VS/ Cinnamic acid
DSM 70847	+	+	+
TUM PI BB 121	+	+	+
TUM 3-D-2	+	+	+
TUM 1-H-7	+	+	+
TUM 71	+	+	+
TUM 3-H-2	+	+	+
TUM PI BA 124	–	–	–

the average value of 2.0 mg L^{-1} for commercial German wheat beers (Back 2005).

Fermentation by-products

There was a variation in the production of fermentation by-products for all the yeast strains (Tables 24 and 25). Except for yeast strain TUM PI BA 124, the concentration of higher alcohols is above 100 mg L^{-1}. The highest level of esters was detected in the beer produced by DSM 70487 with a concentration of 63.23 ± 2.83 mg L^{-1}. With concentrations of 6.13 mg L^{-1} for isoamyl acetate and 2.93 mg L^{-1} for 4-VG, DSM 70487 had the highest levels of isoamyl acetate and the second highest concentration of 4-VG. In addition to *S. cerevisiae* var. *diastaticus* strain DSM 70487, TUM 3-H-2 and TUM 3-D-2 also showed concentrations of esters specific to the production of German wheat beers. The concentrations of these typical German wheat beer fermentation by-products are within the average reference values for regular German wheat beers (2–8 mg L^{-1} isoamyl acetate and 1–4 mg L^{-1} of 4-VG) (Back 2005). Therefore, these *S. cerevisiae* var. *diastaticus* yeast strains seem to be suitable for the production of German wheat beers.

The concentrations of acetaldehyde, 2,3-pentanedione and diacetyl are associated with unmatured, so-called green beer and are mostly taken by brewers as an indication that the maturation phase is complete (Meilgaard 1975; Narziss and Back 2005; Kunze 2011). The ratio of diacetyl to pentanedione is also helpful to indicate whether elevated diacetyl concentrations are due to contaminants or fermentation by-products (Lodolo *et al.* 2008). TUM 71 and TUM 3-H-2 showed concentrations of diacetyl above the individual threshold of 0.15 mg L^{-1} (Meilgaard 1975). The concentration of acetaldehyde is below their individual thresholds of 25 mg L^{-1} for all strains. TUM 71 and TUM 3-H-2 showed concentrations of diacetyl above the individual threshold of 0.15 mg L^{-1} (Meilgaard 1975).

Sulfur dioxide

Table 26 shows the SO_2 concentration of the finished beers produced by the investigated yeast strains. As shown in the table, the concentration of sulfur dioxide (SO_2) during fermentation produced by the *S. cerevisiae* var. *diastaticus* strains is very low in the finished beers. Except for TUM 71 and TUM 3-D-2, the concentration of SO_2 is below the detection limit of 0.5 mg L^{-1}. In contrast to the *S. cerevisiae* var. *diastaticus* yeast strains, *S. pastorianus* strain TUM PI BA 124 produced the highest quantity of SO_2 with a total amount of 8.33 ± 0.53 mg L^{-1} on average. Compared to commonly used *S. pastorianus* and *S. cerevisiae* brewing culture strains, TUM PI BA 124 showed similarly high concentrations of SO_2 to the yeast strain Securitas—TUM 193® (Meier-Dörnberg *et al.* 2017a) and could therefore also be suitable for producing beers with a long-term flavor stability.

Sensory evaluation

The following Figs 11–14 show the average of each flavor intensity judged by all seven panelists and summarized according to the main flavor categories. All the beers produced had no prevailing off-flavors and were rated with a four or a five in every category of the DLG scheme for beer (data not shown). In terms of the descriptive sensory evaluation, the following

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

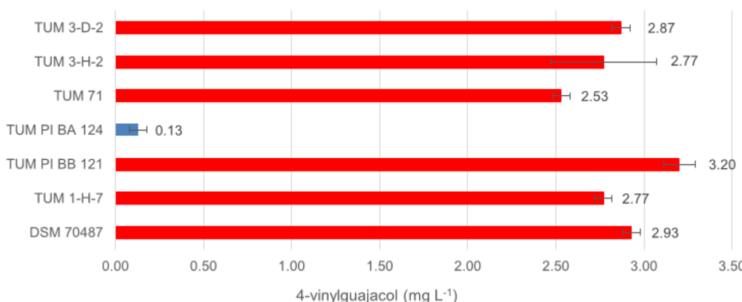


Figure 10. Concentration of 4-VG measured in the finished beers after lagering produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

Table 24. Average of important fermentation by-products measured in triplicate of the final beers produced with DSM 70487, TUM 1-H-7, TUM PI BB 121 and TUM PI BA 124; confidence level 95%.

	Fermentation by-products (mg L^{-1})			
	DSM 70487	TUM 1-H-7	TUM PI BB 121	TUM PI BA 124
Isoamyl acetate	6.13 ± 0.69	1.27 ± 0.05	1.70 ± 0.00	2.50 ± 0.09
Ethyl acetate	57.10 ± 2.26	28.67 ± 1.78	23.97 ± 0.28	34.27 ± 1.47
∑ Ester (E)	63.23 ± 2.83	29.93 ± 1.83	25.67 ± 0.28	36.77 ± 1.56
n-Propanol	28.13 ± 3.83	28.60 ± 2.49	22.07 ± 0.67	11.87 ± 0.14
i-Butanol	16.40 ± 3.66	10.87 ± 0.28	12.13 ± 0.05	7.83 ± 0.19
Amyl alcohols	95.87 ± 9.25	79.03 ± 1.59	79.77 ± 0.37	69.67 ± 0.35
∑ Higher alcohols (HE)	140.40 ± 16.75	118.50 ± 4.35	113.97 ± 0.42	89.37 ± 0.59
4-VG	2.93 ± 0.05	2.77 ± 0.05	3.20 ± 0.09	0.13 ± 0.05
Diacetyl	0.05 ± 0.01	0.04 ± 0 01	0.03 ± 0.01	0.05 ± 0.01
2,3-Pentanedione	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
∑ Vicinal diketones	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
Acetaldehyde	0.23 ± 0.14	0.05 ± 0.04	0.20 ± 0.09	8.37 ± 1.64
Ratio (∑E: ∑HE)	1: 2.22	1: 3.96	1: 4.44	1: 2.43

Table 25. Average of important fermentation by-products measured in triplicate of the final beers produced with TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

	Fermentation by-products (mg L^{-1})		
	TUM 71	TUM 3-H-2	TUM 3-D-2
Isoamyl acetate	1.87 ± 0.05	2.70 ± 0.28	2.57 ± 0.14
Ethyl acetate	33.23 ± .164	29.40 ± 2.91	35.17 ± 1.38
∑ Ester (E)	35.0 ± 1.70	32.10 ± 3.19	37.73 ± 1.50
n-Propanol	21.37 ± 0.69	20.57 ± 2.30	25.87 ± 0.37
i-Butanol	10.03 ± 0.32	17.30 ± 1.91	19.93 ± 0.54
Amyl alcohols	79.07 ± 1.91	86.07 ± 8.77	106.67 ± 1.41
∑ Higher alcohols (HE)	110.47 ± 2.88	123.93 ± 12.76	152.47 ± 2.26
4-VG	2.53 ± 0.05	2.77 ± 0.30	2.87 ± 0.05
Diacetyl	0.23 ± 0.03	0.19 ± 0.02	0.06 ± 0.01
2,3-Pentanedione	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
∑ Vicinal diketones	0.25 ± 0.03	0.21 ± 0.02	0.07 ± 0.01
Acetaldehyde	22.40 ± 4.84	6.93 ± 3.92	0.33 ± 0.11
Ratio (∑E: ∑HE)	1: 3.15	1: 3.86	1: 4.04

Table 26. SO_2 concentration of the final beers produced with the investigated yeast strains; confidence level 95%

SO_2 concentration of the finished beers	
TUM yeast strain	SO_2 (mg L^{-1})
DSM 70847	<0.50 ± 0.00
TUM 1-H-7	<0.50 ± 0.00
TUM PI BB 121	<0.50 ± 0.00
TUM PI BA 124	8.33 ± 0.53
TUM 71	1.00 ± 0.00
TUM 3-H-2	<0.50 ± 0.00
TUM 3-D-2	0.67 ± 0.27

Figs 11–14 show the aroma profiles of the investigated *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM 71, TUM 3-H-2 and TUM 3-D-2 as well as the aroma profile of the investigated *S. pastorianus* yeast strain TUM PI BA 124. The overall flavor impression (main flavor) is shown in a solid black line, and the most distinct individual flavor attributes (main aroma attributes) are shown in a dotted black line. The individual flavor attributes represent the most noted and highest rated flavors by all panelists within the seven main aroma categories. The average values of the single flavor attributes are summarized in main categories and represent the overall flavor impression.

As shown in the figures, all *S. cerevisiae* var. *diastaticus* yeast strains have a clove-like aroma. This is in accordance with the

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

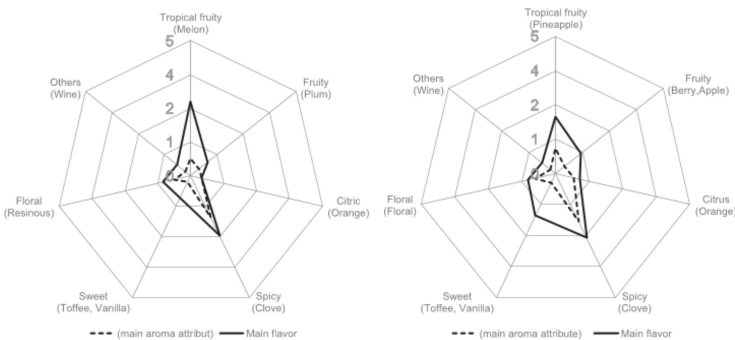


Figure 11. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487 (left) and TUM PI BB 121 (right).

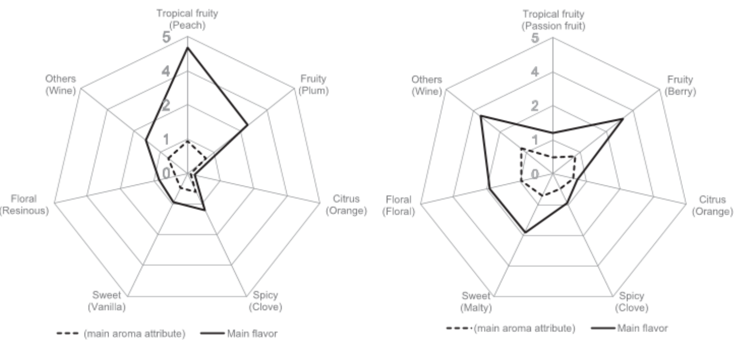


Figure 12. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains TUM 3-H-2 (left) and TUM 71 (right).

POF results obtained by sniffing and the analytically detected concentrations. Even if the detected concentrations in the finished beers are above the individual threshold of 4-VG for all investigated *S. cerevisiae* var. *diastaticus* yeasts, only the strains DSM 70487 and TUM PI BB 121 had a very distinct clove-like aroma that was recognized by the panelists. TUM 1-H-7, TUM 71, TUM 3-H-2 and TUM 3-D-2 had a very slight clove-like aroma with main flavor impressions of fruits and other flavors. Caused by the distinct clove-like aroma of the beers brewed with DSM 70487 and TUM PI BB 121, over 50% of the tasters associated the produced and tasted beers with German wheat beer. Compared with strain DSM 70487, TUM PI BB 121 also produces fruity flavors reminiscent of melon and other tropical fruity flavors (flavor intensity of 2.63, e.g. Fig. 11). In addition to a German wheat beer, 37% of the panelists also recommended strain TUM PI BB 121 for brewing a kölsch style beer. TUM 3-H-2 and TUM 71 seemed to be suitable for more than one beer type. Even if the aroma profile differed between these two strains from a very tropical fruity flavor from the beers produced with TUM 3-H-2 (flavor intensity of 4.43, e.g. Fig. 11) to fruity flavors as well as a flavor reminiscent of wine for strain TUM 71, both strains also had a spicy flavor reminiscent of the clove-like flavor in German wheat beers. In conclusion, 42.85% of the panelists could not clearly assign this beer to a wheat nor to an ale style beer. These strains may be suitable for brewing a 'Bavarian Ale' beer, which was suggested by Meier-Dörnberg as a beer type for brewing a beer with the fruitiness of an ale style, underlined by the slightly spicy and yeasty flavors of a wheat beer (Meier-Dörnberg *et al.* 2017c). TUM 3-D-2 shows a well-balanced flavor profile. The panelists also could not clearly associate the beer to a specific beer style, but at 28.5%, most of the panelists associated the beers produced with TUM 3-D-2 with a wheat beer style. The yeast TUM 1-H-7 was judged as an ale beer style, and the beer produced was very fruity. The *S. pastorianus* yeast strain TUM PI BA 124 is POF negative with no analytically detected concentrations of 4-VG and was associated by the panelists as an ale style beer, mainly caused by the distinct sweet and fruity flavor impressions reminiscent of chocolate and apples.

CONCLUSION

The purpose of this work was to investigate different spoilage yeast strains/isolates of the species *S. cerevisiae* var. *diastaticus* according to their spoilage potential and their application for brewing. It could be shown that the spoilage potential of *S. cerevisiae* var. *diastaticus* and therefore the super-attenuating power is strain dependent. Furthermore, the results show that *S. cerevisiae* var. *diastaticus* yeast strains are suitable for brewing

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Figure 13. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains TUM 1-H-7 (left) and TUM 3-D-2 (right).

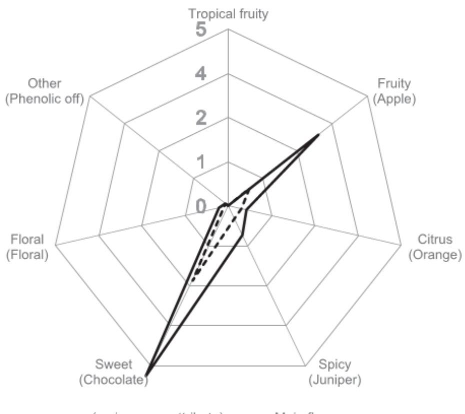


Figure 14. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. pastorianus* yeast strain TUM PI BA 124.

under similar conditions compared with classical brewing culture strains, resulting in desirable, appealing beers with clear taste. For this purpose, 19 spoilage yeast cultures, isolated from different beverages of various breweries (beer, beer-mixed beverages and lemonade), were genetically identified using RT-PCR and tested on their phenolic off-flavor and spoilage potential. Eighteen isolates were identified as belonging to the species *S. cerevisiae* var. *diastaticus,* all capable of building phenolic off-flavors. With the exception of yeast isolate TUM 3-H-2, all *S. cerevisiae* var. *diastaticus* isolates showed super-attenuating ability with differences in their spoilage potential caused by the time needed to metabolize residual wort sugars such as higher dextrins and starch (modified Durham tube test with fermented beer medium). As the results show, the investigated super-attenuating yeasts have a distinct beer spoilage potential. The metabolism of dextrin and starch degradation is strain dependent which could be confirmed by the developed starch agar plate test. Under anaerobic conditions this starch agar plate test can be used to detect super-attenuating *S. cerevisiae* var. *diastaticus* yeast strains to achieve reliable results within a shorter time period. Compared to that, traditional brewing culture strains did not grow. In conclusion, qPCR of the *STA1* gene provides reliable results for the species identification of *S. cerevisiae* var. *diastaticus* but did not correlate with the respective super-attenuating ability. To detect spoilage and super-attenuating power, strains also need to be checked by physiological tests. One spoilage yeast culture, isolated from a brewery, was identified as the *S. pastorianus* strain TUM PI BA 124. This strain is phenolic off-flavor negative and did not show any super-attenuating power. Based on the obtained results, yeast isolates DSM 70487, TUM 71, TUM PI BB 121, TUM PI BA 124, TUM 1-H-7, TUM 3-H-2 and TUM 3-D-2 were investigated further and used for brewing trials. TUM PI BA 124 was selected as a negative and DSM 70487 as a positive control strain. TUM 3-D-2, TUM PI BB 121, TUM 1-H-7 and TUM 71 were selected according to their super-attenuation power. TUM 3-H-2 showed no super-attenuation even when the strain was identified as *S. cerevisiae* var. *diastaticus* by RT-PCR to detect the *STA1* gene. By using capillary electrophoresis of the IGS2-314 loci and a sequence analysis of the D1/D2 26S and the ITS1-5.8S-ITS2 ribosomal DNA, the species of the selected strains were identified and the resulting unique banding patterns showed that each isolate represents a genetically different strain. In addition to the genetic analysis and the detection of their super-attenuating power and spoilage potential, the strains were investigated for morphological differences and on their sporulation behavior by phase microscopy. The *S. cerevisiae* var. *diastaticus* yeast cells appeared singly or in pairs from one to four cells and exhibited a smaller cell size in contrast to lager and ale brewing yeast strains as already reported by Powell and Kerruish (2017). The cells of the *S. cerevisiae* var. *diastaticus* yeast strains show faster and higher spore formation than the lager and ale strains. Under identical experimental conditions, the *S. cerevisiae* var. *diastaticus* yeast strains showed a spore formation of mostly one to three spores contained within an ascus with a main spore formation of two spores compared to lager and ale strains with mainly one spore within an ascus. In addition to the phenolic off-flavor ability and the spoilage potential due to the fermentation of higher dextrins, all six investigated *S. cerevisiae* var. *diastaticus* yeast strains as well as the *S. pastorianus* yeast strain TUM PI BA 124 showed different phenotypic characteristics and flavor profiles. The most interesting differences are presented in the following Table 27. All strains varied in their fermentation rates and degrees of apparent attenuation. With the exception

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

Table 27. Summary of the results obtained by qPCR, sugar metabolism test, POF test, brewing trials and sensory evaluation of the investigated yeast strains DSM 70487, TUM 71, TUM PI BB 121, TUM PI BA 124, TUM 1-H-7, TUM 3-H-2 and TUM 3-D-2; fermentation degree described as super fermenter (SF), high fermenter (HF), weak fermenter (WF), sporulation behavior described according to the percentage of total sporulated cells as low (<20%) medium (20%–30%) high (>30%).

TUM identifier	Species identification by qPCR	Sugar metabolism		Fermentation degree	Spoilage potential	Phenolic off flavor	Flocculation behaviour	Sporulation behaviour	Sensory acceptance	Recommended beer style
		Wort sugars	Higher dextrins							
DSM 70487	*S. cerevisiae* var. *diastaticus*	+	+	SF	↑	+	flocculent	low	√	Wheat beer
TUM PI BB 121	*S. cerevisiae* var. *diastaticus*	+	+	SF	↑	+	powdery	medium	√	Wheat beer
TUM 3-D-2	*S. cerevisiae* var. *diastaticus*	+	+	SF	↑	+	powdery	medium	√	Wheat beer
TUM 1-H-7	*S. cerevisiae* var. *diastaticus*	+	+	SF	→	+	flocculent	high	√	Ale
TUM 71	*S. cerevisiae* var. *diastaticus*	+	+	SF	↑	+	powdery	medium	√	Wheat beer/Ale ('Bavarian Ale')
TUM 3-H-2	*S. cerevisiae* var. *diastaticus*	–	–	WF	↓	+	flocculent	high	√	Wheat beer/Ale ('Bavarian Ale')
TUM PI BA 124	*S. pastorianus*	+	–	HF	↓	–	flocculent	low	√	Dark beer/Ale

of TUM 3-H-2, all investigated *S. cerevisiae* var. *diastaticus* yeast strains ferment almost all wort sugars to the full extent and are also able to metabolize higher dextrins and starch as shown in the modified Durham tube test with fermented beer medium and the starch agar plate test. Even if TUM 3-H-2 is identified as being a genetically different strain of the species *S. cerevisiae* var. *diastaticus*, this strain only fermented a low level of maltotriose (45.14% ± 09.05%) and no higher dextrins and starch. Therefore, as the *STA1* genes of the strain did not encode for glucoamylase enzymes and also did not utilize maltose, this strain could be classified as a weak fermenter (see fermentation degree, Table 27). In the case of non-fermentation of higher dextrins and starch, beers produced with strain TUM PI BA 124, identified as *S. pastorianus*, reached their apparent attenuation of 84.47 ± 0.14% after 96 h and needed less time to achieve the final gravity of 2.1 °P compared to the investigated *S. cerevisiae* var. *diastaticus* yeast strains. In comparison with the low fermentation performance, TUM 3-H-2 flocculated continuously and showed the lowest concentration of cells in suspension over the complete fermentation period. As Powell reported, it would be expected for *S. cerevisiae* var. *diastaticus*, as a beer-spoiling yeast, to show more powdery flocculation behavior than classical ale and lager brewing strains (Powell and Kerruish 2017). The investigated *S. cerevisiae* var. *diastaticus* yeast strains did not clearly show a more powdery flocculation behavior compared with common ale brewing strains. Compared with the characterized ale brewing strains by Meier-Dörnberg, the *S. cerevisiae* var. *diastaticus* yeast strains also did not flocculate below the pitching concentration by reaching their final gravity. However, the number of flocculated cells compared to the maximum achieved yeast cells in suspension is much higher (Meier-Dörnberg *et al.* 2017a,c). The higher concentration of yeast cells in suspension is hereby due to the yeast propagation caused by their super-attenuating property. The pH of the final beer (5.2 in the pitched wort) was within the range of normal beers of 4.4–4.6. The total amino acid utilization was different for each investigated strain and lower compared to the metabolized FAN and total AS amount for common lager and ale brewing yeast strains previously shown by Meier-Dörnberg in 2017 (Meier-Dörnberg *et al.* 2017a,c). In conclusion, the investigated *S. cerevisiae* var. *diastaticus* strains need a lower FAN concentration in the wort to achieve a fermentation performance comparable to classical culture brewing yeast strains. The individual and main flavor impression of the *S. cerevisiae* var. *diastaticus* beers ends in a mainly dry and winey body with noticeable phenolic off-flavors underlined by plenty of fruity flavors. All *S. cerevisiae* var. *diastaticus* yeast strains were capable of building phenolic off-flavors, which was confirmed by the concentrations of 4-VG in the finished beers, which were above the individual threshold. Except for the TUM 3-H-2, TUM 71 and TUM 1-H-7 strain, all panelists recognized the clove-like flavor and therefore referred to these beers as wheat style beers. TUM 3-H-2 and TUM 71 also produced a concentration of 4-VG above the threshold (e.g. 2.77 and 2.53 m L^{-1}), but the flavor was not recognized by the panelists (flavor intensity of 0.71 and 0.57, e.g. Fig. 12), which may have been caused and suppressed by synergistic effects. Based on the many fruity aroma impressions and slightly spicy flavors (4-VG respectively), *S. cerevisiae* var. *diastaticus* yeast strain TUM 3-H-2 and TUM 71 may be suitable for brewing a 'Bavarian Ale', as already suggested in former yeast characterization of different *Saccharomyces* brewing yeasts by Meier-Dörnberg (Meier-Dörnberg *et al.* 2017a,c). In conclusion, 42.8% of the panelists could not clearly assign this beer to a wheat or to an ale style. The beers produced using strain TUM 1-H-7 were assigned by the panelists to an ale beer style (50%). The production of fermentation by-products, as well as the resulting flavor composition in the finished beers was

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

strain-dependent and followed no defined order. The formation of sulfur dioxide (SO_2) during fermentation could only be detected in TUM 71, TUM 3-D-2 and the *S. pastorianus* yeast strain TUM PI BA 124. Only in the beers produced with TUM PI BA 124 could a sulfur dioxide concentration comparable to common lager brewing yeast strains (Securitas—TUM 193®) be detected. As a result of the higher fementation temperature and pitching rate compared with classical lager brewing strains (Meier-Dörnberg *et al.* 2017), the panelists assigned the beers produced with TUM PI BA 124 to a dark style and ale style. Besides the phenotypic brewing characteristics, it may be possible to use this yeast strain as a high-performance yeast strain for lager beer production with a special aroma profile and a high flavor stability. In conclusion, yeast strains of the species *S. cerevisiae* var. *diastaticus* are suitable for producing tasty beers under classical batch fermentation conditions. None of the panelists could detect any unpleasant taste or prevailing off-flavors in the beers produced. Among the investigated *S. cerevisiae* var. *diastaticus* yeasts, strain TUM 3-H-2 shows no spoilage potential and super-attenuating ability and may be used for full-bodied beers with fruity aromas. *Saccharomyces cerevisiae* var. *diastaticus* yeast strains show high potential in brewing in batches and can be also used in secondary or mixed fermentations to produce beers with special flavors and/or a low carbohydrate content. Also, the use of *S. cerevisiae* var. *diastaticus* yeast strains in high-gravity brewing can be of great interest for the beverage industry to increase economic efficiency and profitability.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

Conflict of interest. None declared.

REFERENCES

Adam AC, Latorre-García L, Polaina J. Structural analysis of glucoamylase encoded by theSTA1 gene of *Saccharomyces cerevisiae* (var.diastaticus). *Yeast* 2004;**21**:379–88.

Altschul S-F, Madden T-L, Schäffer A-A *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;**25**:3389–402.

Amin G, de Mot R, van Dijck K *et al.* Direct alcoholic fermentation of starchy biomass using amylolytic yeast strains in batch and immobilized cell systems. *Appl Microbiol Biotechnol* 1985;**22**:237–45.

Andrews J, Gilliland RB. Super-attenuation of beer: A study of three organisms capable of causing abnormal attenuations. *J Inst Brew* 1952;**58**:189–96.

Back W. *Ausgewählte Kapitel der Brauereitechnologie*. Nürnberg: Hans Carl, 2005.

Balogh I, Maráz A. Presence of STA gene sequences in brewer's yeast genome. *Lett Appl Microbiol* 1996;**22**:400–4.

Bamforth CW, Bokulich NA (eds). *Brewing Microbiology: Current Research, Omics and Microbial Ecology*. Norfolk, UK: Caister Academic Press, 2017.

Bayly JC, Douglas LM, Pretorius IS *et al.* Characteristics of Flo11-dependent flocculation in. *FEMS Yeast Res* 2005;**5**:1151–6.

Begrow W. Fighting quality threats: notable microbiological contaminations of craft beer in the United States. *Brewing and Beverage Industry International* 2017;**5**:10–3.

Boulton C, Quain D. *Brewing Yeast and Fermentation*. Oxford, Malden, MA, Ames, Iowa: Blackwell Science; Iowa State University Press [distributor], 2009.

Brandl A. *Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien*. Weihenstephan: Dissertation, Technische Universität München, 2006.

Büchl N-R, Hutzler M, Mietke-Hofmann H *et al.* Differentiation of probiotic and environmental *Saccharomyces cerevisiae* strains in animal feed. *J Appl Microbiol* 2010;**109**:783–91.

Chant J. Patterns of bud-site selection in the yeast *Saccharomyces cerevisiae*. *J Cell Biol* 1995;**129**:751–65.

Davidow LS, Goetsch L, Byers B. Preferential occurrence of nonsister spores in two-spored asci of *Saccharomyces cerevisiae*: evidence for regulation of spore-wall formation by the spindle pole body. *Genetics* 1980;**94**:581–95.

Erratt JA. Glucoamylases from *Saccharomyces diastaticus*. *Crit Rev Biotechnol* 1987;**5**:95–104.

Erratt JA, Stewart GG. Genetic and biochemical studies on glucoamylase from *Saccharomyces diastaticus*. In: Stewart GG, Russell I (eds). *Advances in Biotechnology: Proceedings of the Fifth International Yeast Symposium Held in London, Canada, July 20-25, 1980*. Burlington: Elsevier Science, 2013, 177–83.

European Bioinformatic Institut Cambridge, 2010. http://www.ebi.ac.uk/interpro/IEntry?ac=IPR004507 (Accessed 15 November 2016).

Faust U, Präve P, Schlingmann M. An integral approach to power alcohol. *Process Biochem* 1983;**3**:31–7.

Favaro L, Basaglia M, Casella S. Processing wheat bran into ethanol using mild treatments and highly fermentative yeasts. *Biomass Bioenerg* 2012;**46**:605–17.

Folz R, Hofmann R, Stahl U. Impact of permeation of O_2 and CO_2 on the growth behaviour of *Saccharomyces diastaticus* in beer. *BrewingScience* 2011;**64**:52–60.

Gilliand RB. *Saccharomyces diastaticus* a starch fermenting yeast. *J Inst Brew* 1966;**72**:271–5.

Hittinger CT. *Saccharomyces* diversity and evolution: a budding model genus. *Trends Genet* 2013;**29**:309–17.

Hutzler M. *Entwicklung und Optimierung von Methoden zur Identifizierung und Differenzierung von getränkerelevanten Hefen*. Weihenstephan: Dissertation, Technische Universität München, 2009.

Hutzler M. *Getränkerelevante Hefen - Identifizierung und Differenzierung: Wie können Hefen praxisrelevant unterschieden werden, und wie können Identifizierungsergebnisse technologisch bewertet werden?* Südwestdt. Verl. für Hochschulschriften: SIA OmniScriptum Publishing, 2010.

Hutzler M, Geiger E, Jacob F. Use of PCRDHPLC (Polymerase Chain Reaction Denaturing High Performance Liquid Chromatography) for the rapid differentiation of industrial *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* strains. *J Inst Brew* 2010;**116**:464–74.

Hutzler M, Riedl R, Koob J *et al.* Fermentation and spoilage yeasts and their relevance for the beverage industry - a review. *BrewingScience* 2012;**65**:33–52.

Innis M-A. *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, 1990.

Jacob F (ed.). *MEBAK brautechnische Analysenmethoden - Würze, Bier, Biermischgetränke: Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission*. Freising: Selbstverl. der Mebak, 2012.

Janderová B, Davaasurengijn T, Vondrejs V *et al.* A new killer brewing yeast capable of degrading dextrin and starch. *J Basic Microbiol* 1986;**10**:627–31.

Jespersen L, van der Aa Kühle A, Petersen KM. Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

relationship with brewing yeasts. *Int J Food Microbiol* 2000;**60**: 43–53.
Jones M, Pierce J-S. Absorption of amino acids from wort by yeasts. *J Inst Brew* 1964;**2**:307–15.
Josepa S, Guillamon J-M, Cano J. PCR differentiation of *Saccharomyces cerevisiae* from *Saccharomyces bayanus/Saccharomyces pastorianus* using specific primers. *FEMS Microbiol Lett* 2000;**193**:255–9.
Kunze W. *Technologie Brauer und Mälzer*. Berlin: Versuchs- u. Lehranstalt f. Brauerei, 2011.
Kurtzman C-P, Robnett C-J. Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. *FEMS Yeast Res* 2003;**3**:417–32.
Laluce C, Mattoon JR. Development of rapidly fermenting strains of *Saccharomyces diastaticus* for direct conversion of starch and dextrins to ethanol. *Appl Environ Microb* 1984;**48**:17–25.
Latorre-García L, Adam AC, Manzanares P *et al.* Improving the amylolytic activity of *Saccharomyces cerevisiae* glucoamylase by the addition of a starch binding domain. *J Biotechnol* 2005;**118**:167–76.
Latorre-García L, Adam AC, Polaina J. Overexpression of the glucoamylase-encoding STA1 gene of *Saccharomyces cerevisiae* var. diastaticus in laboratory and industrial strains of Saccharomyces. *World J Microbiol Biotechnol* 2008;**24**:2957–63.
Liti G, Carter DM, Moses AM *et al.* Population genomics of domestic and wild yeasts. *Nature* 2009;**458**:337–41.
Lodolo EJ, Kock JLF, Axcell BC *et al.* The yeast *Saccharomyces cerevisiae*- the main character in beer brewing. *FEMS Yeast Res* 2008;**8**:1018–36.
Lomander L, Gundersen K. Nonsporulating, respiration-deficient variants of *Saccharomyces pastorianus* induced by cycloheximide. *J Bacteriol* 1963;**86**:956–65.
Marín-Navarro J, Gurgu L, Alamar S *et al.* Structural and functional analysis of hybrid enzymes generated by domain shuffling between Saccharomyces cerevisiae (var. diastaticus) Sta1 glucoamylase and *Saccharomycopsis fibuligera Bgl1* β-glucosidase. *Appl Microbiol Biotechnol* 2011;**89**: 121–30.
Marín-Navarro J, Polaina J. Glucoamylases: structural and biotechnological aspects. *Appl Microbiol Biotechnol* 2011;**89**:1267–73.
Meaden P, Ogden K, Bussey H *et al.* A DEX gene conferring production of extracellular amyloglucosidase on yeast. *Gene* 1985;**34**:325–34.
Meier-Dörnberg T, Hutzler M, Michel M *et al.* The importance of a comparative characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* strains for brewing. *Fermentation* 2017a;**3**:41.
Meier-Dörnberg T, Jacob F, Michel M *et al.* Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry: cases of contamination, 2008–2017. *MBAA TQ (Technical Quarterly)* 2017b;**54**. DOI: 10.1094/TQ-54-4-1130-01.
Meier-Dörnberg T, Michel M, Wagner R-S *et al.* Genetic and phenotypic characterization of different top-fermenting *Saccharomyces cerevisiae* ale yeast isolates. *BrewingScience* 2017c;**70**:9–25.
Meilgaard M-C. Flavour chemistry of beer. Part 2. Flavour and threshold of 239 aroma volatiles. *Technical Quarterly Master Brewers Association of the Americans* 1975;**12**:151–68.
Merritt AE, Hurley R. Evaluation of sporulatioin media for yeasts obtained from pathological material. *J Med Microbiol* 1972;**5**:21–30.
Mukai N, Masaki K, Fujii T *et al.* Single nucleotide polymorphisms of *PAD1* and *FDC1* show a positive relationship with ferulic acid decarboxylation ability among industrial yeasts used in alcoholic beverage production. *J Biosci Bioeng* 2014;**118**:50–5.
Müller-Auffermann K, Caldera A, Jacob F *et al.* Characterization of different bottom fermenting *Saccharomyces pastorianus* brewing yeast strains. *BrewingScience* 2015;**68**:46–57.
Narziss L, Back W. *Abriss der Bierbrauerei*. Weinheim: Wiley-VCH, 2005.
Powell CD, Kerruish DWM. Beer-spoiling yeasts: genomics, detection, and control. In: Bamforth CW, Bokulich NA (eds). *Brewing Microbiology: Current Research, Omics and Microbial Ecology*. Norfolk, UK: Caister Academic Press, 2017.
Priest FG, Campbell I. Rapid identification of microorganisms. In: Priest FG, Campbell I (eds). *Brewing Microbiology*. Boston, MA: Springer US, 2003, 305–28.
Procopio S, Brunner M, Becker T. Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation. *Eur Food Res Technol* 2014;**239**:421–39.
Rees R. 10 Barrel recalls Swill; Bend brewery cooking up a secret replacement beer, partner says. 2014. http://www.bendbulletin.com/business/2282468-151/10-barrel-recalls-swill (Accessed 10 March 2018).
Richard P, Viljanen K, Penttilä M. Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*. *AMB Expr* 2015;**5**:12.
Rousseau P, Halvorson HO, Bulla LA *et al.* Germination and outgrowth of single spores of *Saccharomyces cerevisiae* viewed by scanning electron and phase-contrast microscopy. *J Bacteriol* 1972;**109**:1232–8.
Soares EV. Flocculation in *Saccharomyces cerevisiae*: a review. *J Appl Microbiol* 2011;**110**:1–18.
Tamura K, Stecher G, Peterson D *et al.* MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;**30**:2725–9.
The Denver Post. Left Hand Brewing recalls Milk Stout Nitro, says foreign yeast put too much fizz in the bottle – The Denver Post, 2016. http://www.denverpost.com/2016/09/12/left-hand-brewing-recalls-milk-stout-nitro/ (Accessed 12 September 2016).
The Denver Post. Longmont's Left Hand Brewing suing yeast provider over $2 million of recalled beer, 2017. http://www.denverpost.com/2017/11/21/left-hand-brewing-lawsuit-nitro-milk-stout-yeast/ (Accessed 16 December 2017).
Vanderhaegen B, Coghe S, Vanbeneden N *et al.* Yeasts as postfermentation agents in beer. *Monatsschrift für Brauwissenschaft* 2002;**55**:218–32.
Vaughan-Martini A, Martini A. *Saccharomyces* Meyen ex Reess. In: Kurtzman CP, Fell JW (eds). *The yeasts, a taxonomic study*. Amsterdam: Elsevier, 2011, 733–46.
Vidgren V. 125th Anniversary Review: yeast flocculation and sedimentation in brewing. *J Inst Brew* 2011;**117**:475–87.
Yamashita I, Fukui S. Molecular cloning of a glucoamylase producing gene in the yeast *Saccharomyces*. *Agric Biol Chem* 1983;**47**:2689–92.
Yamashita I, Hatano T, Fukui S. Subunit structure of glucoamylase of *Saccharomyces diastaticus*. *Agric Biol Chem* 1984;**48**: 1611–6.
Zepf M. Die Bedeutung des Absetzverhaltens der Hefe: Flokkulationsverhalten. *Der Doemensianer* 2010;**1**:51.

Downloaded from https://academic.oup.com/femsyr/article-abstract/18/4/foy023/4923030
by Technische Universitaet Muenchen user
on 18 June 2018

3 Discussion

The yeast strain used in the brewery significantly impacts the aroma and the effectiveness of the brewing process (63, 67, 124, 181). In addition to yeast strain selection, the fermentation process is influenced and driven by the composition of the fermentation substrate, as well as by technical and technological factors (91, 155). The yeast cells react to changes in parameters such as head pressure, convection, temperature and aeration rate, which modify the fermentation performance and the resulting aroma profile of the beer (156). This makes it possible to offset fluctuations and create a more fruity or neutral overall aromatic impression of the beer (168). The fermentation process and the overall sensory impression of the finished beer can therefore be adapted, but not fundamentally altered, to the requirements and conditions of the brewery. With the exception of specialty malts, hop varieties and dosing techniques, the use of a new yeast strain represents the simplest way of manufacturing an alternative or new beer type with individual aromas. The selection of a yeast strain with the same aroma profile, but with process-optimized properties, can also make beer production more efficient and cost-effective without losing the “house flavor”. Selecting an alternative or new yeast strain is often very difficult for brewers. Previous investigations into the properties of individual yeast strains relevant to brewing primarily address the impact of varying individual fermentation parameters or consider brewery-specific yeast strains that are not commercially available to other brewers (54, 111, 141, 171). It is without doubt that successful implementation of a “new” yeast strain for the brewery is only possible using in-house applications under practice conditions. A lack of capacity, economic interests or microbiological uncertainties mean that these new strains are usually not implemented, with the result that breweries continue to rely on proven yeast strains (44, 152).

The aim of this paper was therefore to generate statements that can be transferred to practice regarding the expected range of aromas and process-relevant properties of different yeast strains to enable targeted and innovated application in breweries. The collection of specific characteristics of selected *Saccharomyces* yeasts thereby forms the basis for targeted selection of brewing yeasts according to the brewing method and desired beer type. A characterization platform was created and implemented for this purpose, combining current molecular biological identification and classification methods with brewery-related phenotypic methods under standardized and consistent conditions, providing results that can be transferred to brewing practice (see Section 2.2).

In contrast to the previous characterization of brewing yeast strains by DONHAUSER and MÜLLER-AUFFERMANN, the use of a standardized wort and standardized fermentation conditions make it possible to obtain diverse phenotypic properties and compare these with each other in a

meaningful way for the first time. In 1987 DONHAUSER et al. investigated 24 bottom-fermenting yeast strains as well as 7 top-fermenting yeast strains in 1991 (31–33) . The high proteolytic solution in 1986 brewing malt resulted in a low zinc content of the brewing wort used as the fermentation substrate, which had a negative effect on the fermentation performance of the investigated bottom-fermenting yeast strains. In addition, a study characterizing 7 bottom-fermenting yeast strains, conducted and published in 2017 by MÜLLER-AUFFERMANN, did not used a standardized wort as the fermentation substrate (111). MÜLLER-AUFFERMANN used cast-out wort obtained from an industrial brewery, which makes it difficult to reproduce or compare results with further characterized yeast strains, as the wort composition is influenced by a number of environmental and technological factors (113).

The fermentation performance of an individual brewing yeast strain represents one of the most important factors for the implementation of new yeast strains in industrial brewing applications. The fermentation performance includes the final degree of attenuation and the necessary total fermentation time. While keeping the output as high as possible, the length of fermentation currently represents the limiting factor in industrial beer manufacture due to economic factors or plant-specific production bottlenecks. Fermentation periods of approximately 4 days for German wheat beer or 7–10 days for bottom-fermenting lager beers usually should not be exceeded.

For this reason, the single fermentations within this study took place until the strain-specific final degree of attenuation was achieved. In this way, all other strain-specific parameters such as flocculation behavior, sugar metabolism and amino acid utilization etc. also provided results and statements. MÜLLER-AUFFERMANN analyzed phenotypic properties after 4 days of fermentation and compared all investigated parameters with one reference strain, which makes it difficult to establish transferable statements. Compared with the investigations of DONHAUSER and MÜLLER-AUFFERMANN, the characterization model shown in this paper also provides genetic analysis to guarantee the genetic distinctiveness of the strains involved to ensure reliable and reproducible results.

Every brewer's ultimate goal is the final desirable taste of the produced beer. The choice of yeast strain is therefore directly linked to the individual and special flavors created when developing new beer types and styles. For this reason, the produced beers are evaluated analytically and in terms of their sensory properties according to a specially prepared tasting sheet. This enables statements to be made for the first time on individual aroma profiles and the overall flavor impression of each individual strain. Table 5 sums up the main differences between the brewing yeast characterization model developed in this study compared with the characterization of DONHAUSER and MÜLLER-AUFFERMANN (31, 32, 111)

Table 5: Main differences of the developed brewing yeast characterization model to former yeast characterization studies of DONHAUSER 1987 and MÜLLER-AUFFERMANN 2015 (31, 32, 111)

Criteria	**Donhauser et al.**	**Müller-Auffermann et al.**	**Meier-Dörnberg et al.**
Investigated yeast type: Bottom-fermenting (BF), top-fermenting (TF), spoilage yeast (SY)	BF, TF	BF	BF, TF, SY
Reference	(31–33)	(111)	(98, 96, 97)
Pilot plant	Pilot brewery (main fermentation); 20 L cornelius keg (maturation and lagering)	2 L small scale fermenter	2 L small scale fermenter
Genetic pre-analysis	Partially on strain level	×	Species and strain level
Wort composition	Industrial wort	Industrial wort	Standardized wort
Constant pitching rate	Only for BF strains	✔	✔
Fermentation time: Final apparent attenuation (FAA)	Different (BF: FAA TF: 3 days)	4 days	FAA
Forced maturation	×	✔	✔
Bunging (0.5 bar)	Single	Row	Single
Sensory analyis	DLG	DLG, descriptive	DLG, descriptive, beer type, triangle test
Phenolic off-flavor test	Only OG	×	✔
Data comparison	✔	To reference strain	✔

Ten commercially available yeast strains to produce traditional beer varieties such as German wheat beer, koelsch, alt, trapist, ale and lager were used to evaluate the characterization model developed in this study (see Section 2.3). Their frequent application on an industrial scale and their specific use for particular beer types supports the suitability of these strains for a direct comparison of the brewing properties under standardized, consistent conditions.

As a result of the increasing microbiological contamination in brewing practice with over-fermenting yeasts of the species *Saccharomyces cerevisiae* var. *diastaticus*, the incidence of *S. cerevisiae* var. *diastaticus* in the beverage industry during the period 2008-2017 has been investigated and listed (see Section 2.4). As the analysis shows, the number of positive contaminations has increased since 2015. The majority of positive findings were determined in the third quarter of the year. This is presumably caused by warmer temperatures in the course of the summer season and by increased production at the expense of various basic cleaning intervals within the beverage manufacturers and breweries. Most of the positive findings occur as so-called secondary contamination in the bottling plant and are traced back to contamination in the filling environment and/or biofilms in the pipework supplying the

bottler. In order to assess the expected spoilage potential and the resulting product changes, as well as the possible suitability of specific strains to manufacture low-calorie beers or add a specific aroma, yeast isolates from various brewing and beverage sectors were analyzed using the characterization model and further investigated in terms of their sporulation capacity and spoilage potential in specially developed tests.

The first step of the characterization model involves genetic identification and taxonomic classification of the yeast isolates to the strain level to ensure the purity of the used yeast strains and subsequently consistent and reproducible results from these strains. The species identification of the investigated yeast isolates and strains was first determined by using species specific RT-PCR systems and sequence analysis of the D1/D2 domain of the 26S rRNA gene and the ITS1-5.8S-ITS2 ribosomal rRNA gene. A total of 32 different yeast strains were genetically identified as belonging to the *Saccharomyces* genus including three *S. pastorianus* lager yeast strains, eleven *S. cerevisiae* ale strains and eighteen *S. cerevisiae* var. *diastaticus* spoilage yeast strains. All yeasts were further classified as being genetically different strains by means of PCR of the IGS2-314 loci combined with capillary electrophoresis of the amplicon fragments and subsequent Bionumerics fingerprint analyses. The PCR amplicons of the ITS1-5.8S-ITS2 region could not be unambiguously sequenced. The ITS1-5.8S-ITS2 of the yeast strains TUM 213, TUM 511® (ale), DSM 70487 and TUM 3-H-2 (*S. cer*. var. *diastaticus*) delivered short nucleotide sequences as a result of different polymorphic repeats in the genome. This suggests that each rDNA domain does not have the same and specific nucleotide sequence and differ in their different loci in the genome of the strain. Sanger sequencing of the ITS1-5.8S-ITS2 ribosomal rRNA region does not therefore appear useful for the reliable genetic analysis of different *Saccharomyces* yeast strains. Genetic identification and taxonomic classification is not only necessary to ensure the fermented product has a reliable and consistent quality, it can also provide an initial assessment of the expected fermentation behavior of the investigated yeast strain (61). However, brewing properties and characteristics are strain dependent regardless of whether two yeast strains are the same, similar or different, and genetic identification/classification does not provide any transferable information on the phenotypic (brewing) properties of a strain. *Saccharomyces* brewing yeast strains can be very heterogeneous, and distinct phenotypic tools used to determine brewing-relevant properties (see Table 3) can produce different results as a result of different test conditions, culture media with different compositions or genetic drifts due to long storage times or repeated inoculation (65). In addition, most of them do not provide any information on the overall brewing potential for industrial applications and the beer type for which the yeast strain is most suitable. To give reliable results about the specific phenotypic brewing properties and the suitable beer type, single phenotypic methods were combined and used in small-scale fermentation trials. The fermentation substrate and the fermentation conditions

were kept constant so that only strain-specific brewing properties and the impact on the aroma profile were recorded. The fermentation profiles were differentiated into the production of bottom-fermenting (lager) and top-fermenting (ale) beers, to maintain practical conditions and therefore obtain representative results (see Section 2.3). To determine which sensory properties are characteristic of the relevant yeast strain, an internally developed tasting scheme was used, which ensured classification to a beer type along with individual aroma attributes.

Out of the 32 genetically identified yeast strains, a total of 19 different strains including three lager, ten ale and six *S. cerevisiae* var. *diastaticus* were screened for phenotypic characteristics in terms of their fermentation performance, flavor, and aroma profiles. All investigated yeast strains showed different brewing properties and flavor characteristics. It could be shown that differences in the fermentation rates and degrees of apparent attenuation are mainly caused by a strain-specific ability to ferment maltotriose, higher dextrins and starch. In terms of sugar utilization, the top-fermenting ale yeast strain Mel - TUM 211® and the *S. cerevisiae* var. *diastaticus* yeast strain TUM 3-H-2 only fermented a low level of maltotriose while the LunaBavaria - TUM 127® yeast strain could not ferment any maltotriose. In the case of non-fermentation of maltotriose, LunaBavaria - TUM 127® beers reached their apparent attenuation faster than Mel-TUM 211® and TUM 3-H-2 and needed less time for fermentation. Interestingly, investigations of other authors showed that some yeast strains such as LunaBavaria - TUM 127®, are not maltotriose-negative and can be adapted to utilize maltotriose by repitching multiple times, resulting in fermentation rates and apparent attenuations comparable with traditional brewing culture strains (18, 63). The *S. cerevisiae* var. *diastaticus* strain TUM 3-H-2 fermented a low level of maltotriose but did not ferment any higher dextrins or soluble starch. Residual carbohydrate metabolization in beer is found to be a major characteristic of *S. cerevisiae* var. *diastaticus* yeast strains due to extracellular degradation by the enzyme glucoamylase. The glucoamylase enzyme for starch degradation is encoded by at least one of the *STA* (*STA1*, *STA2* and *STA3*) or *DEX* genes (81, 183). (94). Accordingly, and even if the *STA1* gene could be verified by RT-PCR, the super-attenuation ability of the investigated *S. cerevisiae* var. *diastaticus* yeast strains can be due to the *STA2*, *STA3* or *DEX* gene, which were not identified by the used RT-PCR system, or the *STA1* gene is inactive and did not encode for glucoamylase enzymes in strain TUM 3-H-2. It can be concluded that strains also need to be checked in physiological tests to detect the specific spoilage and super-attenuating power. The metabolism of dextrin and starch is strain dependent, which could be confirmed in a developed starch agar plate test and a modified Durham tube test with a fermented beer medium (see Section 2.5). The starch agar plate test in particular can be used to detect super-attenuating *S. cerevisiae* var. *diastaticus* yeast strains to achieve reliable results within a short period. This requires an additional cell-washing step

to remove any remaining adherent wort carbohydrate, which ensures that the cells could not use this as an energy source for cell growth. Furthermore, bromophenol blue can be added to the agar for better detection of cell growth under anaerobic conditions. For the dextrin agar plate test, dextrin prepared by digesting starch with alpha-amylase should be used because commercially available dextrin cannot guarantee the necessary level of purity for reliable test results (81). Dextrin is a superordinate term for a starch degradation product. Its molecular size is defined only as a size between starch and oligosaccharides, and it is conceivable that dextrin would undergo at least partial degradation by non-super-attenuating yeast strains. In addition to the genetic analysis and detection of their super-attenuating power and spoilage potential, the *S. cerevisiae* var. *diastaticus* strains were investigated for morphological differences and their sporulation behavior by phase microscopy. Compared with lager and ale brewing strains, yeast cells of *S. cerevisiae* var. *diastaticus* exhibited a smaller cell size as confirmed by POWELL et al. (128). The cells appeared singly or in pairs from one to four cells. The cells of the *S. cerevisiae* var. *diastaticus* strains show faster and higher spore formation than the lager and ale strains. Under identical experimental conditions, *S. cerevisiae* var. *diastaticus* yeast strains displayed spore formation of mostly one to three spores within an ascus with a main spore formation of two spores compared with lager and ale strains which had mainly one spore within an ascus. To classify all the investigated strains according to their specific flocculation behavior, the cell concentration was measured during the main fermentation phase and maturation phase. Strains were classified according to their flocculation behavior as flocculent or non-flocculent ("powdery") yeast strains according to the difference in the number of cells at the strain-specific final gravity compared with the maximum cell concentration reached during fermentation. This did not take into account the influence of the different used fermentation profiles for lager and ale beers. Overall, each investigated lager strain showed greater flocculent behavior than the ale and *S. cerevisiae* var. *diastaticus* strains. Within the ale strains, not every top-fermenting strain showed powdery behavior or less flocculation behavior than the lager strains. LunaBavaria - TUM 127® and Vetus - TUM 184® showed flocculent behavior similar to the bottom-fermenting strains Frisinga - TUM 34/70®, Securitas - TUM 193® and TUM PI BA 124. However, the flocculation potential in yeast differs from strain to strain and seems to be due to the physiological properties of each strain. It can be concluded that, with the exception of Mel - TUM 211® and Tropicus - TUM 506®, "powdery" yeast strains ferment beer faster than "flocculent" strains, resulting in similar final attenuations. It was expected that *S. cerevisiae* var. *diastaticus* strains would exhibit more powdery flocculation behavior than classical ale and lager brewing strains although this could not be confirmed (128). In comparison with the ale brewing strains they did not flocculate below the pitching concentration by reaching their final gravity. However, the number of flocculated cells

compared with the maximum cells in suspension was much higher. The higher concentration of yeast cells in suspension is hereby due to the yeast propagated as a result of super-attenuation and can also be caused by the smaller cell size compared with ale brewing strains. Differences were observed in the total amino acid utilization for each investigated yeast strain, and no conclusion can be drawn as to cell growth. The total amino acid utilization was different for each investigated *S. cerevisiae* var. *diastaticus* strain and lower compared with the metabolized FAN and total AS amount for the investigated lager and ale brewing yeast strains in Sections 2.2 and 2.3. In conclusion, the investigated *S. cerevisiae* var. *diastaticus* strains need a lower FAN concentration in the wort to achieve a fermentation performance comparable to classical culture brewing yeast strains. With the exception of Mel - TUM 211® and Tropicus - TUM 506®, the capacity for acidification (ΔpH) of all investigated strains was within the range of average beers and resulted in the finished beer having a pH value of 4.4 to 4.6 (5.2 in the pitched wort). The weaker capacity of acidification of Mel - TUM 211® and Tropicus - TUM 506® might be due to the excretion of yeast metabolites and the uptake and metabolization of pyruvate as a result of cell autolysis caused by the low fermentation performance (3).

In order to make statements on the expected aroma profile of individual yeast strains and thus give a reliable recommendation for the most suitable beer type, there was a focus on the resulting overall sensory properties, and the individual and main flavor impression of the final beers as well as the production/concentration of single fermentation by-products. The production of phenolic off-flavors in particular was shown to be the main specific aroma compound for German wheat beers. The performed phenolic off-flavor tests made it possible to predict phenolic flavors coming from the decarboxylation of coumaric-, ferulic- or cinnamic acid. It could be shown that if the panelists recognized a clove-like flavor resulting from the decarboxylation of ferulic acid to the aroma-active 4-vinylguaiacol, they referred to these beers as wheat-style beers. This individual flavor impression is therefore not directly linked to a concentration of 4-VG above the threshold. Depending on the overall flavor profile of the beer, these phenolic (off-) flavors are suppressed by synergistic effects and thus are not perceptible despite increased concentration in the final product. The flavor and aroma assessment in this case further showed that the top-fermenting ale strain Harmonia - TUM 511® and the *S. cerevisiae* var. *diastaticus* strains TUM 3-H-2 and TUM 71 offered desired flavor and aroma impressions and may be suitable for brewing a "Bavarian Ale". This newly proposed beer type is characterized by the fruitiness of an ale-style brewed beer, underlined by the slightly spicy and yeasty flavors of a German wheat beer. Not all of the investigated top-fermenting *S. cerevisiae* brewing strains were capable of producing POF. The panelists could only detect aroma-active components formed by the top-fermenting yeast strains LeoBavaricus - TUM 68® and LunaBavaria - TUM 127®, commonly used for German

wheat beer production, and Harmonia-TUM 511® (ale beer production) and for Monacus - TUM 381® (trappist beer production). The top-fermenting *S. cerevisiae* strains Mel - TUM 211®, Colonia - TUM 177®, Tropicus - TUM 506® and Vetus - TUM 184® are POF negative, which explains and confirms their commercial use for top-fermented aroma intensive and fruity beer types alongside German wheat beers (91). The use of the *S. pastorianus* brewing strains resulted in typically POF-negative lager beers with a clean flavor profile and a relatively low level of fruity or floral flavors compared with ale strains (12, 39). Within the lager strains, Securitas - TUM 193® showed unique strain properties. The formation of sulfur dioxide (SO_2) during fermentation could be detected in all strains, but concentrations differed from 0.50 ± 0.00 mg/L to a considerably higher level on average of 9.47 ± 0.68 mg/L for the *S. pastorianus* lager strain Securitas - TUM 193®. The beers produced with *S. pastorianus* Securitas - TUM 193® showed a more intensive aroma and fruity flavor profile compared with the beers produced with Frisinga - TUM 34/70® and TUM PI BA 124. Securitas - TUM 193® can therefore be highly suitable for producing fruity lager beers with high flavor stability. The SO_2 formation for the investigated *S. cerevisiae* var. *diastaticus* strains did not exceed 1.00 ± 0.00 mg/L, which was much lower than the average produced by the investigated lager and ale brewing strains. The main and individual flavor impressions of the *S. cerevisiae* var. *diastaticus* beers ends in a mainly dry and winey body with noticeable POF underlined by lots of fruity flavors. All *S. cerevisiae* var. *diastaticus* strains were capable of producing POF above the individual threshold. The results show that *S. cerevisiae* var. *diastaticus* strains are suitable for brewing under identical conditions compared with classical ale brewing culture strains, resulting in desirable, appealing beers with a clear taste. Strains of *S. cerevisiae* var. *diastaticus* show high potential as pure starter cultures and can also be used in secondary or mixed fermentations to produce beers with special flavors and/or low carbohydrate content or they can be used in high gravity brewing to increase economic efficiency and profitability.

As the results show, all investigated yeast strains have distinct fermentation properties resulting in beers with a great variety of different aroma impressions and intensities. The isolation and characterization of different yeast strains for application in breweries is an underestimated opportunity to develop new beer styles and flavors without violating the German purity law. In addition, by combining the pure culture with other yeast strains in mixed fermentations or by varying the pitching time, the resulting flavor variety is almost limitless (181). The characterization model for genetic and phenotypic characterization of selected *Saccharomyces* strains as beer fermentation starter cultures presented in this dissertation can hereby be used to distinguish brewing yeasts and make statements on their suitability and application potential for brewing. By creating a data set of brewing yeasts with unique fermentation properties and aroma profiles we want to place the focus on using yeasts

beyond the limited number of strains used currently in the brewing industry. The use of a limited number of strains has resulted in a limited beer diversity (18, 61, 99, 156). The results obtained are collected and summarized for comparison. An illustrative example of these results is shown for the German wheat beer yeast strain LeoBavaricus - TUM 68® in Figure 4.

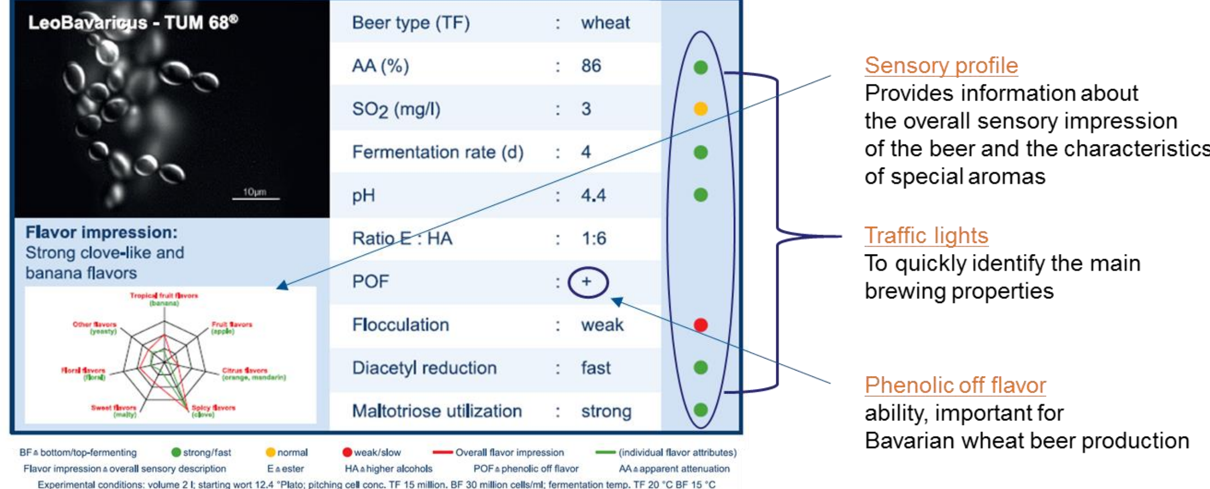


Figure 4: Visualized characterization of top-fermenting brewing strain *S. cerevisiae* LeoBavaricus - TUM 68® (63)

The visualized main characteristics allows brewers around the world access to a simplified and targeted selection of brewing yeast strains suitable for their specific purposes. Breweries can be given customized advice regarding the desired type of beer, irrespective of whether they want to replace their existing yeast strain to improve the aroma profile of existing beer styles, develop new beer styles, or optimize their fermentation process. Knowledge of the different yeast strain characteristics can, in particular, promote the competitiveness of small and medium-sized breweries and, if necessary, secure their existence by being part of the steadily increasing market for beer specialties (185). This model for a comparative characterization can further be used to identify and classify newly acquired or unknown yeasts in term of their brewing ability. According to an extrapolation, there are about 669,000 different yeast species of which 1,500 are currently known and only about 20 are used industrially (62, 75, 170). The richness of nature in new yeasts is far from exhausted (89, 176). Since each species consists of a large number of strains, which may have potential for beer fermentation, the brewer has no limits in terms of yeast. Only the effort to characterize these yeasts strains stands between a great beer diversity and the brewer

4 References

1. Abdel-Aty, L. M. (1991), Immunchemische und molekularbiologische Untersuchungen an verschiedenen *Saccharomyces*- und *Schizosaccharomyces* Hefen, TU München, Freising-Weihenstephan.

2. Albergaria, H. and Arneborg, N. (2016), Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. Appl. Microbiol. Biotechnol. 100, pp. 2035–2046. DOI: 10.1007/s00253-015-7255-0.

3. Annemüller, G. and Manger, H.-J. (2013), Gärung und Reifung des Bieres. VLB, Berlin. ISBN: 978-3-921690-73-4.

4. Annemüller, G., Manger, H.-J., Lietz, P. (2013), Die Hefe in der Brauerei. VLB, Berlin. ISBN: 978-3-921690-76-5.

5. Back, W. (1987), Nachweis und Identifizierung von Fremdhefen in der Brauerei. BRAUWELT, pp. 735–737.

6. Back, W. (1994), Kultivierung/Methoden, Brauerei, Winzerei. Carl Getränke-Fachverl., Nürnberg. ISBN: 978-3418007045.

7. Back, W. (2006), Colour atlas and handbook of beverage biology. Carl, Nürnberg. ISBN: 978-3418007991.

8. Baleiras Couto, M. M., van der Vossen, J.M.B.M., Hofstra, H., and Huis in`t Veld, J.H.J. (1994), RAPD analysis: a rapid technique for differentiation of spoilage yeasts. International Journal of Food Microbiology. 24, pp. 249–260.

9. Barnett, J. A. (1992), The taxonomy of the genus *Saccharomyces* meyen *ex reess*. Yeast. 8, pp. 1–23. DOI: 10.1002/yea.320080102.

10. Barnett, J. A. (2000), A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850-1880. Yeast, pp. 755–771. DOI: 10.1002/1097-0061.

11. Berlowska, J., Kregiel, D., Rajkowska, K. (2015), Biodiversity of brewery yeast strains and their fermentative activities. Yeast. 32, pp. 289–300. DOI: 10.1002/yea.3041.

12. Bing, J., Han, P.-J., Liu, W.-Q., Wang, Qi-Ming, and Bai, F.-Y. (2014), Evidence for a Far East Asian origin of lager beer yeast. Curr. Biol. 24, pp. R380-1. DOI: 10.1016/j.cub.2014.04.031.

13. Blättel, V., Petri, A., Rabenstein, A., Kuever, J., and König, H. (2013), Differentiation of species of the genus *Saccharomyces* using biomolecular fingerprinting methods. Appl. Microbiol. Biotechnol. 97, pp. 4597–4606. DOI: 10.1007/s00253-013-4823-z.

14. Bleoanca, I., Silva, Ana Rita Courelas, Pimentel, C., Rodrigues-Pousada, Claudina, and Menezes, Regina de Andrade (2013), Relationship between ethanol and oxidative stress in laboratory and brewing yeast strains. J. Biosci. Bioeng. 116, pp. 697–705. DOI: 10.1016/j.jbiosc.2013.05.037.

15. Bokulich, N. A., Bamforth, C. W., Mills, D. A. (2012), Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. PLoS ONE. 7, pp. e35507. DOI: 10.1371/journal.pone.0035507.

16. Bolat, I. C., Turtoi, M., Walsh, M. C. (2009), Influence of yeast drying process on different lager brewing strains viability. Journal of Agroalimentary Processes and Technologies. 3, pp. 370–377.

17. Borneman, A. R. and Pretorius, I. S. (2015), Genomic insights into the *Saccharomyces* sensu stricto complex. Genetics. 199, pp. 281–291. DOI: 10.1534/genetics.114.173633.

18. Boulton, C. and Quain, D. (2009), Brewing yeast and fermentation. Blackwell Science; Iowa State University Press [distributor], Oxford, Malden, MA, Ames, Iowa. ISBN: 0-632-05475-1.

19. Brandl, A. (2006), Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien, Technische Universität München, Weihenstephan.

20. Buchner, E. (1907), Cell-free fermentation. Nobel Lecture, pp. 103–120.

21. Butler, P. R., Brown, M., Oliver, S. G. (1996), Improvement of antibiotic titers from *Streptomyces* bacteria by interactive continuous selection. Biotechnol. Bioeng. 49, pp. 185–196. DOI: 10.1002/(SICI)1097-0290(19960120)49:2<185::AID-BIT7>3.0.CO;2-M.

22. Canonico, L., Comitini, F., Ciani, M. (2014), Dominance and influence of selected *Saccharomyces cerevisiae* strains on the analytical profile of craft beer refermentation. J. Inst. Brew. 120, pp. 262–267. DOI: 10.1002/jib.133.

23. Cocolin, L., Campolongo, S., Gorra, R., Rolle, L., and Rantsiou, K. (2011), *Saccharomyces cerevisiae* Biodiversity During the Brewing Process of an Artisanal Beer: A Preliminary Study. J. Inst. Brew. 3, pp. 352–358.

24. Crauwels, S., Steensels, J., Aerts, G., Willems, A., Verstrepen, K., Lievens, B. (2015), *Brettanomyces bruxellensis*, essential contributor in spontaneous beer fermentations providing novel opportunities for the brewing industry. BrewingScience, pp. 110–121.

25. Curran, B. P. and Bugeja, V. C. (1996), Protoplast fusion in *Saccharomyces cerevisiae*. Methods Mol Biol. 53, pp. 45–49. DOI: 10.1385/0-89603-319-8:45.

26. D´amore, T. (1992), Cambridge prize lecture improving yeast fermentation performance. J. Inst. Brew. 98, pp. 375–382.

27. Deutsche Presse-Agentur (2012), Das Reinheitsgebot beim Bier schmeckt nicht jedem, Kölnische Rundschau. https://www.rundschau-online.de/das-reinheitsgebot-beim-bier-schmeckt-nicht-jedem-12465174 (last accessed 18.04.2018).

28. Deutscher Brauer-Bund e.V. (2014), Bierausstoß 1960 - 2011. http://www.brauer-bund.de/aktuell/brauwirtschaft-in-zahlen.html (last accessed 18.04.2018).

29. Deutscher Brauer-Bund e.V., B. (2018), Zahl deutscher Brauereien wächst weiter. BRAUWELT, pp. 418–419.

30. Deželak, M., Gebremariam, M. M., Cadež, N., Zupan, Jure, Raspor, Peter, Zarnkow, Martin, Becker, Thomas, and Košir, I. Jože (2014), The influence of serial repitching of *Saccharomyces pastorianus* on its karyotype and protein profile during the fermentation of gluten-free buckwheat and quinoa wort. International Journal of Food Microbiology. 185, pp. 93–102. DOI: 10.1016/j.ijfoodmicro.2014.05.023.

31. Donhauser, S., Wagner, D., Gordon, D. (1987), Hefestämme und Bierqualität. BRAUWELT, pp. 1654–1664.

32. Donhauser, S., Wagner, D., Guggeis, H. (1987), Hefestämme und Bierqualität. BRAUWELT, pp. 1273–1279.

33. Donhauser, S., Wagner, D., Springer, H. (1991), Obergärige Hefestämme und Bierqualität. Der Weihenstephaner. 3, pp. 131.

34. Dunham, M. J., Gartenberg, M. R., Brown, G. W. (2015), Methods in yeast genetics and genomics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. ISBN: 978-1621821342.

35. Dunn, B. and Sherlock, G. (2008), Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. Genome Res. 18, pp. 1610–1623. DOI: 10.1101/gr.076075.108.

36. Elena, S. F. and Lenski, R. E. (2003), Evolution experiments with microorganisms. Nat Rev Genet. 4, pp. 457–469. DOI: 10.1038/nrg1088.

37. Finn, D. and Stewart, G. G. (2002), Fermentation characteristics of dried brewers yeast. ASBC, pp. 135–138. DOI: 10.1094/ASBCJ-60-0135.

38. Francesco, G. de, Turchetti, B., Sileoni, V., Marconi, Ombretta, and Perretti, G. (2015), Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. J. Inst. Brew. 121, pp. 113–121. DOI: 10.1002/jib.185.

39. Gallone, B., Mertens, S., Gordon, J. L., Maere, Steven, Verstrepen, Kevin J., and Steensels, J. (2017), Origins, evolution, domestication and diversity of *Saccharomyces* beer yeasts. Curr Opin Biotechnol. 49, pp. 148–155. DOI: 10.1016/j.copbio.2017.08.005.

40. Gallone, B., Steensels, J., Prahl, T., Soriaga, Leah, Saels, Veerle, Herrera-Malaver, Beatriz, Merlevede, Adriaan, Roncoroni, Miguel, Voordeckers, Karin, Miraglia, Loren, Teiling, Clotilde, Steffy, Brian, Taylor, Maryann, Schwartz, Ariel, Richardson, Toby, White, Christopher, Baele, Guy, Maere, Steven, and Verstrepen, K. J. (2016), Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. Cell. 166, pp. 1397-1410.e16. DOI: 10.1016/j.cell.2016.08.020.

41. Garcia Sanchez, R., Solodovnikova, N., Wendland, J. (2012), Breeding of lager yeast with *Saccharomyces cerevisiae* improves stress resistance and fermentation performance. Yeast. 29, pp. 343–355. DOI: 10.1002/yea.2914.

42. Gayevskiy, V. and Goddard, M. R. (2016), *Saccharomyces eubayanus* and *Saccharomyces arboricola* reside in North Island native New Zealand forests. Environ Microbiol. 18, pp. 1137–1147. DOI: 10.1111/1462-2920.13107.

43. Gibson, B., Geertman, J.-M. A., Hittinger, C. T., Krogerus, K., Libkind, D., Louis, E. J., Magalhães, F., and Sampaio, J. P. (2017), New yeasts-new brews. FEMS Yeast Res. 17. DOI: 10.1093/femsyr/fox038.

44. Gibson, B. and Liti, G. (2015), *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. Yeast. 32, pp. 17–27. DOI: 10.1002/yea.3033.

45. Gibson, B. R., Storgårds, E., Krogerus, K., and Vidgren, V. (2013), Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species *Saccharomyces eubayanus*. Yeast. 30, pp. 255–266. DOI: 10.1002/yea.2960.

46. Gierds, J. H. and Harms, D. (2014), Klassifizierung brauereirelevanter Hefen mittels MALDI-TOF-MS. BRAUWELT, pp. 1218–1221.

47. Giusto, C., Iacumin, L., Comi, G., Buiatti, Stefano, and Manzano, M. (2006), PCR-TTGE and RAPD-PCR techniques to analyze *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* isolated from craft beers. Journal of the Institute of Brewing. 112, pp. 340–345. DOI: 10.1002/j.2050-0416.2006.tb00740.x.

48. Gonçalves, M., Pontes, A., Almeida, P., Barbosa, Raquel, Serra, Marta, Libkind, Diego, Hutzler, Mathias, Gonçalves, Paula, and Sampaio, J. Paulo (2016), Distinct domestication trajectories in top-fermenting beer yeasts and wine yeasts. Curr. Biol. 26, pp. 2750–2761. DOI: 10.1016/j.cub.2016.08.040.

49. Haberger, N. (2016), 500 Jahre Reinheitsgebot, Bayerischer Rundfunk. http://www.br.de/br-fernsehen/sendungen/unser-land/menschen-und-handwerk/500-jahre-bier-reinheitsgebot-feiern-oder-abschaffen100.html ().

50. Hansen, E.C., Klöcher, A. (1911), Gesammelte theoretische Abhandlungen über Gärungsorganismen. G. Fischer, Jena.

51. Hanske, P.-P. (2016), Warum man das Reinheitsgebot endlich kippen sollte, WeltN24 GmbH. https://www.welt.de/icon/article153626650/Warum-man-das-Reinheitsgebot-endlich-kippen-sollte.html (last accessed 18.04.2018).

52. Heine, F., Stahl, F., Sträuber, H., Wiacek, Claudia, Benndorf, Dirk, Repenning, Cornelia, Schmidt, Frank, Scheper, Thomas, Bergen, Martin von, Harms, Hauke, and Müller, S. (2009), Prediction of flocculation ability of brewing yeast inoculates by flow cytometry, proteome analysis, and mRNA profiling. Cytometry A. 75, pp. 140–147. DOI: 10.1002/cyto.a.20661.

53. Hibbett, D. S., Binder, M., Bischoff, J. F., Blackwell, Meredith, Cannon, Paul F., Eriksson, Ove E., Huhndorf, Sabine, James, Timothy, Kirk, Paul M., Lücking, Robert, Thorsten Lumbsch, H., Lutzoni, François, Matheny, P. Brandon, McLaughlin, David J., Powell, Martha J., Redhead, Scott, Schoch, Conrad L., Spatafora, Joseph W., Stalpers, Joost A., Vilgalys, Rytas, Aime, M. Catherine, Aptroot, André, Bauer, Robert, Begerow, Dominik, Benny, Gerald L., Castlebury, Lisa A., Crous, Pedro W., Dai, Yu-Cheng, Gams, Walter, Geiser, David M., Griffith, Gareth W., Gueidan, Cécile, Hawksworth, David L., Hestmark, Geir, Hosaka, Kentaro, Humber, Richard A., Hyde, Kevin D., Ironside, Joseph E., Kõljalg, Urmas, Kurtzman, Cletus P., Larsson, Karl-Henrik, Lichtwardt, Robert, Longcore, Joyce,

Miadlikowska, Jolanta, Miller, Andrew, Moncalvo, Jean-Marc, Mozley-Standridge, Sharon, Oberwinkler, Franz, Parmasto, Erast, Reeb, Valérie, Rogers, Jack D., Roux, Claude, Ryvarden, Leif, Sampaio, José Paulo, Schüssler, Arthur, Sugiyama, Junta, Thorn, R. Greg, Tibell, Leif, Untereiner, Wendy A., Walker, Christopher, Wang, Zheng, Weir, Alex, Weiss, Michael, White, Merlin M., Winka, Katarina, Yao, Yi-Jian, and Zhang, N. (2007), A higher-level phylogenetic classification of the Fungi. Mycol Res. 111, pp. 509–547. DOI: 10.1016/j.mycres.2007.03.004.

54. Hiralal, L., Olaniran, A. O., Pillay, B. (2014), Aroma-active ester profile of ale beer produced under different fermentation and nutritional conditions. J. Biosci. Bioeng. 117, pp. 57–64. DOI: 10.1016/j.jbiosc.2013.06.002.

55. Hough, J. S. (1959), Flocculation characteristics of strains present in some typical british pitching yeasts. J. Inst. Brew., pp. 479–482.

56. Husnik, J. I., Volschenk, H., Bauer, J., Colavizza, Didier, Luo, Zongli, and van Vuuren, H. J. J. (2006), Metabolic engineering of malolactic wine yeast. Metab Eng. 8, pp. 315–323. DOI: 10.1016/j.ymben.2006.02.003.

57. Husnik, J.I., Delaquis, P.J., Cliff, M. A., and van Vuuren, H.J.J. (2007), Functional analyses of the malolactic wine yeast ML01. American Journal of Enology and Viticulture. 58, pp. 42–52.

58. Hutzler, M. (2010), Getränkerelevante Hefen - Identifizierung und Differenzierung. SIA OmniScriptum Publishing, Südwestdt. Verl. für Hochschulschriften. ISBN: 3838114825.

59. Hutzler, M. (2015), Großes Potenzial Die Vielfältige Aromenwelt der Hefen. BRAUINDUSTRIE, pp. 28–31.

60. Hutzler, M., Geiger, E., Jacob, F. (2010), Use of PCRDHPLC (polymerase chain reaction denaturing high performance liquid chromatography) for the rapid differentiation of industrial *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* strains. J. Inst. Brew. 116, pp. 464–474.

61. Hutzler, M. and Jacob, F. (2015), Immer bessere Kontrolle. BRAUINDUSTRIE, pp. 76–80.

62. Hutzler, M., Koob, J., Riedl, R., Schneiderbanger, H., Müller-Auffermann, K., and Jacob, F. (2015), Yeast identification and characterization (Chapter 6), pp. 65–105. Hill, A. E., ed. ElsevierElsevier.

63. Hutzler, M., Meier-Dörnberg, T., Stretz, D., Englmann, J., Zarnkow, M., and Jacob, F. (2017), TUM-Hefen – die Geburtsstunde von LeoBavaricus – TUM 68®. BRAUWELT. 157, pp. 266–269.

64. Hutzler, M., Riedl, R., Koob, J., and Jacob, F. (2012), Fermentation and Spoilage Yeasts and their Relevance for the Beverage industry - A Review. BrewingScience - Monatschrift für Brauwissenschaft. 65, pp. 33–52.

65. Jens Pfannebecker (2008), Entwicklung und Anwendung molekularbiologischer Methoden zur Art- und Stamm-Identifizierung pro- und eukaryotischer Organismen, Johannes Gutenberg-Universität Mainz, Mainz.

66. Jespersen, L. and Jakobsen, M. (1996), Specific spoilage organisms in breweries and laboratory media for their detection. International Journal of Food Microbiology. 33, pp. 139–155. DOI: 10.1016/0168-1605(96)01154-3.

67. Jespersen, L., van der Aa Kühle, Alis, Petersen, K. M. (2000), Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeasts. International Journal of Food Microbiology. 60, pp. 43–53. DOI: 10.1016/S0168-1605(00)00326-3.

68. Jones, M. and Pierce, J.-S. (1964), Absorption of amino acids from wort by yeasts. J. Inst. Brew. 2, pp. 307–315.

69. Kobi, D., Zugmeyer, S., Potier, S., and Jaquet-Gutfreund, L. (2004), Two-dimensional protein map of an "ale"-brewing yeast strain: proteome dynamics during fermentation. FEMS Yeast Research. 5, pp. 213–230. DOI: 10.1016/j.femsyr.2004.07.004.

70. Kopecká, J., Němec, M., Matoulková, D. (2016), Comparison of DNA-based techniques for differentiation of production strains of ale and lager brewing yeast. J Appl Microbiol. 120, pp. 1561–1573. DOI: 10.1111/jam.13116.

71. Krogerus, K. and Gibson, B. R. (2013), 125 th Anniversary Review: Diacetyl and its control during brewery fermentation. J. Inst. Brew, pp. n/a. DOI: 10.1002/jib.84.

72. Krogerus, K., Magalhães, F., Vidgren, V., and Gibson, B. (2015), New lager yeast strains generated by interspecific hybridization. J Ind Microbiol Biotechnol. 42, pp. 769–778. DOI: 10.1007/s10295-015-1597-6.

73. Kurtzman, C. P. (2003), Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces*, and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. FEMS Yeast Research. 4, pp. 233–245. DOI: 10.1016/S1567-1356(03)00175-2.

74. Kurtzman, C. P. (2015), Identification of food and beverage spoilage yeasts from DNA sequence analyses. International Journal of Food Microbiology. 213, pp. 71–78. DOI: 10.1016/j.ijfoodmicro.2015.05.023.

75. Kurtzman, C. P. and Fell, J. W. (2006), Yeast systematics and phylogeny – implications of molecular identification methods for studies in ecology, pp. 11–30. Gábor, P.; Rosa, C., ed. Springer-VerlagSpringer-Verlag. Berlin/Heidelberg.

76. Kurtzman, C. P. and Robnett, C. J. (1998), Identification and phylogeny of *ascomycetous* yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leeuwenhoek, pp. 331–3771.

77. Kurtzman, C. P. and Robnett, C. J. (2013), Relationships among genera of the *Saccharomycotina* (*Ascomycota*) from multigene phylogenetic analysis of type species. FEMS Yeast Res. 13, pp. 23–33. DOI: 10.1111/1567-1364.12006.

78. Kurtzman, C.-P. and Robnett, C.-J. (2003), Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Research. 3, pp. 417–432. DOI: 10.1016/S1567-1356(03)00012-6.

79. Kurtzman, Cletus P.; Fell, Jack W. (2011), The yeasts, a taxonomic study5th ed. rev. enl. Elsevier, Amsterdam. ISBN: 0444813128.

80. Kurtzman, C. P., and Sugiyama, J. (2015), *Saccharomycotina* and *Taphrinomycotina*: The yeasts and yeast like fungi of the Ascomycota. In The Mycota: A comprehensive treatise on fungi as experimental systems for basic and applied research, 2, pp. 3–33. McLaughlin, D. J.; Spatafora, J. W., ed. Springer. Berlin, Heidelberg.

81. Laluce, C. and Mattoon, J. R. (1984), Development of rapidly fermenting strains of *Saccharomyces diastaticus* for direct conversion of starch and dextrins to ethanol. Appl Environ Microbiol, pp. 17–25.

82. Latorre-García, L., Adam, A. C., Polaina, J. (2008), Overexpression of the glucoamylase-encoding *STA1* gene of *Saccharomyces cerevisiae* var. *diastaticus* in laboratory and industrial strains of *Saccharomyces*. World Journal of Microbiology and Biotechnology. 24, pp. 2957–2963. DOI: 10.1007/s11274-008-9837-9.

83. Lauterbach, A., Usbeck, J. C., Behr, J., and Vogel, R. F. (2017), MALDI-TOF MS typing enables the classification of brewing yeasts of the genus *Saccharomyces* to major beer styles. PLoS ONE. 12, pp. e0181694. DOI: 10.1371/journal.pone.0181694.

84. Lei, H., Xu, H., Feng, L., Yu, Zhimin, Zhao, Haifeng, and Zhao, M. (2016), Fermentation performance of lager yeast in high gravity beer fermentations with different sugar supplementations. J Biosci Bioeng. 122, pp. 583–588. DOI: 10.1016/j.jbiosc.2016.05.004.

85. Li, H., Liu, Y., Zhang, W. (), Method for preparing non-alcoholic beer by Candida shehatae. CN102220198 B.

86. Li, Q., Zhao, X.-Q., Chang, A. K., Zhang, Qiu-Mei, and Bai, F.-W. (2012), Ethanol-induced yeast flocculation directed by the promoter of *TPS1* encoding trehalose-6-phosphate synthase 1 for efficient ethanol production. Metab Eng. 14, pp. 1–8. DOI: 10.1016/j.ymben.2011.12.003.

87. Libkind, D., Hittinger, C. T., Valério, E., Gonçalves, Carla, Dover, Jim, Johnston, Mark, Gonçalves, Paula, and Sampaio, J. Paulo (2011), Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc Natl Acad Sci U S A. 108, pp. 14539–14544. DOI: 10.1073/pnas.1105430108.

88. Link, B. (2010), Bier und Bierbrauen im Alten Ägypten – Von der Vorzeit bis zum Neuen Reich, Ludwigs Maximilian Universität München, München.

89. Liti, G., Carter, D. M., Moses, A. M., Warringer, Jonas, Parts, Leopold, James, Stephen A., Davey, Robert P., Roberts, Ian N., Burt, Austin, Koufopanou, Vassiliki, Tsai, Isheng J., Bergman, Casey M., Bensasson, Douda, O'Kelly, Michael J T, van Oudenaarden, Alexander, Barton, David B H, Bailes, Elizabeth, Nguyen, Alex N., Jones, Matthew, Quail, Michael A., Goodhead, Ian, Sims, Sarah, Smith, Frances, Blomberg, Anders, Durbin, Richard, and Louis, E. J. (2009), Population genomics of domestic and wild yeasts. Nature. 458, pp. 337–341. DOI: 10.1038/nature07743.

90. Liti, G., Peruffo, A., James, S. A., Roberts, Ian N., and Louis, E. J. (2005), Inferences of evolutionary relationships from a population survey of LTR-retrotransposons and telomeric-associated sequences in the *Saccharomyces sensu stricto* complex. Yeast. 22, pp. 177–192. DOI: 10.1002/yea.1200.

91. Lodolo, E. J., Kock, J. L. F., Axcell, B. C., and Brooks, M. (2008), The yeast *Saccharomyces cerevisiae*- the main character in beer brewing. FEMS Yeast Res. 8, pp. 1018–1036. DOI: 10.1111/j.1567-1364.2008.00433.x.

92. Lumbsch, H.T., Huhndorf, S. (2007), Outline of *Ascomycota* - 2007. MYCONET. 13, pp. 1–58.

93. Manzano, M., Giusto, C., Bartolomeoli, I., Buiatti, Stefano, and Comi, G. (2005), Microbiological analyses of dry and slurry yeasts for brewing. Journal of the Institute of Brewing. 111, pp. 203–208. DOI: 10.1002/j.2050-0416.2005.tb00667.x.

94. Meaden, P., Ogden, K., Bussey, H., and Tubb, R. S. (1985), A *DEX* gene conferring production of extracellular amyloglucosidase on yeast. Gene. 34, pp. 325–334. DOI: 10.1016/0378-1119(85)90141-6.

95. Meier-Dörnberg, T., Hutzler, M., Jacob, F., and Schneiderbanger, H. (2015), Geschmacklich ansprechend Mit der richtigen Hefe anregende alkoholfreie Biere produzieren. BRAUINDUSTRIE, pp. 12–15.

96. Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, Frank-Jürgen, and Jacob, F. (2017), The importance of a comparative characterization of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* strains for brewing. Fermentation 3. 41, pp. 1-25. DOI: 10.3390/fermentation3030041.

97. Meier-Dörnberg, T., Kory, O. I., Jacob, F., Michel, Maximilian, and Hutzler, M. (2018), *Saccharomyces cerevisiae* variety *diastaticus* friend or foe? Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* variety *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization. FEMS Yeast Res. 18, pp. 1-27. DOI: 10.1093/femsyr/foy023.

98. Meier-Dörnberg, T., Michel, M., Wagner, R.-S., Jacob, F., and Hutzler, M. (2017), Genetic and phenotypic characterization of different top-fermenting *Saccharomyces* cerevisiae ale yeast isolates. BrewingScience - Monatschrift für Brauwissenschaft. 70, pp. 9–25. DOI: 10.23763/BRSC16-25MEIER-DOERNBERG.

99. Meier-Dörnberg, T., Schneiderbanger, H., Michel, M., Hutzler, M., and Jacob, F. (2016), Hefe als neuer Geschmacksmotor der Brauindustrie. Der Weihenstephaner. 84, pp. 22–26.

100. Mertens, S., Steensels, J., Saels, V., Rouck, Gert de, Aerts, Guido, and Verstrepen, K. J. (2015), A large set of newly created interspecific *Saccharomyces* hybrids increases aromatic diversity in lager beers. Appl Environ Microbiol. 81, pp. 8202–8214. DOI: 10.1128/AEM.02464-15.

101. Mertens, S., Steensels, J:, Gallone, B., Souffriau, B., Malcorps, P., and Verstrepen, K. (2017), Rapid screening method for phenolic off-flavor (POF) production in yeast. ASBC. DOI: 10.1094/ASBCJ-2017-4142-01.

102. Methner, F.-J. (1987), Über die Aromabildung beim Berliner Weissbier unter besonderer Berücksichtigung von Säuren und Estern, Technische Universität Berlin, Berlin.

103. Meußdoerffer, F. and Zarnkow, M. (2014), Das Bier, C.H.Beck. ISBN: 9783406666681.

104. Michel, M., Kopecká, J., Meier-Dörnberg, T., Zarnkow, Martin, Jacob, Fritz, and Hutzler, M. (2015), Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. Yeast. DOI: 10.1002/yea.3146.

105. Michel, M., Meier-Dörnberg, T., Jacob, F., Methner, F.-J., Wagner, R.-S., and Hutzler, M. (2016), Review: Pure non- *Saccharomyces* starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. J. Inst. Brew. 122, pp. 569–587. DOI: 10.1002/jib.381.

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

107. Monerawela, C. and Bond, U. (2017), Brewing up a storm. Biotechnol Adv. 35, pp. 512–519. DOI: 10.1016/j.biotechadv.2017.03.003.

108. Mott, A. C. (2017), The relationship between very high gravity fermentations and oxidative stress in the lager yeast *Saccharomyces pastorianus*., University of Nottingham.

109. Mukai, N., Masaki, K., Fujii, T., Kawamukai, Makoto, and Iefuji, H. (2010), *PAD1* and *FDC1* are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 109, pp. 564–569. DOI: 10.1016/j.jbiosc.2009.11.011.

110. Müller, S., Hutter, K. J., Bley, T., Petzold, L., and Babel, W. (1997), Dynamics of yeast cell states during proliferation and non proliferation periods in a brewing reactor monitored by multidimensional flow cytometry. Bioprocess Engineering. 17, pp. 287. DOI: 10.1007/s004490050387.

111. Müller-Auffermann, K., Caldera, A., Jacob, F., and Hutzler, M. (2015), Characterization of different bottom fermenting *Saccharomyces pastorianus* brewing yeast strains. BrewingScience - Monatschrift für Brauwissenschaft. 68, pp. 46–57.

112. Müller-Auffermann, K., Silva, W., Hutzler, M., and Jacob, F. (2014), Evaluation and Development of an alternative analysis method for rapid determination of yeast vitality. BrewingScience, pp. 72–80.

113. Narziss, L. and Back, W. (2005), Abriss der Bierbrauerei. Wiley-VCH, Weinheim. ISBN: 3527310355.

114. Naseeb, S., James, S. A., Alsammar, H., Michaels, Christopher J., Gini, Beatrice, Nueno-Palop, Carmen, Bond, Christopher J., McGhie, Henry, Roberts, Ian N., and Delneri, D. (2017), *Saccharomyces jurei* sp. nov., isolation and genetic identification of a novel yeast species from Quercus robur. International Journal of Systematic and Evolutionary Microbiology. 67, pp. 2046–2052. DOI: 10.1099/ijsem.0.002013.

115. Naumov, G. I., James, S. A., Naumova, E. S., Louis, E. J., and Roberts, N. (2000), Three new species in the *Saccharomyces sensu stricto* complex : *Saccharomyces cariocanus* , *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. International Journal of Systematic and Evolutionary Microbiology, pp. 1931–1942.

116. Naumov, G. I., Naumova, E. S., Masneuf-Pomarède, I. (2010), Genetic identification of new biological species *Saccharomyces arboricolus* Wang et Bai. Antonie van Leeuwenhoek. 98, pp. 1–7. DOI: 10.1007/s10482-010-9441-5.

117. Nguyen, H.-V. and Boekhout, T. (2017), Characterization of *Saccharomyces uvarum* (Beijerinck, 1898) and related hybrids. FEMS Yeast Res. 17. DOI: 10.1093/femsyr/fox014.

118. Nguyen, H.-V. and Gaillardin, C. (2005), Evolutionary relationships between the former species *Saccharomyces uvarum* and the hybrids *Saccharomyces bayanus* and *Saccharomyces pastorianus*; reinstatement of *Saccharomyces uvarum* (Beijerinck) as a distinct species. FEMS Yeast Res. 5, pp. 471–483. DOI: 10.1016/j.femsyr.2004.12.004.

119. Nguyen, H.-V., Legras, J.-L., Neuvéglise, C., and Gaillardin, C. (2011), Deciphering the hybridisation history leading to the Lager lineage based on the mosaic genomes of *Saccharomyces bayanus* strains NBRC1948 and CBS380. PLoS ONE. 6, pp. e25821. DOI: 10.1371/journal.pone.0025821.

120. Nier, H. (2017), Boom der kleinen Brauereien. https://de.statista.com/infografik/9215/craft-bier-boom-der-kleinen-brauereien-in-oesterreich/ (last accessed 06.04.2018).

121. Nier, H. (2017), Zahl der Brauereien steigt auf über 1.400. https://de.statista.com/infografik/8700/zahl-der-brauereien-in-deutschland/ (last accessed 06.04.2018).

122. Pérez-Través, L., Lopes, C. A., Querol, A., and Barrio, E. (2014), On the complexity of the *Saccharomyces bayanus* taxon. PLoS ONE. 9, pp. e93729. DOI: 10.1371/journal.pone.0093729.

123. Peris, D., Sylvester, K., Libkind, D., Gonçalves, Paula, Sampaio, José Paulo, Alexander, William G., and Hittinger, C. Todd (2014), Population structure and reticulate evolution of *Saccharomyces eubayanus* and its lager-brewing hybrids. Mol Ecol. 23, pp. 2031–2045. DOI: 10.1111/mec.12702.

124. Pires, E. J., Teixeira, J. A., Brányik, T., and Vicente, A. A. (2014), Yeast: the soul of beer's aroma--a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl. Microbiol. Biotechnol. 98, pp. 1937–1949. DOI: 10.1007/s00253-013-5470-0.

125. Piskur, J., Rozpedowska, E., Polakova, S., Merico, Annamaria, and Compagno, C. (2006), How did *Saccharomyces* evolve to become a good brewer? Trends Genet. 22, pp. 183–186. DOI: 10.1016/j.tig.2006.02.002.

126. Polaina, J. (2002), Brewer's yeast: genetics and biotechnology, 1. Aufl., pp. 1–17. Khachatourians, G. G.; Arora, D. K., ed. Elsevier professionalElsevier professional. s.l.

127. Pope, G. A., MacKenzie, D. A., Defernez, M., Aroso, Miguel A. M. M., Fuller, Linda J., Mellon, Fred A., Dunn, Warwick B., Brown, Marie, Goodacre, Royston, Kell, Douglas B., Marvin, Marcus E., Louis, Edward J., and Roberts, I. N. (2007), Metabolic footprinting as a tool for discriminating between brewing yeasts. Yeast. 24, pp. 667–679. DOI: 10.1002/yea.1499.

128. Powell, C. D. and Kerruish, D. W.M. (2017), Beer-Spoiling Yeasts, pp. 289–328. Bamforth, C. W.; Bokulich, N. A., ed. Caister academic press. Norfolk,UK.

129. Priest, F. G. and Campbell, I. (2003), Rapid identification of microorganisms, pp. 305–328. Priest, F. G.; Campbell, I., ed. Springer US. Boston, MA.

130. Procopio, S., Brunner, M., Becker, T. (2014), Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation. Eur Food Res Technol. 239, pp. 421–439. DOI: 10.1007/s00217-014-2236-6.

131. Procopio, S., Krause, D., Hofmann, T., and Becker, T. (2013), Significant amino acids in aroma compound profiling during yeast fermentation analyzed by PLS regression. LWT - Food Science and Technology. 51, pp. 423–432. DOI: 10.1016/j.lwt.2012.11.022.

132. Pulvirenti, A., Nguyen, H.-V., Caggia, C., Giudici, P., Rainieri, S., and Zambonelli, C. (2000), *Saccharomyces uvarum*, a proper species within *Saccharomyces sensu stricto*. FEMS Microbiol. Lett. 192, pp. 191–196.

133. Rainieri, S., Kodama, Y., Kaneko, Y., Mikata, Kozaburo, Nakao, Yoshihiro, and Ashikari, T. (2006), Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. Appl Environ Microbiol. 72, pp. 3968–3974. DOI: 10.1128/AEM.02769-05.

134. Rainieri, S., Kodama, Y., Nakao, Y., Pulvirenti, Andrea, and Giudici, P. (2008), The inheritance of mtDNA in lager brewing strains. FEMS Yeast Res. 8, pp. 586–596. DOI: 10.1111/j.1567-1364.2008.00363.x.

135. Rainieri, S., Zambonelli, C., Hallsworth, J. E., Pulvirenti, A., and Giudici, P. (1999), *Saccharomyces uvarum*, a distinct group within *Saccharomyces sensu stricto*. FEMS Microbiol. Lett. 177, pp. 177–185.

136. Rainieri, S., Zambonelli, C., Kaneko, Y. (2003), *Saccharomyces sensu stricto*: Systematics, genetic diversity and evolution. J. Biosci. Bioeng. 96, pp. 1–9. DOI: 10.1016/S1389-1723(03)90089-2.

137. Riu-Aumatell, M., Miró, P., Serra-Cayuela, A., Buxaderas, S., and López-Tamames, E. (2014), Assessment of the aroma profiles of low-alcohol beers using HS-SPME–GC-MS. Food Research International. 57, pp. 196–202. DOI: 10.1016/j.foodres.2014.01.016.

138. Röcken, W. and Schulte, S. (1986), Nachweis von Fremdhefen. BRAUWELT, pp. 1921–1927.

139. Rong, L., Peng, L.-J., Ho, C.-T., Yan, Shou-He, Meurens, Marc, Zhang, Zheng-Zhu, Li, Da-Xiang, Wan, Xiao-Chun, Bao, Guan-Hu, Gao, Xue-Ling, and Ling, T.-J. (2016), Brewing and volatiles analysis of three tea beers indicate a potential interaction between tea components and lager yeast. Food Chemistry. 197, pp. 161–167. DOI: 10.1016/j.foodchem.2015.10.088.

140. Saerens, S. and Swiegers, J. H. (), Production of low-alcohol or alcohol-free beer with *Pichia kluyveri* yeast strains. WO2014135673.

141. Saerens, S.-M.-G., Verbelen, P.-J., Vanbeneden, N., Thevelein, J.-M., and Delvaux, F.-R. (2008), Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. Appl. Microbiol. Biotechnol. 80, pp. 1039–1051. DOI: 10.1007/s00253-008-1645-5.

142. Saerle, B. A. and Tubb, R. S. (1981), A rapid method for recognising strains of yeast able to hydrolyse starch or dextrin. FEMS Microbiol. Lett. 111, pp. 211–212.

143. Sampaio, J. P., Pontes, A., Libkind, D., and Hutzler, M. (2017), Taxonomy, diversity, and typing of brewing yeasts, pp. 85–118. Bamforth, C. W.; Bokulich, N. A., ed. Caister academic press. Norfolk,UK.

144. Scherer, A. (2003), Entwicklung von PCR-Methoden zur Klassifizierung industriell genutzter Hefen, Technische Universität München, Weihenstephan.

145. Schmid, R. D. (2016), Taschenatlas der Biotechnologie und Gentechnik. Wiley-VCH, s.l. ISBN: 978-3527335145.

146. Schneiderbanger, H., Hutzler, M., Jacob, F. (2013), Aromaprofile ausgewählter Weizenbier-Hefestämme. BRAUWELT. 153, pp. 267–270.

147. Schöneborn, H. (2001), Differenzierung und Charakterisierung von Betriebshefekulturen mit genetischen und physiologischen Methoden, TU München, Freising-Weihenstephan.

148. Schönenberg, S. (31.05.2010), Der physiologische Zustand und der Sauerstoffbedarf von Bierhefen unter brautechnologischen Bedingungen, Technische Universität München, Weihenstephan.

149. Schönenberg, S., Thiele, F., Back, W. (2011), Hefewissenschaft unter brautechnologischen Bedingungen (2). BRAUWELT. 151, pp. 96–100.

150. Soares, E. V. (2011), Flocculation in *Saccharomyces cerevisiae*: a review. J. Appl. Microbiol. 110, pp. 1–18. DOI: 10.1111/j.1365-2672.2010.04897.x.

151. Spitaels, F., Wieme, A. D., Janssens, M., Aerts, Maarten, Daniel, Heide-Marie, van Landschoot, Anita, Vuyst, Luc de, and Vandamme, P. (2014), The microbial diversity of traditional spontaneously fermented lambic beer. PLoS ONE. 9, pp. e95384. DOI: 10.1371/journal.pone.0095384.

152. Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, Martina, Aslankoohi, Elham, Christiaens, Joaquin F., Gemayel, Rita, Meert, Wim, New, Aaron M., Pougach, Ksenia, Saels, Veerle, van der Zande, Elisa, Voordeckers, Karin, and Verstrepen, K. J. (2012), Selecting and generating superior yeasts for the brewing industry. Cerevisia. 37, pp. 63–67. DOI: 10.1016/j.cervis.2012.08.001.

153. Steensels, J., Snoek, T., Meersman, E., Picca Nicolino, Martina, Voordeckers, Karin, and Verstrepen, K. J. (2014), Improving industrial yeast strains. FEMS Microbiol. Rev. 38, pp. 947–995. DOI: 10.1111/1574-6976.12073.

154. Steensels, J. and Verstrepen, K. J. (2014), Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annu. Rev. Microbiol. 68, pp. 61–80. DOI: 10.1146/annurev-micro-091213-113025.

155. Stewart, G. (2016), *Saccharomyces* species in the production of beer. Beverages. 2, pp. 34. DOI: 10.3390/beverages2040034.

156. Stewart, G. G., Hill, A. E., Russell, I. (2013), 125 th Anniversary Review: Developments in brewing and distilling yeast strains. J. Inst. Brew. 119, pp. 202–220. DOI: 10.1002/jib.104.

157. Stewart, G. G. and Russel, I. (1986), One hundred years of yeast research and development in the brewing industry. J. Inst. Brew, pp. 537–558.

158. Stratford, M. and Keenan, M.H.J. (1988), Yeast flocculation: Quantification. Yeast. 4, pp. 107–115.

159. Takahashi, M., Kita, Y., Kusaka, K., Mizuno, A., and Goto-Yamamoto, N. (2015), Evaluation of microbial diversity in the pilot-scale beer brewing process by culture-dependent and culture-independent method. J. Appl. Microbiol. 118, pp. 454–469. DOI: 10.1111/jam.12712.

160. Timke, M., Wang-Lieu, N. Q., Altendorf, K., and Lipski, A. (2008), Identity, beer spoiling and biofilm forming potential of yeasts from beer bottling plant associated biofilms. Antonie van Leeuwenhoek. 93, pp. 151–161. DOI: 10.1007/s10482-007-9189-8.

161. Timmins, É. M., Quain, D. E., Goodacre, R. (1998), Differentiation of brewing yeast strains by pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. Yeast. 14, pp. 885–893. DOI: 10.1002/(SICI)1097-0061(199807)14:10<885::AID-YEA286>3.0.CO;2-G.

162. Török, T., Rockhold, D., King, A. D. (1993), Use of electrophoretic karyotyping and DNA-DNA hybridization in yeast identification. International Journal of Food Microbiology. 19, pp. 63–80. DOI: 10.1016/0168-1605(93)90124-Y.

163. van der Aa Kühle, Alis, Jesperen, L., Glover, R. L.K., Diawara, Bréhima, and Jakobsen, M. (2001), Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. Yeast. 18, pp. 1069–1079. DOI: 10.1002/yea.756.abs.

164. van Holle, A., Machado, M. D., Soares, E. V. (2012), Flocculation in ale brewing strains of *Saccharomyces cerevisiae*: re-evaluation of the role of cell surface charge and hydrophobicity. Appl. Microbiol. Biotechnol. 93, pp. 1221–1229. DOI: 10.1007/s00253-011-3502-1.

165. van Oevelen, D., Spaepen, M., Timmermans, P., Verachtert, H. (1977), Microbiological aspects of spontaneous wort fermentation in the production of Lambic and Geuze. J. Inst. Brew. 83, pp. 356–360.

166. Vanbeneden, N., Gils, F., Delvaux, F., and Delvaux, F. R. (2008), Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: Occurrence of volatile phenolic flavour compounds in beer and distribution of *Pad1*-activity among brewing yeasts. Food Chemistry. 107, pp. 221–230. DOI: 10.1016/j.foodchem.2007.08.008.

167. Vaughan-Martini, A. and Martini, A. (2011), *Saccharomyces Meyen ex Reess*, 5th ed. rev. enl, pp. 733–746, ed. ElsevierElsevier. Amsterdam.

168. Verstrepen, K. J., Derdelinckx, G., Dufour, J.-P., Winderickx, Joris, Thevelein, Johan M., Pretorius, Isak S., and Delvaux, F. R. (2003), Flavor-active esters: Adding fruitiness to beer. J. Biosci. Bioeng. 96, pp. 110–118. DOI: 10.1016/S1389-1723(03)90112-5.

169. Verstrepen, K. J., Derdelinckx, G., Verachtert, H., and Delvaux, F. R. (2003), Yeast flocculation: what brewers should know. Appl. Microbiol. Biotechnol. 61, pp. 197–205. DOI: 10.1007/s00253-002-1200-8.

170. Verstrepen, K.-J., Chambers, P.-J., Pretorius, I.-S. (2006), The development of superior yeast strains for the food and beverage industries: Challenges, opportunities and potential benefits (Chapter 13), pp. 399–444. Querol, A.; Fleet, G.-H., ed. Springer. Berlin, Heidelberg.

171. Vidgren, V., Multanen, J.-P., Ruohonen, L., and Londesborough, J. (2010), The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. FEMS Yeast Research. 10, pp. 402–411. DOI: 10.1111/j.1567-1364.2010.00627.x.

172. Wainwright, T. (1973), Diacetyl - A review. J. Inst. Brew. 79, pp. 451–470.

173. Walker, G. and Stewart, G. (2016), *Saccharomyces cerevisiae* in the production of fermented beverages. Beverages. 2, pp. 30. DOI: 10.3390/beverages2040030.

174. Walker, G. M. (2011), *Pichia anomala*. Antonie van Leeuwenhoek. 99, pp. 25–34. DOI: 10.1007/s10482-010-9491-8.

175. Walther, A., Hesselbart, A., Wendland, J. (2014), Genome sequence of *Saccharomyces carlsbergensis*, the world's first pure culture lager yeast. G3 (Bethesda). 4, pp. 783–793. DOI: 10.1534/g3.113.010090.

176. Wang, Q.-M., Liu, W.-Q., Liti, G., Wang, Shi-An, and Bai, F.-Y. (2012), Surprisingly diverged populations of *Saccharomyces cerevisiae* in natural environments remote from human activity. Mol Ecol. 21, pp. 5404–5417. DOI: 10.1111/j.1365-294X.2012.05732.x.

177. Wang, S.-A. and Bai, F.-Y. (2008), *Saccharomyces arboricolus* sp. nov., a yeast species from tree bark. International Journal of Systematic and Evolutionary Microbiology. 58, pp. 510–514. DOI: 10.1099/ijs.0.65331-0.

178. Wang, Z.-Y., He, X.-P., Liu, N., and Zhang, B.-R. (2008), Construction of self-cloning industrial brewing yeast with high-glutathione and low-diacetyl production. International Journal of Food Science & Technology. 43, pp. 989–994. DOI: 10.1111/j.1365-2621.2007.01546.x.

179. Wang, Z.-Y., He, X.-P., Zhang, B.-R. (2007), Over-expression of *GSH1* gene and disruption of *PEP4* gene in self-cloning industrial brewer's yeast. International Journal of Food Microbiology. 119, pp. 192–199. DOI: 10.1016/j.ijfoodmicro.2007.07.015.

180. Wenning, M., Seiler, H., Scherer, S. (2002), Fourier-transform infrared microspectroscopy, a novel and rapid tool for identification of yeasts. Appl Environ Microbiol. 68, pp. 4717–4721. DOI: 10.1128/AEM.68.10.4717-4721.2002.

181. White, C. and Zainasheff, J. (2010), Yeast. Brewers Publications, Boulder, CO. ISBN: 978-0937381960.

182. Winston, F. (2008), EMS and UV mutagenesis in yeast. Curr Protoc Mol Biol. Chapter 13, pp. Unit 13.3B. DOI: 10.1002/0471142727.mb1303bs82.

183. Yamashita, I., Hatano, T., Fukui, S. (1984), Subunit structure of glucoamylase of *Saccharomyces diastaticus*. Agric. Biol. Chem. 48, pp. 1611–1616.

184. Yarrow, D. (2011), Methods for the isolation, maintenance and identification of yeasts, 5th ed. rev. enl, pp. 77–100, ed. ElsevierElsevier. Amsterdam.

185. Zocher, H. (2017), Bierspezialitäten beleben den Biermarkt. BRAUINDUSTRIE. 102, pp. 9.

5 Appendix

5.1 Peer-reviewed papers as co-author

- 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
- 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
- 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
- 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

5.2 Non-reviewed papers

- Michel, M., Meier-Dörnberg, T., Hutzler, M., Jacob, F.: „Alternative Bierhefen – Was erwartet uns?", Brauwelt 10, 2018, S.266-268
- Hutzler, M., Meier-Dörnberg, T., Stretz, D., Englmann, J., Zarnkow, M., Jacob, F.: "TUM Hefen die Geburtstunde von LeoBavaricus – TUM 68®", Brauwelt 10, 2017, S.1556-1559
- Michel, M., Meier-Dörnberg, Jacob, F., Hutzler, M. "Die Suche nach neuen Brauhefen abseits der *Saccharomyces"*, Brauwelt 19-20, 2016, S.555-558
- 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 1, Jgh.84, 2016, S.22-25
- Meier-Dörnberg, T., Hutzler, M., Jacob, F., Schneiderbanger, H., "Geschmacklich ansprechend. Mit der richtigen Hefe anregende alkoholfreie Biere produzieren", BrauIndustrie 7, 2015, jgh.100, S.12-15

- Hutzler, M., Stretz, D., Meier-Dörnberg, T., Michel, M., Schneiderbanger, H.: “Großes Potenzial – Die vielfältige Aromenwelt der Hefen.” Brauindustrie 4: S. 28-31, 2015

5.3 Oral presentations

- Meier-Dörnberg, T., Michel, M: „Besondere Hefen“. 6 Seminar Hefe und Mikrobiologie, Weihenstephan, 13.03.2018
- Meier-Dörnberg, T.: “TUM Hefen”. 12. Weihenstephaner Praxisseminar, Kulmbach, 19-20.10.2017
- Meier-Dörnberg, T., Michel, M., Wagner, R.S., Jacob, F., Hutzler, M.: “Genetic and phenotypic characterization of different top-fermenting *Saccharomyces cerevisiae* ale yeast isolates”. 36th European Brewery Convention 2017, 16.05.2017, Ljubljana, Slovenia
- Meier-Dörnberg, T.: “Etablierung von MALDI Barcodes zur optimierten Auswahl und Anwendung von Brauhefen”. 4 Projekttreffen “MALDI Barcode von Brauhefen”, Weihenstephan, 27.04.2017
- Meier-Dörnberg, T.: “TUM Hefen – Stars und Sternchen-”. 5 Seminar Hefe und Mikrobiologie, Weihenstephan, 15.03.2017
- Meier-Dörnberg, T.: “Hefevielfalt und Biervariation”. 11. Weihenstephaner Praxisseminar, Ingolstadt, 20-21.10.2016
- Meier-Dörnberg, T., Lauterbach, A., Behr, J., Hutzler, M., Vogel, R.F.: “Etablierung von MALDI Barcodes zur optimierten Auswahl und Anwendung von Brauhefen”. 3. Projekttreffen “MALDI Barcode von Brauhefen”, Weihenstephan, 12.10.2016
- Meier-Dörnberg, T.: “*Saccharomyces* Brauereihefen – Vielfalt und Einsatz”. 4 Seminar Hefe und Mikrobiologie, Weihenstephan, 2016
- Meier-Dörnberg, T.: “Etablierung von MALDI Barcodes zur optimierten Auswahl und Anwendung von Brauhefen”. 2 Projekttreffen “MALDI Barcode von Brauhefen” , Weihenstephan, 26.11.2015
- Meier-Dörnberg, T.: “Praxisrelevante Charakterisierung von *Saccharomyces* Hefen”. 3. Seminar Hefe und Mikrobiologie, Weihenstephan, 2015
- Meier-Dörnberg, T.: “Etablierung von MALDI Barcodes zur optimierten Auswahl und Anwendung von Brauhefen”. 1 Projekttreffen “MALDI Barcode von Brauhefen” , Weihenstephan, 23.10.2014

5.4 Poster presentations

- Mathias Hutzler, Tim Meier-Dörnberg, Dominique Stretz, Fritz Jacob: "TUM Hefen". Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität, TU München published by Hans Carl Verlag, 2016
- Lauterbach, A.*, Meier-Dörnberg, T.*, Hutzler, M., Behr, J., Vogel, F.: "The potential of MALDI-TOF MS for sensotyping brewing yeasts". WBC 2016, 13-17.08.16, Denver, Colorado, USA
- 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 2016, 13-17.08.16, Denver, Colorado, USA
- Meier-Dörnberg, T., Schneiderbanger, H., Jacob, F., Hutzler, M.: "Alcohol-free wheat beer with maltose negative yeast strain *Saccharomycodes ludwigii"*. 4th international Young Scientists Symposium on Malting, Brewing, and Distilling, Ghent, Belgium, 2014

5.5 Permission of publishers to reproduce publications

Meier-Dörnberg, T., Michel, M., Wagner, R.S., Jacob, F., Hutzler, M. (2017). "**Genetic and Phenotypic Characterization of Different Top-fermenting *Saccharomyces cerevisiae* Ale Yeast Isolates.**" BrewingScience 70: pp. 9-25. DOI: 10.23763/BRSC16-25MEIER-DOERNBERG

Fachverlag Hans Carl GmbH · Postfach 99 01 53 · 90268 Nürnberg

Forschungszentrum Weihenstephan
für Brau- und Lebensmittelqualität
Herrn Dipl.-Ing. Tim Meier-Dörnberg
Alte Akademie 3
85354 Freising-Weihenstephan

Verlagsanschrift:
Andernacher Straße 33a
90411 Nürnberg
Telefon: +49 (0)911 9 52 85 - 0
E-Mail: info@hanscarl.com
Internet: www.hanscarl.com

Telefax:
Geschäftsltg. 0911 9 52 85 - 81 20
Buchhaltung 0911 9 52 85 - 81 30
Anzeigenabt. 0911 9 52 85 - 81 40
Redaktion 0911 9 52 85 - 81 60
Vertrieb 0911 9 52 85 - 81 42

Ihr Ansprechpartner: Sabine Raab

Telefon: +49 (0) 911-9 52 85 20
Telefax: +49 (0) 911-9 52 85-8120
E-Mail: raab@hanscarl.com

Datum: 28.11.2017

Dear Mr Meier-Dörnberg,

we hereby grant the permission to include the following publication in your theses:

Meier-Dörnberg T., Michel, M., Wagner, R.S., Jacob F., Hutzler M. : "Genetic and Phenotypic Characterization of Different Top-fermenting Saccharomyces cerevisiae Ale Yeast Isolates", BrewingScience 70: 9-25, 2017. DOI 10.23763/BRSC16-25MEIER-DOERNBERG

Mit freundlichen Grüßen
Fachverlag Hans Carl GmbH

FACHVERLAG HANS CARL GmbH Andernacher Straße 33a 90411 Nürnberg

i.V. Sabine Raab
Sabine Raab
Assistentin der Geschäftsführung

Fachverlag Hans Carl GmbH
Sitz Nürnberg, Registergericht HRB 17388
GF: Michael Schmitt
Verkehrs-Nr. Verlag: 11006 Fachbuchhandlung: 48715

Bankverbindung:
Postbank Nürnberg Kto-Nr. 4 100-857 BLZ 760 100 85 IBAN DE 04 7601 0085 0004 1008 57 BIC/Swift PBNKDEFF760
Castellbank Nürnberg Kto-Nr. 04 000 200 BLZ 790 300 01 IBAN DE 37 7903 0001 0004 0002 00 BIC/Swift FUCEDE77XXX
Sparkasse Nürnberg Kto-Nr. 1 116 003 BLZ 760 501 01 IBAN DE 44 7605 0101 0001 1160 03 BIC/Swift SSKNDE77XXX

1/14

Meier-Dörnberg, T., Hutzler, M., Michel M., Methner, F.-J., Jacob F. (2017). "**The Importance of a Comparative Characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* Strains for Brewing.**" Fermentation 3, 41: pp. 1-25. DOI: 10.3390/fermentation3030041

Betreff: Re: unformal permission DOI:10.3390/fermentation3030041
Von: Mayora Li <mayora.li@mdpi.com>
Datum: 11/24/2017 7:54 AM
An: Tim Meier-Dörnberg <tmd@wzw.tum.de>
Kopie (CC): Fermentation Editorial Office <fermentation@mdpi.com>

Dear Dr. Meier-Dörnberg

Thank you very much for your message. /Fermentation/ is an open access journal. The copyright owners are paper authors. Therefore, you need not require the permissions. You could reuse it freely.

By the way, /Fermentation/ could enjoy the open access free of charge in the years of 2017 and 2018. You are always welcome to publish with us in the /Fermentation/. We will surely provide you the best editorial service.

In case of any questions or any information you need, please feel free to contact us.

Kind regards,

Mayora Li
Assistant Editor

On 11/23/2017 5:27 PM, Tim Meier-Dörnberg wrote:

> Dear Ms. Mayora Li,
>
> I am preparing my doctoral (PhD) thesis, which is intended to be a compilation of an overall summary (overview) and research papers – bound together.
>
> I would greatly appreciate your permission to include the following publication in my thesis:
>
> ·Meier-Dörnberg T., Hutzler M., Michel, M., Methner, F.-J., Jacob, F.: "The Importance of a Comparative Characterization of Saccharomyces Cerevisiae and Saccharomyces Pastorianus Strains for Brewing", Fermentation 2017, 3 (3), 41; DOI:10.3390/fermentation3030041
>
> A unformal permission would be just fine as i have to add it as attachment to my thesis.
>
> Yours sincerely,
>
> Tim Meier-Dörnberg
>
> --
> Dipl.-Ing. Tim Meier-Dörnberg
> Mikrobiologie & Hefezentrum / Microbiology& Yeast Center
> Forschungszentrum Weihenstephan
> für Brau- und Lebensmittelqualität
> Technische Universität München
> Alte Akademie 3
> 85354 Freising-Weihenstephan
> DEUTSCHLAND / GERMANY
> Tel. / Phone: +49 8161 71-3417
> Fax: +49 8161 71-4181
> Email: tmd@wzw.tum.de <mailto:tmd@wzw.tum.de>
> Internet: http://www.blq-weihenstephan.de
> Steuernr. / tax no. 143/241/80037
> USt.-Identnr. / VAT-no. DE811193231
>
> ---
>
> Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität
> Direktor: Prof. Dr.-Ing. Fritz Jacob*//*

Meier-Dörnberg T., Jacob, F., Michel, Hutzler M. (2017). "**Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the beverage industry -Cases of contamination with *S. cerevisiae* var. *diastaticus* in the period 2008 to 2017-** " MBAA Technical quarterly 54, 4: pp. 140-148. DOI: 10.1094/TQ-54-4-1130-01

Re: PDF of Your MBAA TQ Article

Betreff: Re: PDF of Your MBAA TQ Article
Von: Susan Freese <SFreese@scisoc.org>
Datum: 1/25/2018 10:55 PM
An: Tim Meier-Dörnberg <tmd@wzw.tum.de>, Mathis Hutzler <m.hutzler@tu.de>

Hello, Dr. Meier-Dörnberg and Dr. Hutzler: The Master Brewers Association of the Americas (MBAA) allows authors to use materials they published with MBAA in other of their publications. You may each reprint the article identified below in your thesis (per email of 1-23-18).

Please include with the article a full bibliographic citation, including the doi number:

doi.org/10.1094/TQ-54-4-1130-01

Good luck completing your theses!

Regards, Sue Freese

Susan Freese
Technical Editor
Scientific Societies
Phone: 651.994.3875
www.scisoc.org

From: Carol Duling <CDuling@scisoc.org>
Date: Tuesday, January 23, 2018 at 12:04 PM
To: Tim Meier-Dörnberg <tmd@wzw.tum.de>
Cc: Susan Freese <SFreese@scisoc.org>
Subject: RE: PDF of Your MBAA TQ Article

Dear Tim,

I am forwarding you to Sue Freese for permission.

Sue, here is the publication information:

Incidence of *Saccharomyces cerevisiae* var. *diastaticus* in the Beverage Industry:
Cases of Contamination, 2008–2017
Tim Meier-Dörnberg, Fritz Jacob, Maximilian Michel, and Mathias Hutzler
MBAA TQ, vol. 54, no. 4, 2017, pp. 140–148

Note: this is for both Tim Meier-Dörnberg and Mathias Hutzler (m.hutzler@tum.de)

Best regards,

Carol

Carol Duling
MBAA Technical Editor
cduling@scisoc.org
Phone: 1-651-994-3869
Fax: 1-651-454-0766

From: Tim Meier-Dörnberg [mailto:tmd@wzw.tum.de]

Meier-Dörnberg T., Kory, O.I., Jacob F., Michel, M., Hutzler M. (2018). "***Saccharomyces cerevisiae* variety *diastaticus* friend or foe? – Spoilage potential and brewing ability of different *Saccharomyces cerevisiae* var. *diastaticus* yeast isolates by genetic, phenotypic and physiological characterization.**" FEMS Yeast Research 18, 4: pp. 1-27. DOI: 10.1093/femsyr/foy023

Re-use of third party content as part of your Oxford Journals article

- As part of your article, you may wish to reuse material sourced from third parties such as other publishers, authors, museums, art galleries etc. To assist with this process, we have a Permission Request form and accompanying Guidelines that specifies the rights required in order for third party material to be published as part of your Article. For a copy of this form, please email.
- Responsibility for clearing these third party permissions must be borne by the Author, and this process completed as soon as possible - preferably before acceptance of the manuscript, but if not possible, before the Article reaches the Production stage of the process.

Rights retained by ALL Oxford Journal authors

- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, for their own personal use, including their own classroom teaching purposes;
- The right, after publication by Oxford Journals, to use all or part of the Article and abstract, in the preparation of derivative works, extension of the article into book-length or in other works, provided that a full acknowledgement is made to the original publication in the journal;
- The right to include the article in full or in part in a thesis or dissertation, provided that this not published commercially;

For the uses specified here, please note that there is no need for you to apply for written permission from Oxford University Press in advance. Please go ahead with the use ensuring that a full acknowledgment is made to the original source of the material including the journal name, volume, issue, page numbers, year of publication, title of article and to Oxford University Press and/or the learned society.

The only exception to this is for the re-use of material for commercial purposes, e.g. republication or distribution of an article by a for-profit publisher or medical communications company etc. Permission for this kind of re-use is required and can be obtained by using Rightslink.

With Copyright Clearance Center's Rightslink ® service it's faster and easier than ever before to secure permission from OUP titles to be republished in a coursepack, book, CD-ROM/DVD, brochure or pamphlet, journal or magazine, newsletter, newspaper, make a photocopy, or translate.

- Simply visit: Oxford Academic and locate your desired content.
- Click on (Order Permissions) within the table of contents and/ or at the bottom article's abstract to open the following page:
- Select the way you would like to reuse the content