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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

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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 *S. pastorianus* *ssp. carlsbergensis,* *S. paradoxus* *S. cariocanus*	Sc-f	Scer	Sce	CAAACGGTGAGAGATTTCTGTGC	[21]
	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)* Main target: Bottom-fermenting culture yeast	BF-LRE-f	BF-LRE	BF-LRE1	ACTCGACATTCAACTACAAGAGTAAAATTT	[21]
	BF-LRE-r			TCTCCGGCATATCCTTCATCA	
S. pastorianus, *S. bayanus (partially)* Main target: Bottom-fermenting culture yeast	BF300E	BF	BF-300	CTCCTTGGCTTGTCGAA	[23]
	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$_2$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. 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$_2$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 [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` -TCCGTAGGTGAACCTGCGG–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$_2$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 [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	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGGGAGGATGCATTAGGAGCATTGTAAGAGAGTCGGAAGTTATCTGCGAAAATGGACTCATTCGAGTGGCCTATTGACGGTCGCCCAAGGTGTCGCA
	IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAATGAGTCCATTTTCGCAGATAACTTCCGACTCTCTTACAATGCTCCTAATGCATCCTCCCACTAGTTCTAGGGTCATCGAATCTATCCATTCTCTAGCA

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.

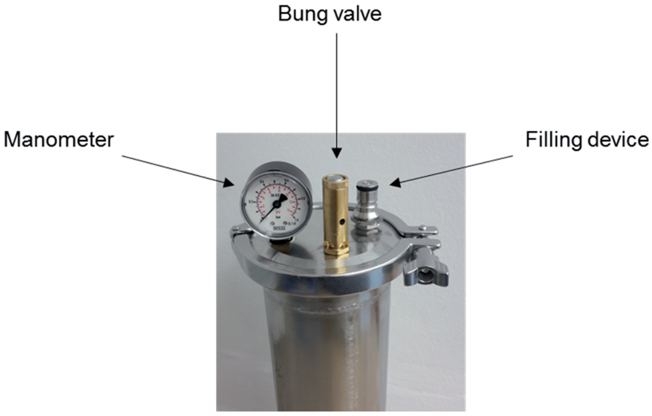


Fig. 1 Modified fermentation vessels

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 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 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

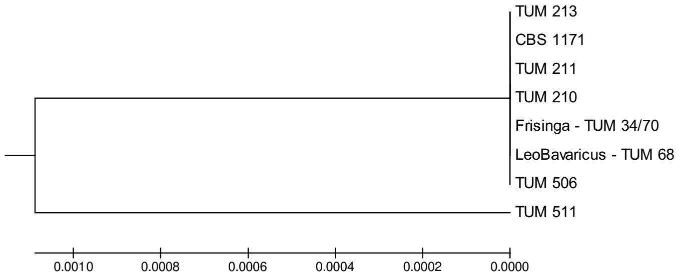


Fig. 2 **Phylogenetic tree of the trimmed single D1/D2 26S rDNA sequences after ClustalW alignment with MEGA6**

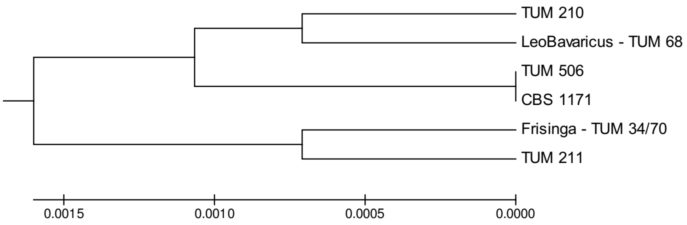


Fig. 3 **Phylogenetic tree of the trimmed single ITS1-5.8S-ITS2 rDNA sequences after ClustalW alignment with MEGA6**

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

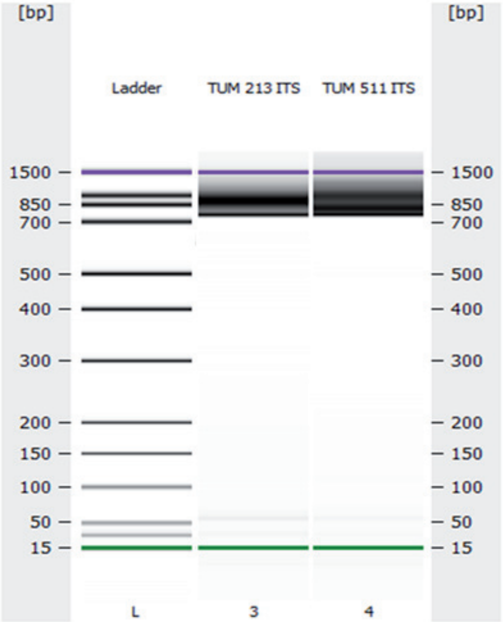


Fig. 4 Capillary electrophoresis ITS1-5.8S-ITS2 patterns for yeast isolates TUM 213 and TUM 511

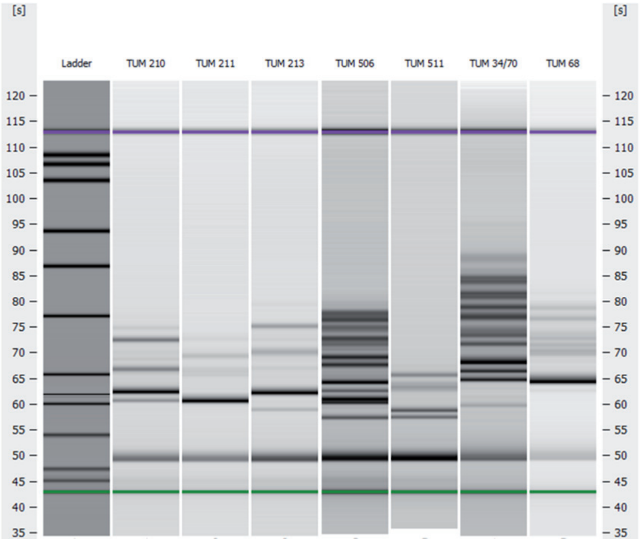


Fig. 5 Capillary electrophoresis IGS2-314 rDNA patterns of all yeast isolates (TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, LeoBavaricus – TUM 68® and Frisinga – TUM 34/70®)

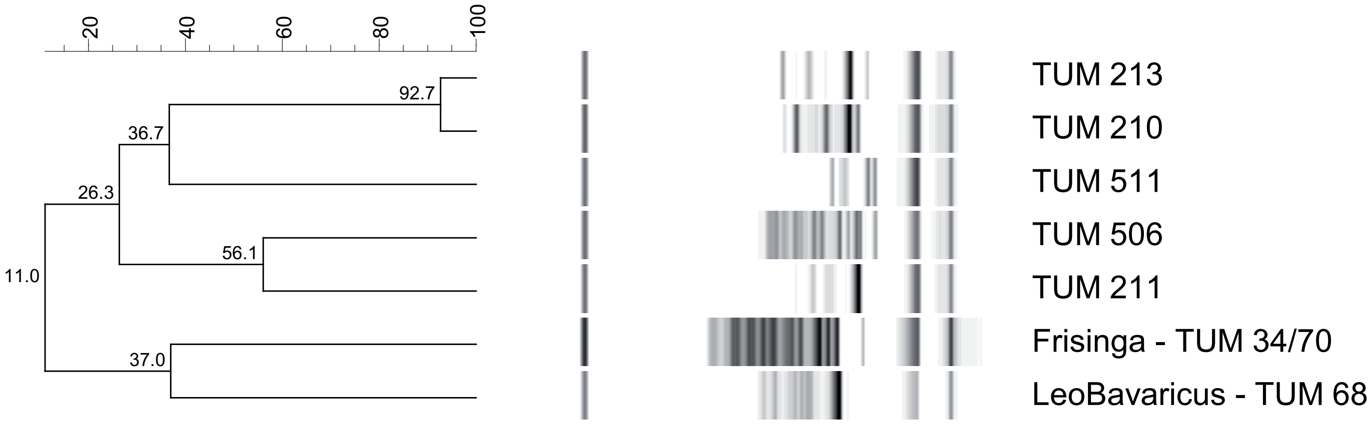


Fig. 6 IGS2-314 rDNA curve-based genetic relationship in percentage between TUM 210, TUM 211, TUM 213, TUM 506, TUM 511, LeoBavaricus – TUM 68® and Frisinga – TUM 34/70® (dendrogram built with Bionumerics 7.6)

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

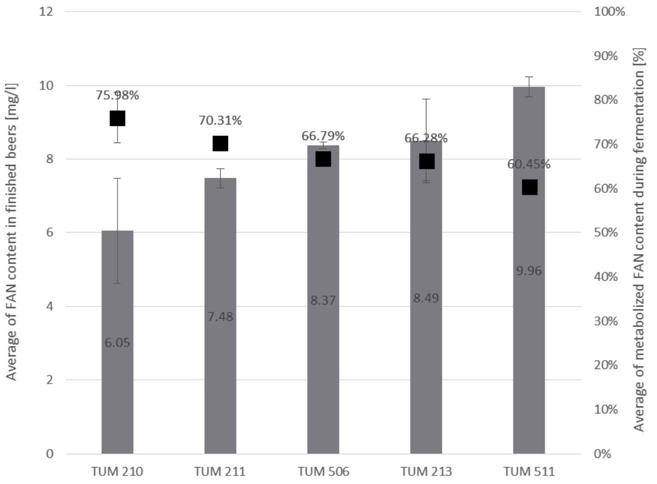


Fig. 8 **Average of metabolized and free amino nitrogen (FAN) content in finished beers produced with yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

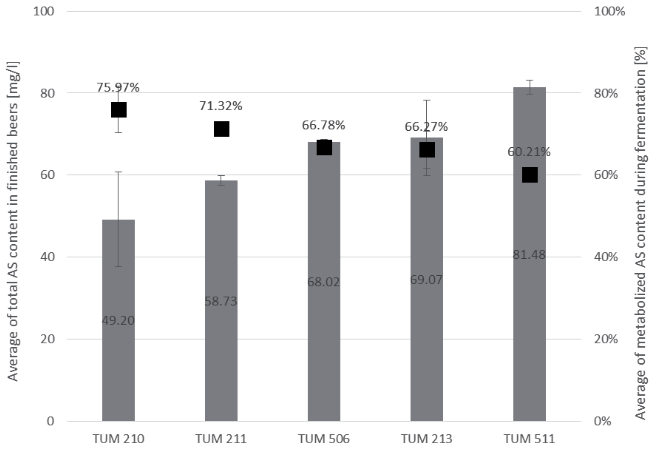
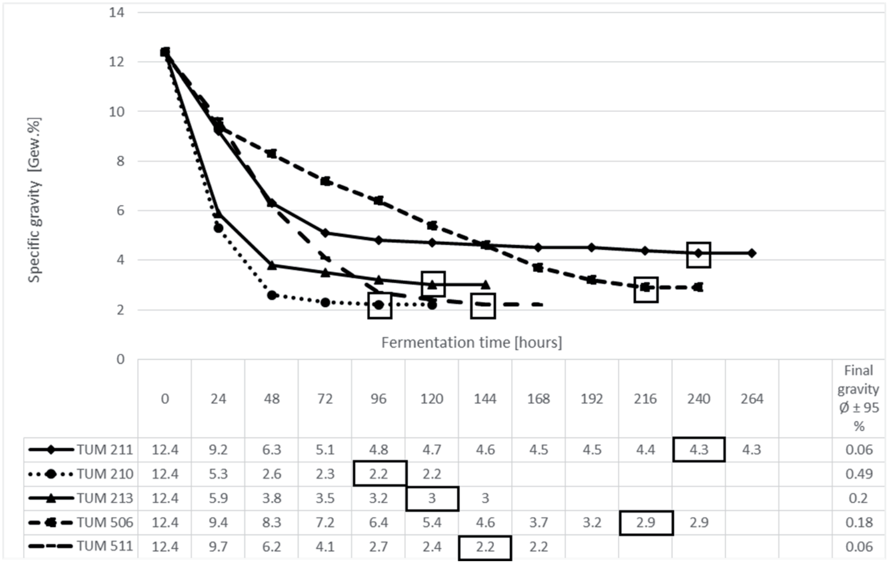


Fig. 9 **Average of metabolized and total amino acid (AS) content in finished beers produced with yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511; confidence level 95 %**

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.



	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 %**

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.

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].

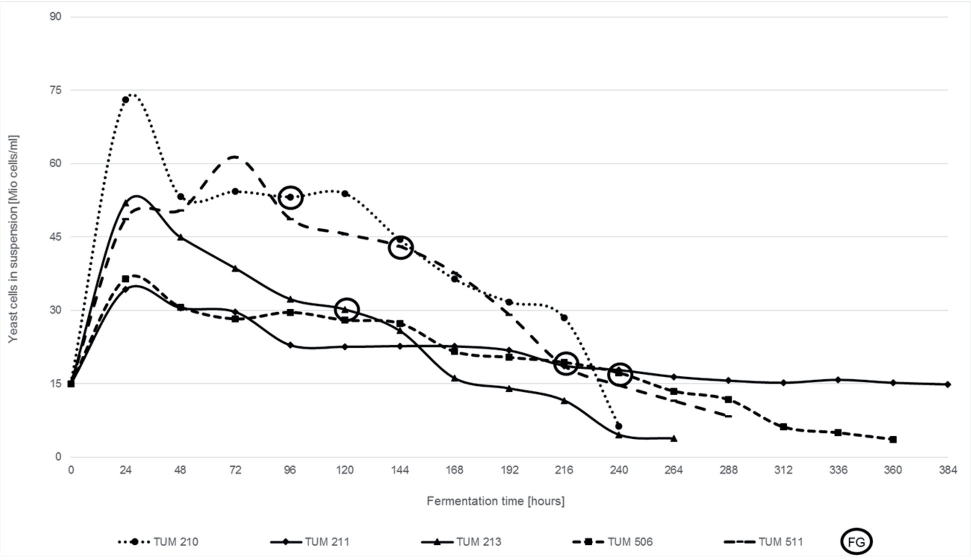


Fig. 11 **Yeast cells in suspension during the main fermentation and maturing phase. The circle marks the specific final gravity of the investigated yeast strains TUM 210, TUM 211, TUM 213, TUM 506 and TUM 511**

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	–	–	+	–	+

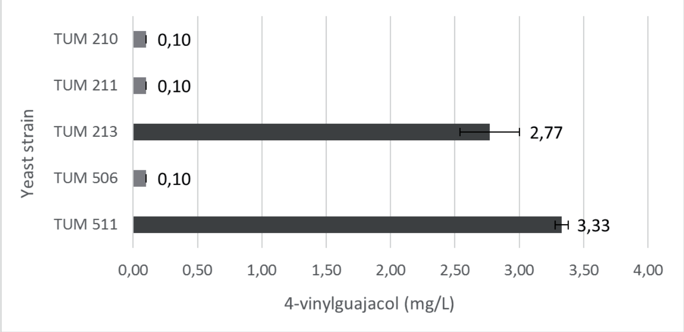


Fig. 12 Phenolic off-flavor ability of the investigated yeast strains; confidence level 95 %

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

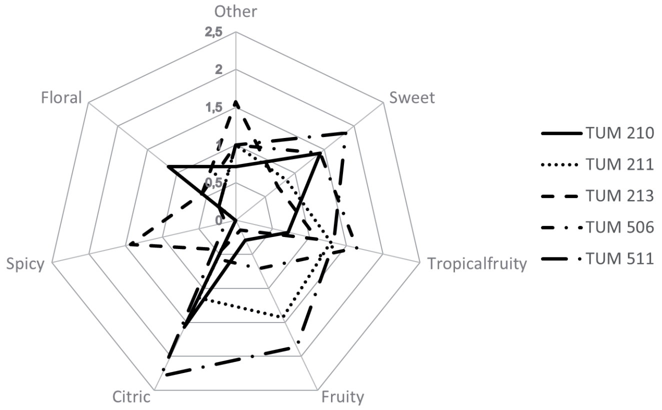


Fig. 13 Comparison of the flavors grouped according to the main categories

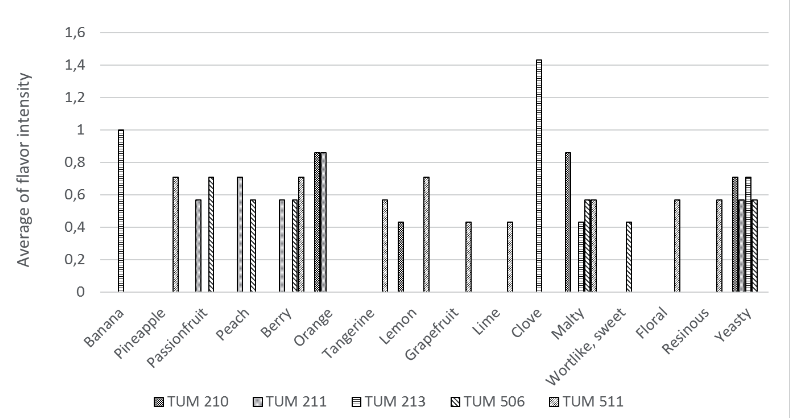


Fig. 14 Average of main flavor intensity of each yeast strain

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 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.

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**	**%**	**Signifi-cance**
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

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