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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

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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.

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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

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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

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$\times$ *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

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_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 (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_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 (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_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 (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.

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).

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 (RedTaq 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

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,

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

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

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

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).

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

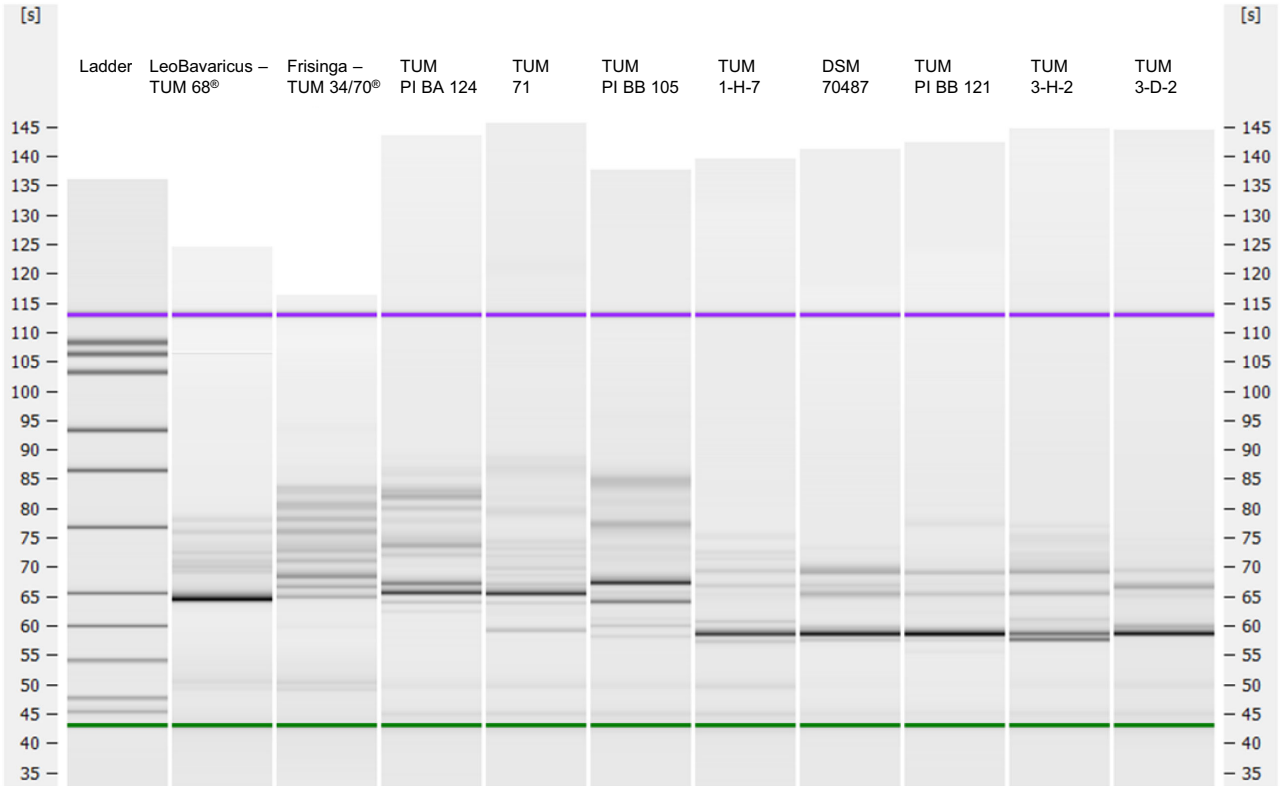

Figure 1. Capillary electrophoresis IGS2-314 rDNA patterns of all yeast isolates (LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2).

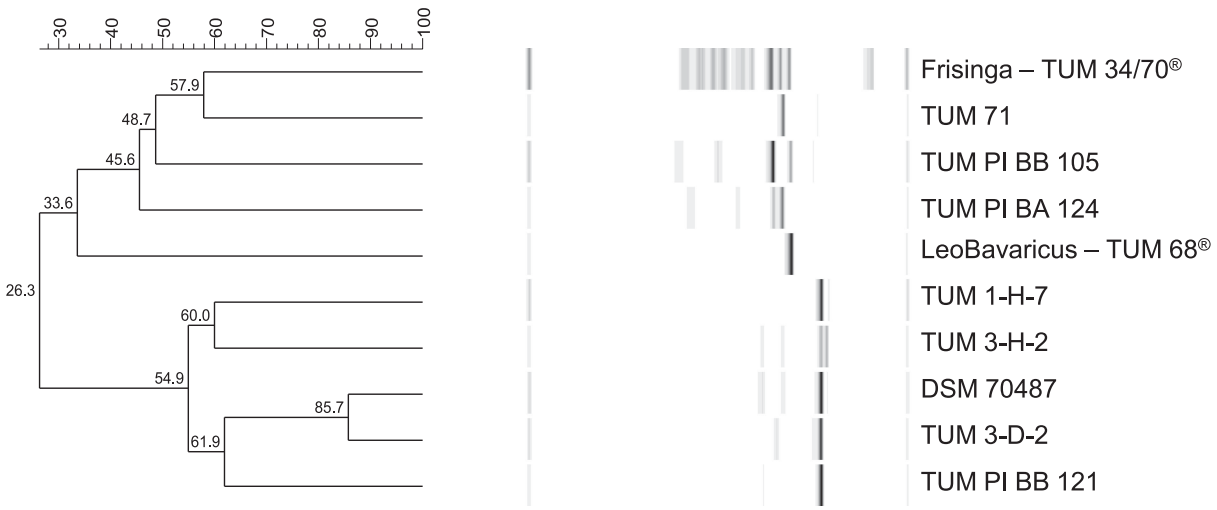

Figure 2. IGS2-314 rDNA band-based genetic relationship in percentage with capillary electrophoresis patterns between LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2 (dendrogram built with Bionumerics 7.6).

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

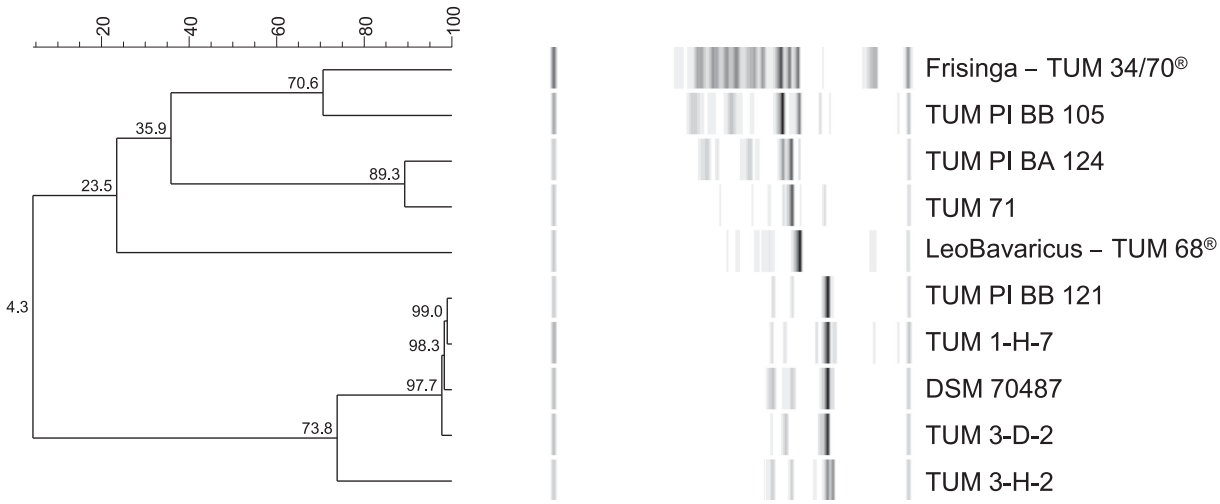

Figure 3. IGS2-314 rDNA curve-based genetic relationship in percentage with capillary electrophoresis patterns between LeoBavaricus—TUM 68®, Frisinga—TUM 34/70®, TUM PI BA 124, TUM 71, TUM PI BB 105, TUM 1-H-7, DSM 70487, TUM PI BB 121, TUM 3-H-2 and TUM 3-D-2 (dendrogram built with Bionumerics 7.6).

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.

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.*

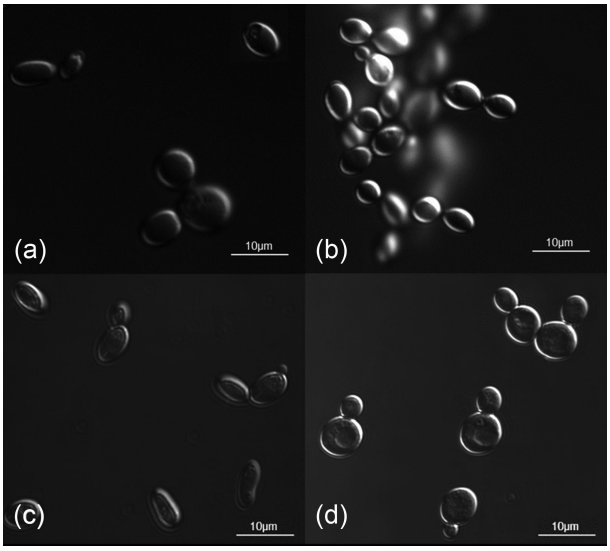

Figure 4. Types of cell morphology associated with *Saccharomyces* yeast species at 100× magnification. Nikon Eclipse Ti microscope images using DIC 100× of (**a**) *S. pastorianus* Frisinga—TUM 34/70® (**b**) *S. cerevisiae* LeoBavaricus—TUM 68® (**c**) *S. cerevisiae* var. *diastaticus* DSM 70487 (**d**)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

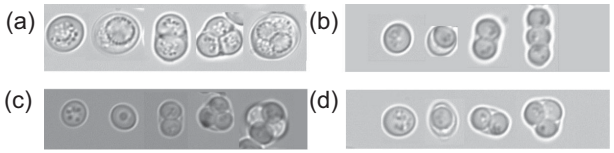

Figure 5. Ascus with ascospores of the *Saccharomyces* yeast strains (**a**) *S. pastorianus* Frisinga—TUM 34/70®, (**b**) *S. cerevisiae* var. *diastaticus* TUM 3-D-2, (**c**) *S. cerevisiae* var. *diastaticus* DSM 70487 and (**d**) *S. cerevisiae* var. *diastaticus* TUM 1-H-7 at 100× magnification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

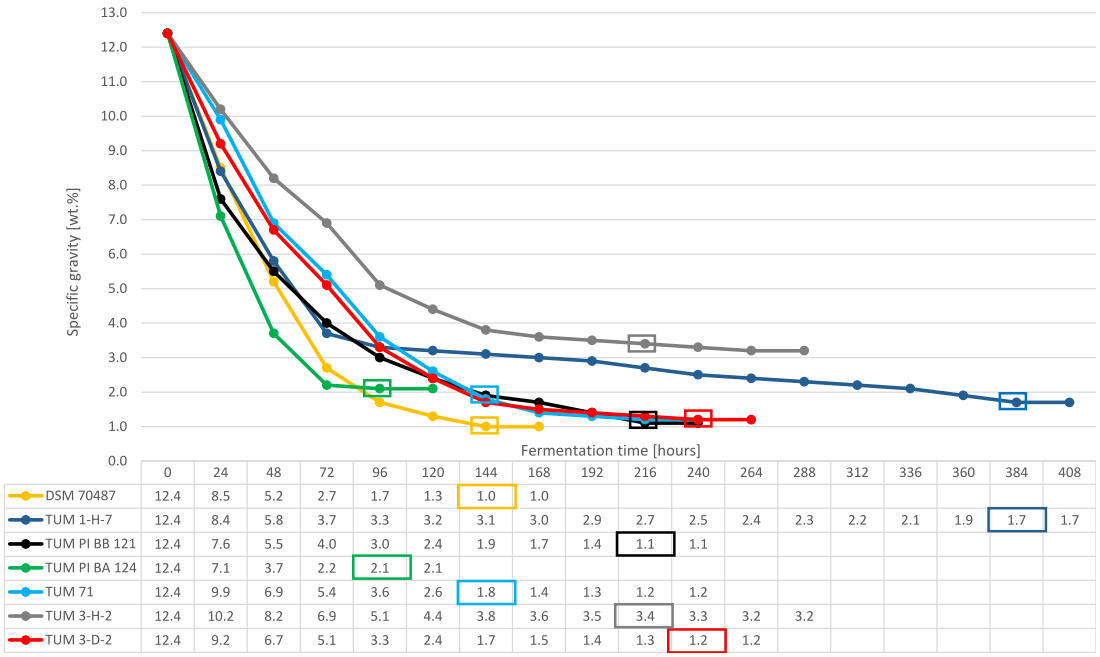

Figure 6. Drop in specific gravity measured in a single reference vessel compared with the average in final gravity (marked with box) measured in triplicate for the tested yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

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

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

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

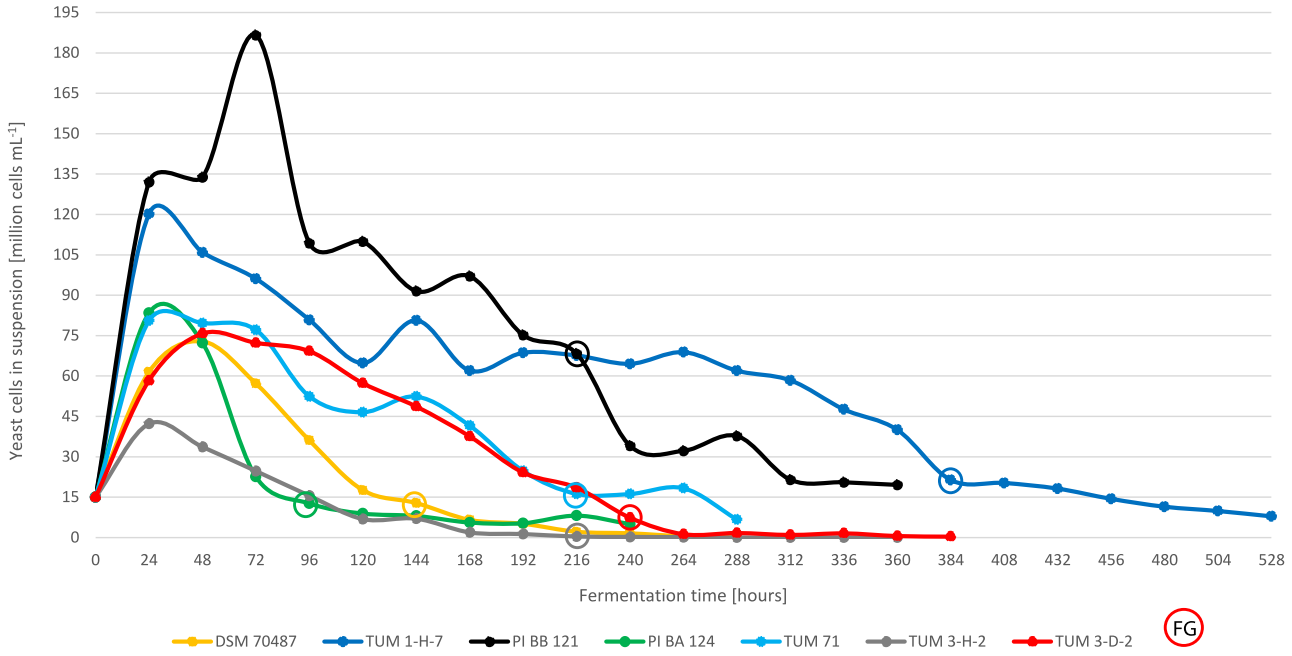

Figure 7. Yeast cells in suspension during the main fermentation and maturing phase. The circle marks the specific final gravity of the investigated yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2.

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.

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

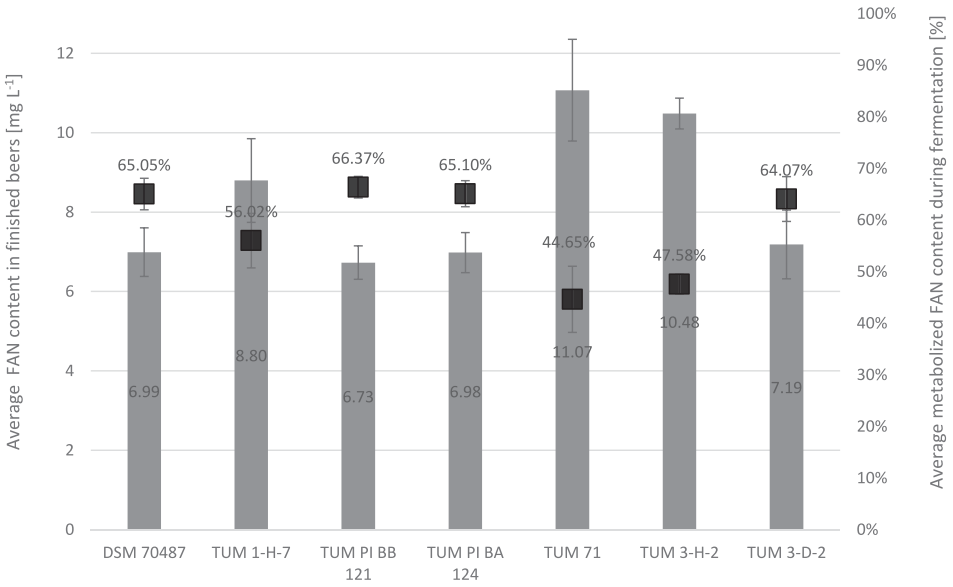

Figure 8. Average metabolized and FAN content in finished beers produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

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

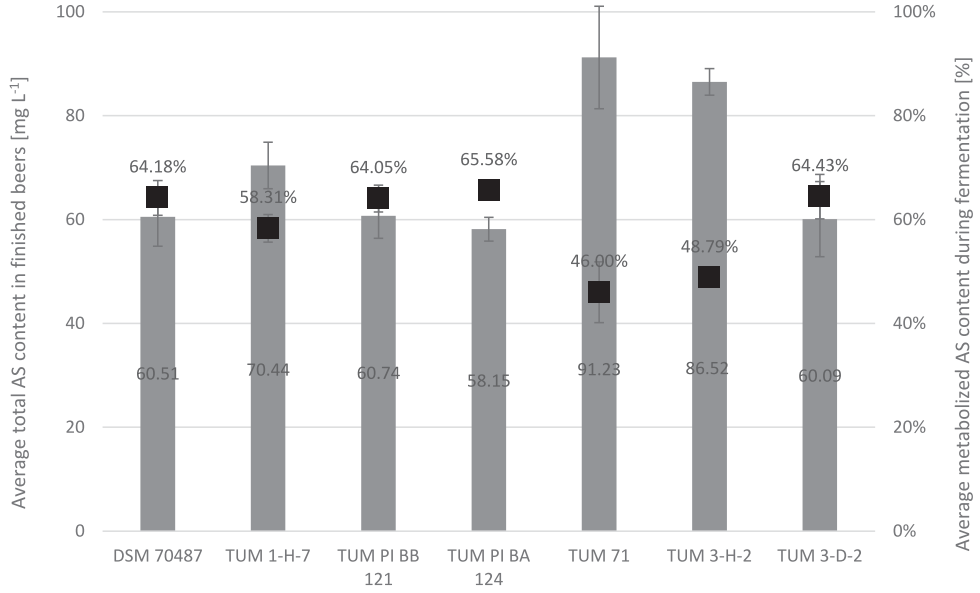

Figure 9. Average metabolized and total amino acid (AS) content in finished beers produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

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

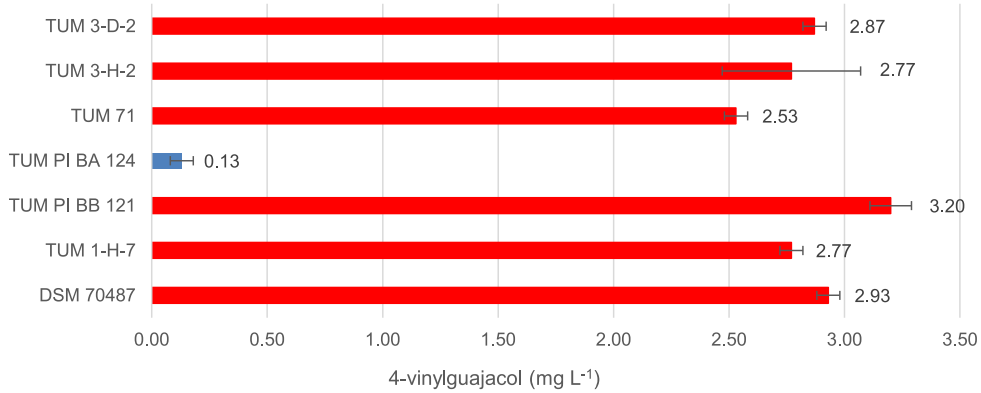

Figure 10. Concentration of 4-VG measured in the finished beers after lagering produced with yeast strains DSM 70487, TUM 1-H-7, TUM PI BB 121, TUM PI BA 124, TUM 71, TUM 3-H-2 and TUM 3-D-2; confidence level 95%.

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

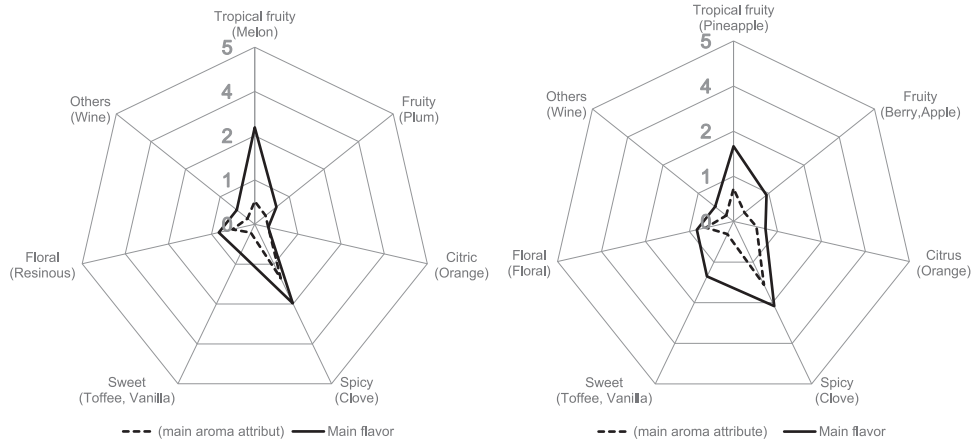

Figure 11. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains DSM 70487 (left) and TUM PI BB 121 (right).

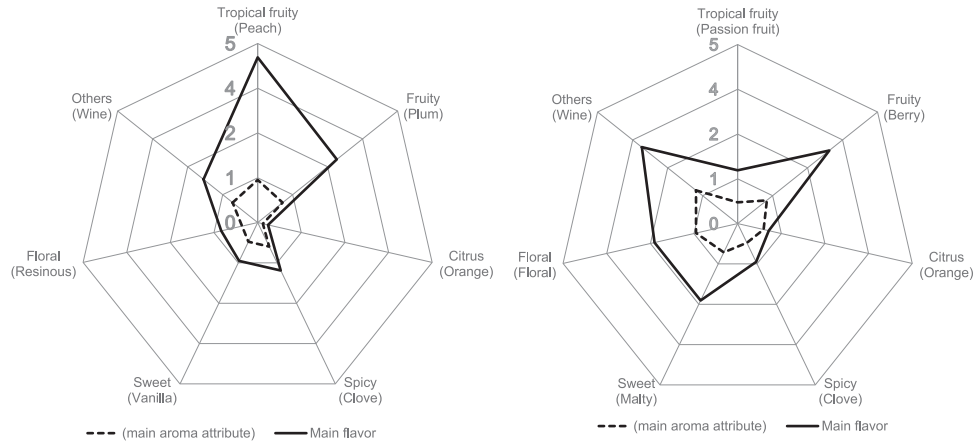

Figure 12. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains TUM 3-H-2 (left) and TUM 71 (right).

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

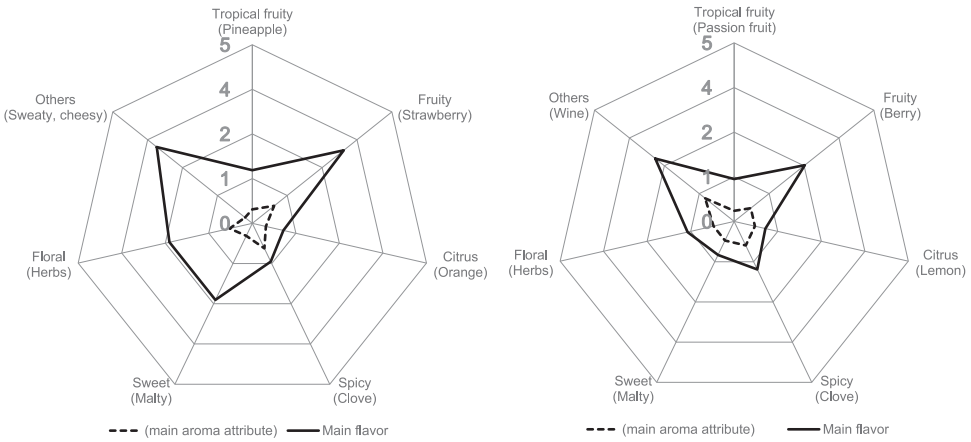

Figure 13. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. cerevisiae* var. *diastaticus* yeast strains TUM 1-H-7 (left) and TUM 3-D-2 (right).

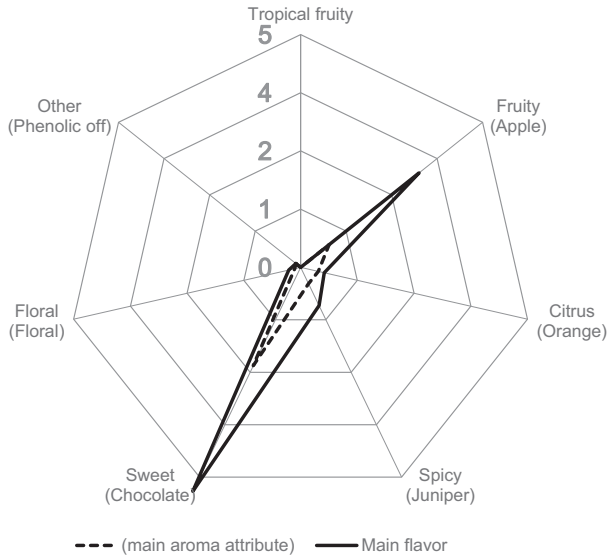

Figure 14. Comparison of the flavors grouped according to the main categories and the respective main aroma attributes for *S. pastorianus* yeast strain TUM PI BA 124.

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

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 accep-tance	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

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.

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