Technische Universität München - Lehrstuhl für Technische Mikrobiologie

Molecular classification of yeasts of the genus *Saccharomyces* with respect to their application potential

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

Vorsitzender: Hon.-Prof. Dr. Fritz Jacob

Prüfende der Dissertation:

1. Prof. Dr. Rudi F. Vogel

2. Prof. Dr. Ralf Kölling

Die Dissertation wurde am 14.01.2019 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 28.03.2019 angenommen.

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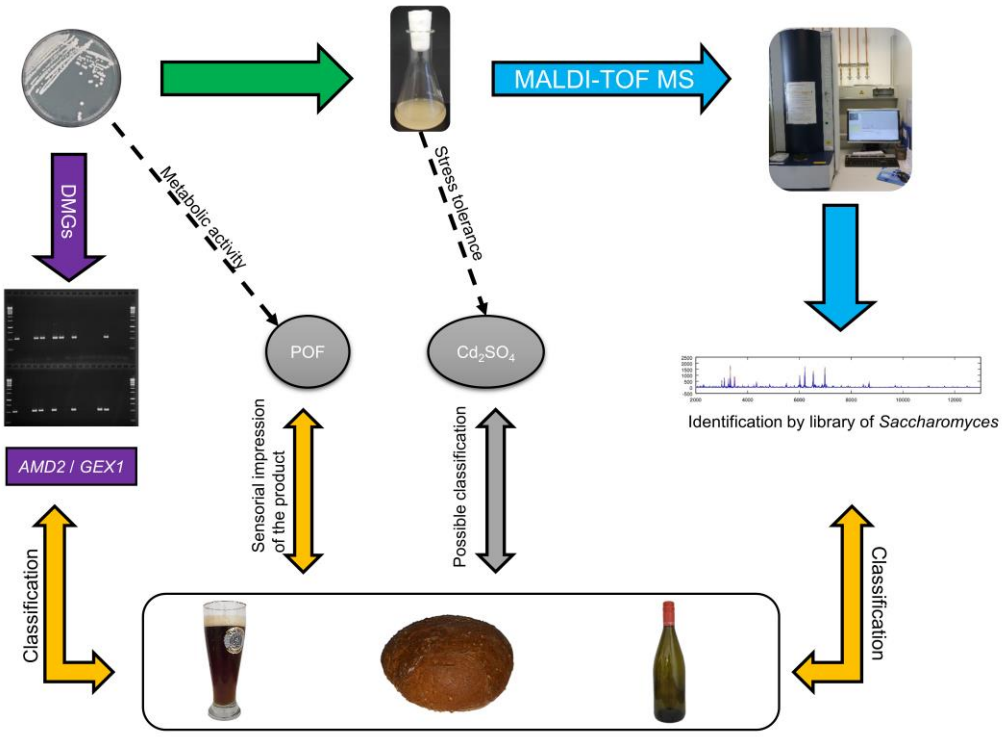

Österreichischer Bäckerspruch

„Der Bäcker und der Brauer, betrachtet man's genauer,

sind dieselben Herren, denn beide lassen's gären."

Vorwort

Die Vorliegende Arbeit entstand im Rahmen eines durch Haushaltsmittel des BMWi über die AiF-Forschungsvereinigung der Deutschen Brauwirtschaft e.V. geförderten Projektes (AiF 17698 N).

Teilergebnisse der vorliegenden Arbeit wurden vorab in Fachzeitschriften publiziert. Siehe Kapitel 10: „List of Publications“

Mein persönlicher Dank gilt:

Prof. Dr. Rudi F. Vogel für die Bereitstellung des Promotionsthemas mit der Organisation der Finanzierung und umfassende Betreuung sowie Unterstützung ist mit großem Dank zu erwähnen.

Prof. Dr. Ralf Kölling-Paternoga für die Begutachtung der Arbeit als auch Prof. Dr. Fritz Jakob für die Übernahme des Prüfungsvorsitzes.

Dr. Jürgen Behr und Dr. Andreas Geissler für eine tolle Zusammenarbeit als auch die vielen Diskussionen, die im Rahmen der Arbeit aufkamen.

Lara Eisenbach, Carolin Schiffer und Viktor Eckel für das Korrekturlesen.

Prof. Dr. Ludwig Niessen, Prof. Dr. Matthias Ehrmann, Andrea Pape, Sabine Forster, Maggie Schreiber, Monika Engel und Angela Seppeur für viel Diskussionsbereitschaft, aber auch ihrer fachlichen sowie organisatorischen Unterstützung.

Dr. Mathias Hutzler und Tim Meier-Dörnberg vom Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität für die Bereitstellung der Hefestämme. Als auch bei den vielen Telefonaten die wir führten, um Ideen bzw. Ergebnisse miteinander zu diskutieren.

All meinen Kollegen und Freunden, die für eine tolle Zeit am Lehrstuhl, in Weihenstephan und in Göppingen sorgten.

Einen besonderen Dank gilt meiner ganzen Familie, die mich über all die Jahre unterstützt haben und die immer für einen da sind.

Kristina Ruhland, die mir besonders am Ende der Arbeit, zur Seite stand und mich stets wieder auf die Beine half.

Vielen Dank euch allen!

Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… date

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Abbreviations

°C	degree Celsius
µ	Micro (10^{-6})
2,5-DHAP	2,5-Dihydroxyacetophenone
2,5-DHB	2,5-Dihydroxybenzoic acid
$3Cd_2SO_4$*8 H_2O	cadmium sulfate hydrate
3-HPA	3-Hydroxypicolinic acid
3MH	3-mercaptohexan-1-ol
3MHA	3-mercaptohexyl acetate
4MMP	4-mercapto-4-methylpentan-2-one
ABySS	Assembly By Short Sequences
AFLP	amplified fragment-length polymorphism
AK	German Alt-Kölsch
ATCC	American Type Culture Collection
BADGE	BIAst Diagnostic Gene findEr
BC	Before Christ
BF	bottom-fermenting
BIC	Bayesian Information Criterion
blastp	protein basic local alignment search tool
BLQ	Research Center Weihenstephan for Brewing and Food Quality
bp	base pair
BTS	bacterial test standard
c	concentration (g/l) or molar concentration (mol/l)
CBS	Central Bureau of Fungal Cultures
Cd_2SO_4	cadmium sulfate
CHCA	α-cyano-4-hydroxycinnamic acid
CLEN	cadaverine, lysine, ethylamine, and nitrate as the sole nitrogen source
CO_2	carbon dioxide
Cu_2SO_4	copper sulfate
d	distance of travelling
DAPC	discriminant analysis of principal components
DMG	diagnostic marker gene
DMS	dimethyl sulfide

DMSO	dimethyl sulphoxid
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
e.g.	lat: *exemplī grātiā*, for example
EDTA	ethylenediaminetetraacetic acid
EST	Expressed sequence tags
et al.	lat.: *et alii*, and other
***FLO*-gene**	Flocculation-gene
FTIR	Fourier transform infrared
g	gram
h	hour
H_2O	water
H_2S	hydrogen sulfide
HiT-MDS	high-throughput multidimensional scaling
HPLC	high performance liquid chromatography
HT	high attenuator
Hz	hertz
ID	identifier
k	kilo (10^3)
l	Liter
LioAc	lithium acetate
LWYM	Lin's wild yeast medium
M	molar concentration (see c)
m	meter, milli (10^{-3})
m/z	mass to charger ratio
MALDI	matrix-assisted-laser-desorption/ionization
MALDI-TOF MS	matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry
MASCAP	mass spectrometry comparative analysis package
MDS	multidimensional scaling
ME	malt extract
MgCl	magnesium chloride
min	Minute
n	nano (10^{-9}), number of samples
NBAP-B	non-brewing application potential of bread
NBAP-W	non-brewing application potential of wine

NCBI	National Center of Biotechnology Information
OS	organic solvent
PCR	polymerase chain reaction
PDR	peak detection rate
POF	phenolic off-flavor
ppm	parts per million
RAPD	random amplified polymorphic DNA
rpm	revolutions per minute
RTC	Real Time Classification
S.	*Saccharomyces*
S. c.	*Saccharomyces cerevisiae*
S. p.	*Saccharomyces pastorianus*
S. para.	*Saccharomyces paradoxus*
SA	Sinapinic acid
SDS	sodium dodecylsulfate
sec	second
SGD	*Saccharomyces* genomce database
SO_2	sulfur dioxide
t	Time
TMW	Technische Mikrobiologie Weihenstephan
TOF	time-of-flight
Tris	Tris-(hydroxymethyl)-aminomethane
TUM	coding of Research Center Weihenstephan for Brewing and Food Quality
U	accelerating voltage
USA	United States of America
V	voltage
var.	lat.: *varietas*, variety
WB	Wheat beer
WBAP	what beer application potential
WPGMA	weighted pair group method with averaging
YPD	yeast peptone dextrose
λ	wavelength

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1 Introduction

About 160 years ago, yeast was discovered as being the primary organism governing alcoholic fermentation by Louis Pasteur (Pasteur, 1860). This might be seen as one of the starting points of the yeast research for the future, however the process of fermentation has been applied unconsciously to make various edible and drinkable products for thousands of years. In the time of ancient China (7000 before Christ (BC)), Mesopotamia (5000 BC) and Egypt (1500 BC), fermented products similar to wine, mixed beverage, beer and leavened bread were made (McGovern et al., 1996, McGovern et al., 2004, Samuel, 1996). At this time, no one could have known about yeast or microorganisms in general or about the biological process of fermentation. The product was probably a result of spontaneous fermentation. Nowadays, mankind knows that different bacteria, molds, yeasts or a mixture of these are applied to ferment e.g. dairy products, beverages, meat products, vegetables or baked goods. Especially yeasts of the genus *Saccharomyces* (*S.*) have a great importance to improve the quality of foods as well as beverages (Sicard and Legras, 2011). Unique aroma profiles are characteristic for the different products. Considering *Saccharomyces*, people around the world use almost one specific species of this genus for different applications namely the "baker's yeast", "brewer's yeast", "distiller's yeast" or "wine yeast", *S. cerevisiae* (Landry et al., 2006, Lindegren, 1949).

However, species of the genus *Saccharomyces* containing 10 species (natural and hybrids) are applied for different applications, but some of them are only found in natural habitats (Hittinger et al., 2017, Naseeb et al., 2017, Wendland, 2014). *S. uvarum* is applied in the fermentation of wine and cider (Masneuf-Pomarede et al., 2016). In contrast, *S. eubayanus* is still tested for its application potential in different products like beer (Gibson et al., 2013) and cider (Gonzalez Flores et al., 2017). In the brewing environment two *Saccharomyces* species are established, one of them are the hybrids of *S. pastorianus* (Vaughan-Martini and Martini, 2011). This species is known in the brewing sector under different terms like bottom-fermenting yeast or lager yeast, which refers the property of the yeast to settle down or its application for Lager beer styles (Bokulich and Bamforth, 2013, Lindegren, 1949). On the other hand, brewers also use the species *S. cerevisiae*, which is described as top-fermenting yeast or Ale yeast (Bokulich and Bamforth, 2013, Lindegren, 1949). The term top-fermenting defines the property of *S. cerevisiae* to rise to the top of the vessel during the fermentation (Kunze and Manger, 2011, Lindegren, 1949). In contrast, the term Ale indicates more or less various top-fermenting beer styles like wheat beer, Alt beer or Kölsch. Furthermore, strains of this species are not only applied in the brewing environment, but are also found e.g. in the production of wine (Polsinelli

et al., 1996, Török et al., 1996), bread (Damiani et al., 1996, Rossi, 1996), liquor (Russell and Stewart, 2014) or kefir (Loretan et al., 2003, Simova et al., 2002). *S. cerevisiae* is likewise applied for scientific purposes and is one of the most studied yeasts, which served as model organism (Botstein and Fink, 1988, Landry et al., 2006).

For instance, several scientists described in the mid of the 1800s for the first time the process of fermentation (Hansen, 1883, Meyen, 1839, Pasteur, 1860) or Goffeau et al. (1996) sequenced for the first time the complete genome of an eukaryote. Those are just two examples, which display a small overview about for the importance of this yeast species for the understanding of biological processes. In modern days, scientific research concerning *S. cerevisiae* has been very diverse. On the one hand side, *S. cerevisiae* serves as model organism to study elemental processes in eukaryotic cells like chaperones (Lee et al., 1996, Srinivasan et al., 1998), regulation (Neigeborn and Carlson, 1984, Wodicka et al., 1997), expression (Hung et al., 1997, Zitomer and Lowry, 1992) or adaptive evolution (Ferea et al., 1999). On the other hand, research continues to focus on *S. cerevisiae* and its use by humans, e.g. domestication (Gallone et al., 2016, Goncalves et al., 2016, Legras et al., 2007), the finding of new species (Libkind et al., 2011, Naseeb et al., 2017), hybridization events (Gonzalez et al., 2006), and a targeted hybridization for improved aroma-producing strains (Steensels et al., 2014).

Today, several strains of *Saccharomyces* species are offered for focused fermentation performances like brewing, wine-making, bread-making or liquor-making. Some food or beverages firms even isolated their own internal strain, which has adapted to the new industrial environment. This is a kind of domestication, which is observed especially in the brewing sector (Gallone et al., 2016). Because of domestication as well as hybridization events, adapted natural strains, genetic modification or industrially produced strains, a variety of yeast strains are available. In fact, the high number of available strains reflect a major problem of yeast selection. The selection of a suitable strain for specific brewing processes or other applications is elaborate, time-consuming and based on trial and error. In some cases, only the experience of employees is used for the yeast selection. However, a wide characterization of the genus *Saccharomyces* with respect to their application potential is indispensable. The characterization might reflect the relation of strains to each other, but will also show the link of yeast strains to their application potential. Strains used with no further information of their true origin can be characterized and classified to a focused application.

As a consequence, the introduction focuses on the taxonomy of the genus *Saccharomyces*, with a focus on those species, which are used in general for industrial applications. Subsequently,

the relation of brewing yeasts to beer styles is reflected including the impact on the aroma profiles. Thereby, the main focus will be the genetic equipment of brewing yeasts. Lastly, an overview is presented about different molecular methods, which enabled a description of *Saccharomyces* strains on genetic and non-genetic, phenotypic and proteomic levels. The brewing process itself as well as the yeast management will not be considered. With regards to these topics, the reader is referred to Kunze and Manger (2011) or Annemüller et al. (2008).

1.1 *Saccharomyces*

1.1.1 Taxonomy

Yeast of *Saccharomyces* are eukaryotes and are taxonomically classified according to Table 1.1. 11 species including hybrids belong to the genus *Saccharomyces* (Boynton and Greig, 2014, Naseeb et al., 2017, Naumov et al., 2000). *S. cariocanus* was isolated and defined to be an own species (Naumov et al., 2000), but it was shown that this species actually belongs to the well-defined populations of *S. paradoxus* (Liti et al., 2006, Liti et al., 2009). On the other hand, Naseeb et al. (2017) isolated a new *Saccharomyces* from an oak tree bark, which represents a distinct biological species namely *S. jurei*. Its closest relatives are *S. cerevisiae*, *S. paradoxus* and *S. mikatae* (Naseeb et al., 2017). *S. mikatae* and *S. kudriavzevii* have been isolated in Japan (Naumov et al., 2000), but strains of *S. kudriavzevii* were likewise isolated in Portugal (Sampaio and Goncalves, 2008). Another natural, non-hybrid species is *S. uvarum*, which is relevant for industrial fermentations (Almeida et al., 2014, Masneuf-Pomarede et al., 2016, Nguyen and Gaillardin, 2005). *S. paradoxus* is a non-domesticated species with a worldwide distribution. It is isolated on the continents of Europe, Asia and America (Boynton and Greig, 2014) from tree exudates (Brysch-Herzberg and Seidel, 2017), insects (Phaff et al., 1956) and soil (Sniegowski et al., 2002). Furthermore, it is the closest known relative of *S. cerevisiae* (Goddard and Burt, 1999, Johnson et al., 2004, Martini, 1989). By contrast, *S. cerevisiae* is one of the most domesticated species worldwide (Gallone et al., 2016, Legras et al., 2007) and used in different industrial and domestic applications. The species is rarely isolated from natural habitats like vineyards (Mortimer and Polsinelli, 1999) or woodlands (Brysch-Herzberg and Seidel, 2017). The hybridization event between *S. cerevisiae* and the cryotolerant *S. eubayanus* isolated in the forest of Patagonia (Libkind et al., 2011) and Tibetan Plateau (Bing et al., 2014), resulted in the hybrids of *S. pastorianus* (Saaz- / Frohberg-Type) (Gallone et al., 2017). *S. bayanus* (Type I / Type II) displays another hybrid, which resulted from a cross between *S. uvarum* and *S. eubaynus* (Nguyen and Boekhout, 2017, Perez-Traves et al., 2014). The identification of yeast populations of fermenting grape must, cider, and brewing showed new natural hybrids of different *Saccharomyces* species, which were double (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*) and triple (*S. cerevisiae* x *S. uvarum* x *S. kudriavzevii*) hybrids (Gonzalez et al., 2006, Gonzalez et al., 2008, Lopandic et al., 2007, Sipiczki, 2008)

Table 1.1: Yeast Taxonomy of *Saccharomyces* from phylum to species level, which is retraceable to the different references in the last column. Those references applied the different species in their scientific purposes.

Yeast Taxa of *Saccharomyces*		**Reference**
Phylum	*Ascomycota*	(Kurtzman, 2011)
Subphylum	*Saccharomycotina*	
Class	*Saccharomycetes*	
Order	*Saccharomycetales*	
Family	*Saccharomycetaceae*	
Genus	*Saccharomyces* (*S.*)	
Species	*S. arboricola*	(Naumov et al., 2013, Wang and Bai, 2008)
	S. bayanus	(Huang et al., 2008, Nguyen and Gaillardin, 2005)
	S. cariocanus	(Naumov et al., 2000, Vaughan-Martini and Martini, 2011)
	S. cerevisiae	(Gallone et al., 2016, Legras et al., 2007)
	S. eubaynus	(Bing et al., 2014, Libkind et al., 2011)
	S. jurei	(Naseeb et al., 2017)
	S. kudriavzevii	(Naumov et al., 2000, Sampaio and Goncalves, 2008)
	S. mikatae	(Naumov et al., 2000)
	S. paradoxus	(Brysch-Herzberg and Seidel, 2017, Redzepovic et al., 2002)
	S. pastorianus	(Dunn and Sherlock, 2008, Peris et al., 2014)
	S. uvarum	(Masneuf-Pomarede et al., 2016, Nguyen and Boekhout, 2017)

1.2 Specific links between genome and application of yeasts of the genus *Saccharomyces*

1.2.1 General overview of the industrial application

Considering the industrial applications of yeasts of the genus *Saccharomyces*, they are found in different sectors and used as starter cultures. Figure 1.1 shows the usage of natural species (blue boxes) and hybrids (green boxes). The cryotolerant species of *S. uvarum* is applied in wine and cider production (Masneuf-Pomarede et al., 2016). Hybrids of *S. cerevisiae* x *S. kudriavzevii* or *S. cerevisiae* x *S. uvarum* are found within the making of wine, cider, probiotic products, and top-fermenting beers (Cecilia et al., 2017, Peris et al., 2017). Other species like *S. paradoxus*, *S. mikatae*, *S. arboricola*, or *S. jurei* are not likely to play an important role in the food and beverage fermentation. Whereas *S. eubaynus* is tested for his potential application in different fermentation processes of cider (Gonzalez Flores et al., 2017), beer (Gibson et al., 2013), and is part of a traditional fermented beverage in South America called *Mudai* (Rodriguez et al., 2014).

The main starter cultures of the genus *Saccharomyces* for the food and beverage industry are strains from the species of *S. cerevisiae* (var. *diastaticus*) as well as in some cases the hybrids of *S. pastorianus*, which are considered separately (Vaughan-Martini and Martini, 2011). Thereby, specific links between genome and application of those species are considered.

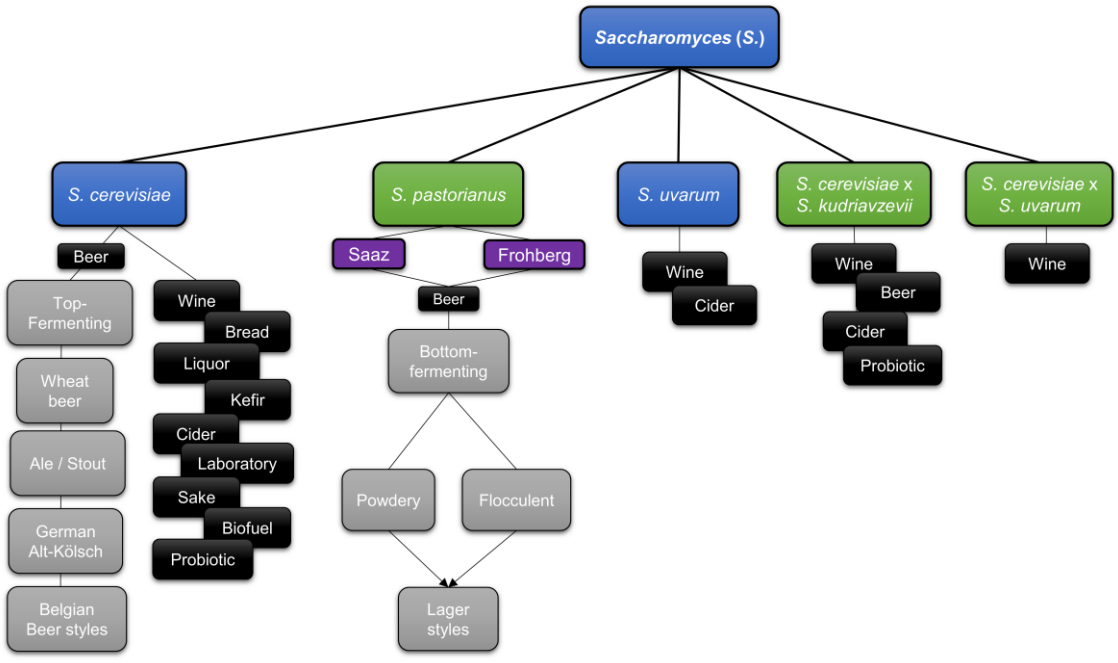

Figure 1.1: Application potentials of some species of the genus *Saccharomyces*. Green boxes = hybrids; blue boxes = natural yeast

1.2.2 *S. cerevisiae*

S. cerevisiae is one out of eight natural species of the genus *Saccharomyces*. Over the years, approx. 80 different names occurred like *S. validus* or *S. sake*, which were abolished and reallocated to *S. cerevisiae* nowadays (Vaughan-Martini and Martini, 2011). A lot of strains are found around the world, which are applied in different food and beverage fermentations (Gallone et al., 2016, Legras et al., 2007). Figure 1.1 visualizes some examples for products fermented with *S. cerevisiae* strains either with pure cultures or combined in mixed microbiota. Furthermore, laboratory strains of *S. cerevisiae* have played an important role for scientific purposes and served as model organisms (Botstein and Fink, 1988, Landry et al., 2006). Due to the many applications of *S. cerevisiae*, it is useful to reflect the scientific past and present of this species as well as the genomic background of industrial ecotypes.

S. cerevisiae was introduced by Meyen (1839), but the work of Louis Pasteur and Emil Christian Hansen gave the first thorough description of this species in the 19th century (Hansen, 1883, Pasteur, 1860, Pasteur, 1876). Louis Pasteur described the process of fermentation and assigned it to yeasts 1860 (Pasteur, 1860). Hansen described yeast strains of *Saccharomyces* isolated in the Carlsberg Brewery, Denmark and introduced techniques of pure culturing (Hansen, 1883). Winge and Lindgren focused separately from each other on their genetic analysis of yeast during the 1930s and 1940s (Lindegren, 1949, Winge and Hjort, 1935). However, Winge analyzed those strains isolated in the brewing environment by Hansen and recovered the stock cultures after 46 years of storage (Szybalski, 2001, Winge and Hjort, 1935). In contrast, Lindgren developed heterothallic strains, which have their origin from natural sources. In 1938, one heterothallic *S. cerevisiae* strain, EM93, was isolated from rotten figs in Mercedes, California and was given to Lindgren for his studies as stated in Mortimer and Johnston (1986). Thereby, *S. cerevisiae* S288c derived from EM93 in the early 1950s and EM93 contributed about 88% of the gene pool of S288c (Landry et al., 2006, Mortimer and Johnston, 1986). Furthermore, the whole genome of S288c was sequenced for the first time for a eukaryotic genome (Goffeau et al., 1996). The genome comprises approx. 6000 genes, which are located in 16 chromosomes (Goffeau et al., 1996). Besides this laboratory strain, *S. cerevisiae* strains from different ecotypes like wine, beer, bakery or sake were completely sequenced to study the biochemistry and genetics of industrial strains (Stewart et al., 2013). Thus a high fraction of genes and genomes could be studied, which made it possible to understand the genetic as well as phenotypic variation of *S. cerevisiae* strains.

Because of these studies, differences between brewing and non-brewing yeast strains could be shown. In general, wine strains are more or less described as the natural ecotype of *S. cerevisiae* with less domestication and brewing yeasts have undergone strong domestication (Gallone et al., 2016). For instance, *S. cerevisiae* strains applied in the fermentation of winery products are diploid (Mortimer, 2000), which means those yeast possess twice the number of chromosomes (2n) (Campbell and Reece, 2006). On the other hand, most of the top-fermenting brewing yeast strains are aneuploid or polyploid (Gallone et al., 2016, Mortimer, 2000). In this case, yeasts own an unusual amount of chromosomes, three (3n) or more sets have been observed (Campbell and Reece, 2006). Furthermore, brewing yeasts have the ability to ferment maltose and maltotriose, which is found in high concentrations in beer wort (Gallone et al., 2016). This trait is linked to the presence of a specific allele of the high affinity maltose transporter *MAL11* (Gallone et al., 2017). This gene is part of the *MAL1* locus including *MAL12* (alpha-D-glucosidase) as well as *MAL13* (MAL-activator protein) (Charron et al., 1986), which is absent in the wine subpopulation (Gallone et al., 2016). Another important phenotypic property of *S. cerevisiae* is the production of phenolic off flavor (POF) (Mertens et al., 2017). The formation of POF is explained in chapter 1.3.2, which shows the impact of brewing yeast on the wheat beer style. Actually, POF is unpleasant in most of the known beer styles as well as in sake and wine (Gallone et al., 2016). The genes responsible for POF are *PAD1* and *FDC1*, which help to decarboxylate phenylacrylic acids resulting in phenolic compounds (Mukai et al., 2010). A lot of industrial strains like brewing yeast strains lost the ability to produce POF, because of loss-of-function mutations (Mukai et al., 2014). However, non-brewing yeast strains applied in the bakery and wine or bio-ethanol production as well as natural isolates still possess the ability to form POF (Gallone et al., 2016, Goncalves et al., 2016, Mukai et al., 2014).

One main difference between brewing and non-brewing strains can be found in the process of fermentation itself. Gallone et al. (2017) described that brewing yeasts are harvested and re-used after the fermentation process to initiate the next fermentation batch. This continued exposure to the brewing environment has resulted in constant selection of these strains that originated from wild yeast (Gallone et al., 2017).

1.2.3 *S. cerevisiae* var. *diastaticus*

S. cerevisiae var. *diastaticus* (Bayly et al., 2005) is described as variety of the species *S. cerevisiae*, which is named over the years as *S. diastaticus* (Gilliland, 1966) or known to be a synonym for *S. cerevisiae* (Vaughan-Martini and Martini, 2011). The major impact to the brewing environment is the complete fermentation of starch and dextrin, which is linked to genes of the *STA*-family (*STA1*, *STA2*

and *STA3*). The *STA* genes encode extracellular glucoamylases (Yamashita et al., 1984), which hydrolyze alpha-D (1–6) bonds beside alpha-D (1–4) ones (Przybyt and Sugier, 1988). This metabolic and fermentation behavior is characteristic for these variety as it is explained by Andrews and Gilliland (1952). Accordingly, it has been shown that *S. cerevisiae* var. *diastaticus* caused low specific gravities (super-attenuation) and an excessive pressure in bottled beer (Andrews and Gilliland, 1952). This is due to a rapid fermentation linked to the formation of high amounts of carbon dioxide. Moreover, *S. cerevisiae* var. *diastaticus* strains produce phenolic off flavor (Spencer and Spencer, 1983), which is explain in chapter 1.3.2. Because of those characteristic, the variety of *S. cerevisiae* is more or less described as a contamination causing product damages and loss of image (Meier-Dörnberg et al., 2017b). Meier-Dörnberg et al. (2017b) showed with real-time polymerase chain reaction that at least six positive contaminations with *S. cerevisiae* var. *diastaticus* have been detected in breweries every year for 2008 to 2017. 71% of these were caused by contamination events during the filling process of beverages (Meier-Dörnberg et al., 2017b). The overview given by Meier-Dörnberg et al. (2017b) reflects the importance of detection of this variety of *S. cerevisiae*.

1.2.4 *S. pastorianus*

S. pastorianus strains are primarily used for the production of bottom-fermenting beer styles (Bokulich and Bamforth, 2013, Lindegren, 1949). These strains are hybrids of *S. cerevisiae* and *S. eubayanus* (Bing et al., 2014, Libkind et al., 2011). Over the years, the naming of these hybrids passed different iterations of *S. carlsbergensis*, *S. monacensis* and *S. cerevisiae* lager type leading to the currently accepted name *S. pastorianus* (Bokulich and Bamforth, 2013, Vaughan-Martini and Martini, 2011). Lindegren (1949) described that bottom-fermenting yeasts are divided into vigorous and weak fermenters. The vigorous group is called Frohberg-type (group 2) and the weak fermenters are assigned to “Saaz-type” (Group 1) (Lindegren, 1949, Monerawela and Bond, 2017a). Those two types are used for the production of lager beer styles, but differentiate in the frequency of application. Breweries of the Czech Republic and Denmark produce beers applying different Saaz strains, which are not used frequently around the world today (Bokulich and Bamforth, 2017, Monerawela and Bond, 2017a). However, “Unterhefe No. 1” (CBS 1513) is one of the first pure cultures of *S. pastorianus* strains isolated from a bottom-fermenting beer and belongs to the Saaz type (Hansen, 1908, Walther et al., 2014). In contrast, Frohberg strains, like TUM 34/70, are more distributed in European or North American breweries (Bokulich and Bamforth, 2017, Monerawela and Bond, 2017a). Those types do not only distinguish within their species, but also show genetic and phenotypic differences. Briefly, both types are a result from the interspecific hybridization of *S. cerevisiae* and *S. eubayanus*, but differentiated within their genome (Bokulich and Bamforth, 2017,

Wendland, 2014). Nakao et al. (2009) presented for the first time the genome sequence of the Frohberg strain TUM 34/70 showing the size of the genome to be 23.6 Mb (36 different chromosome structures, 64 chromosomes in total) (Bokulich and Bamforth, 2017, Walther et al., 2014). Walther et al. (2014) analyzed the genome of CBS 1513 and determined the ploidity for some strains. For one thing, it was shown that the genome is much smaller, with 19.5 Mb, compared to the Frohberg strain (Nakao et al., 2009). CBS 1513 is basically triploid with a diploid *S. eubayanus* and a haploid *S. cerevisiae* genome content having 29 unique chromosomes and 47 chromosomes in total (Walther et al., 2014). Walther et al. (2014) resequenced the Frohberg strain TUM 34/70 and also demonstrated the genome composition of this strain. It is tetraploid, composed of two diploid *S. cerevisiae* and *S. eubayanus* genomes (Walther et al., 2014). Considering the current models of the origin of these two types, three different hypotheses were proposed, which are explained in the review of Gallone et al. (2017). The most popular hypothesis is that multiple and independent hybridization events between distinct diploid *S. cerevisiae* and diploid *S .eubayanus* parental strains resulted in the Frohberg and Saaz groups (Gallone et al., 2017). Besides the genetic differences of these types, phenotypic differences were identified as well in different studies. It was shown that Frohberg strains possess a faster fermentation and higher degree of attenuation than Saaz strains (Gibson et al., 2013, Walther et al., 2014). The bad fermentation performance of Saaz-type strains is limited by an inability to use maltotriose (Gibson et al., 2013).

In contrast to the Frohberg and Saaz classification, brewers distinguish bottom-fermenting strains also based on their flocculation behavior. Terms like flocculation yeast or non-flocculation / powdery yeast are used (Kunze and Manger, 2011). The flocculation is linked to the *FLO*-gene family including genes such as Lg-*FLO1*, *FLO1*, *FLO5* and *FLO9* (Heine et al., 2009, Verstrepen et al., 2003b). The flocculation behavior is one of the most important characteristics for the brewing process. For instance, the usage of flocculation yeasts enable a faster settling of the yeasts, which results in bright beers with a low attenuation (Kunze and Manger, 2011). In contrast, powdery yeasts disperse in the wort and settle down at the end of the fermentation, which results in a higher attenuation and turbid beer (Kunze and Manger, 2011). Furthermore, it is described that the flocculation behavior is not a stable property. Flocculation yeasts of *S. pastorianus* may lose this ability or have a lower flocculation than before, because of crossing-over events, which are caused by frequent re-pitching (Annemüller et al., 2008).

1.3 A closer look at the link between fermentation products and *Saccharomyces* strain

Besides the use of different raw materials, the aroma profile of fermented products e.g. wine, beer styles or sake is likewise influenced by pure cultures of *Saccharomyces* species or mixed microbiota (Landaud et al., 2008, Querol and Fleet, 2006). Those sets of microorganisms give products their diversity, uniqueness and quality (Landaud et al., 2008). In all cases, the genomic features play an important role for all microorganisms, be it bacteria, molds or yeasts. Considering non-brewing applications, products like wine are influenced by different *S. cerevisiae* strains, which become dominant during the fermentation (Landaud et al., 2008). Sulfur compounds have a detrimental or beneficial effect on the aroma profile in wine (Landaud et al., 2008, Mestres et al., 2000). For instance, volatile thiols like 4-mercapto-4-methyl-pentan-2-one (4MMP; cat urine), 3-mercaptohexan-1-ol (3MH; passion fruit) or 3-mercaptohexyl acetate (3MHA; Riesling type-note) define winery products (Landaud et al., 2008, Usbeck et al., 2014). The gene *ATF1* encodes alcohol acetyltransferase and transforms 3MH to 3MHA during the fermentation (Swiegers et al., 2006, Usbeck et al., 2014). In contrast, *IRC7*, which encodes a β-lyase, is responsible for the formation of 4MMP (Roncoroni et al., 2011). Besides these examples for non-brewing products, beer styles are likewise affected by the applied starter cultures of *S. cerevisiae* or *S. pastorianus*. This is reflected below.

The major parameters defining a beer style comprise process parameters and the ingredients malt, hops and yeast (Narziss, 1984). In many countries further parameters can be varied including the use of un-malted grains, enzymes and other additives (Bamforth, 2000). The process of fermentation initiation by a selected strain was unknown in the past and mostly wild fermentation occurred. Along with the discovery of the fermentation of sugars by yeasts and the development of pure yeasts for a monitored brewing, the purity law was expanded within the beer taxes law (Annemüller et al., 2008, Meußdoerffer and Zarnkow, 2014, Meussdoerffer, 2009). Brewers in Germany are only allowed to use malt, hops, water and yeasts by "Vorläufigen Biergesetz §9" (BGBL, 1993). While the variation of malts has a long tradition and the exploitation of new hop varieties for craft beer brewing is upcoming in recent years, most breweries only use one single or a very small number of brewing yeasts. A lot of beer styles are actually influenced by specific brewing yeasts, which is reflected by the examples given in the following section. It shall be noted that besides the described beer styles below, beers like Lambic, Wit beer, Trappist, Saison or African traditional beers are also influenced by pure cultures or mixed microbiota (De Keersmaecker, 1996, Dornbusch, 2010, Focke and Jentsch, 2013, Lyumugabe et al., 2012).

1.3.1 Lager styles

For the fermentation of Lager beer styles different strains of *S. pastorianus* are used, which have either flocculent or powdery properties (chapter 1.2.4). The flavor of lager beers is affected as well by the applied *S. pastorianus* strain. Bottom-fermenting yeast strains have the tendency to produce higher levels of sulphur compounds than *S. cerevisiae* strains (Yoshida et al., 2008).

Sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) are compounds, which make significant contribution to the aroma profile (Bokulich and Bamforth, 2013, Yoshida et al., 2008). SO_2 acts as an antioxidant, which slows the development of oxidant haze as well staling of flavors in beer and has a flavor note reminiscent of burnt matches (Landaud et al., 2008, Yoshida et al., 2008). H_2S has a pungent aroma of rotten eggs and is a precursor of other compounds with negative sensory characteristics (Landaud et al., 2008, Yoshida et al., 2008). Those compounds are produced during the sulfur metabolism. It contains enzymes encoded by the *MET*-gene family, transporter for the uptake of extracellular sulfate by *SUL1* / *SUL2* and a SO_2 efflux pump (*SSU1*), which exports intracellular SO_2 through the plasma membrane (Hansen and Kielland-Brandt, 1996, Nakao et al., 2009, Yoshida et al., 2008).

Another sulphur compound, which occur in lager beer styles is dimethyl sulfide (DMS), which affects the aroma. DMS has sensorial impressions associated with cabbage, corn, onion or blackcurrant (Landaud et al., 2008). This compound may be derived from the thermal degradation of S-methyl methionine during kiln drying of the malt and wort preparation or via the brewing yeast metabolism (Bamforth and Anness, 1981, Hansen et al., 2002, Landaud et al., 2008). Within the fermentation process the oxidized form of dimethyl sulphoxid (DMSO) is enzymatically reduced to DMS (Anness and Bamforth, 1982, Hansen et al., 2002, Landaud et al., 2008). This enzymatic conversion is linked to a methionine sulfoxide reductase encoded by *MXR1* (Hansen, 1999, Hansen et al., 2002, Landaud et al., 2008) and increases the final concentration of DMS in beer.

Considering the utilization of carbohydrates during the production of Lager beer styles, the trisaccharide raffinose is fermented completely by *S. pastorianus* strains (Lindegren, 1949), which is assigned to the genes of *MEL1* and *SUC2* (Lazo et al., 1978, Taussig and Carlson, 1983). This is one of the major differences to top-fermenting *S. cerevisiae* strains, which are incapable to completely utilize raffinose (Lindegren, 1949).

1.3.2 Wheat beer style

Wheat beers are typically top-fermented beer styles of south Germany (Bavaria) (Dornbusch, 2010, Schneiderbanger et al., 2016). A characteristic of wheat beers is a signature flavor, which is associated to notes of clove, banana, apple or vanilla and comes from *S. cerevisiae* wheat beer strains (Dornbusch, 2010, Kunze and Manger, 2011, Schneiderbanger et al., 2016). The impression to a spicy and clove-like aroma of wheat beers is associated to the production of POF by *S. cerevisiae* (Goncalves et al., 2016, Mosher and Trantham, 2017). Substrates like ferulic acid, coumaric acid or cinnamic acid are decarboxylated to volatile compounds such as 4-vinylphenol (medicinal), 4-vinylguaiacol (clove) and vinylbenzene (styrol) by the enzymes phenylacrylic acid decarboxylase and ferulic acid decarboxylase (Goncalves et al., 2016, Richard et al., 2015, Vanbeneden et al., 2006). The genes encoding the respective enzymes *PAD1* and *FDC1* are both located on chromosome IV (Mukai et al., 2010).

The fruitiness can be explained with the formation of aroma-active esters, which are separated into two groups namely acetate esters and ethyl esters (Pires et al., 2014, Verstrepen et al., 2003a). Alcohol acetyl transferases, which are encoded by the genes *ATF1*, *ATF2* and Lg-*ATF1* are responsible for acetate esters like isoamyl acetate (banana aroma) or ethyl acetate (solvent-like aroma) (Pires et al., 2014, Procopio et al., 2011). The biosynthesis of ethyl esters is regulated through a condensation reaction between an acyl-CoA unity and ethanol (Pires et al., 2014). The reaction is catalysed by two acyl-CoA/ethanol O-acylstransferases encoded by *EeBI* and *Eht1* genes (Pires et al., 2014, Saerens et al., 2006). Those esters remind to sweet apple (ethyl hexanoate) or sour apple aroma (ethyl octanoate) (Pires et al., 2014, Procopio et al., 2011, Verstrepen et al., 2003a).

1.3.3 Alt beer and Kölsch style

Another top-fermenting beer style is Alt beer and Kölsch, which are top-fermented beers typically found in north-west Germany (Kunze and Manger, 2011). These beers are fermented at a low temperatures compared to other ales. (Dornbusch, 2010, Kunze and Manger, 2011, White and Zainasheff, 2010). The sensorial impression of these beers is especially influenced by the *S. cerevisiae* strain and described as fruity notes (Dornbusch, 2010, Focke and Jentsch, 2013, Verstrepen et al., 2003a). Moreover, the beers have a tendency to possess a sulphurous flavor, which is similar to bottom-fermenting beer styles (Focke and Jentsch, 2013, White and Zainasheff, 2010).

1.3.4 Ale

Ale beers are primary produced in Great Britain, Ireland and North America (Kunze and Manger, 2011). Moreover, the sensorial impression varies from the application of raw materials (aroma hops, malts) or the use of Ale yeast strains of *S. cerevisiae* (Focke and Jentsch, 2013, Kunze and Manger, 2011). The formation of volatile compounds by yeast, especially esters, can vary between high and low concentration in different Ale strains (Focke and Jentsch, 2013, Meier-Dörnberg et al., 2017c). Higher alcohols define the final aroma profile and are associated with the sensory impression of sweet alcohol, roses or solvent (Meier-Dörnberg et al., 2017c, Pires et al., 2014). Thereby, amino acids are absorbed by the brewing yeast and after a transamination, these by-product are formed in the Ehrlich pathway (Pires et al., 2014). The total count of the most important genes involved in the Ehrlich pathway is approximately 105, which encode dehydrogenases (i.e. *AAD3*, *GPD1*), decarboxylases (i.e. *PAD1*, *THI3*) and reductases (i.e. *AHP1*, *MET10*) (Styger et al., 2011). Meier-Dörnberg et al. (2017c) described Ale strains, which have the property to produce POF and yielded in beers with a clove-like flavor similar to wheat beers.

This shows a wide variation of yeast strains within beer styles, which define the sensorial impression differential of the final products.

1.4 Molecular characterization of *Saccharomyces*

In this chapter an overview is given about the different scientific purposes starting with genetic and non-genetic methods. Subsequently, a closer look is provided on marker genes and this is followed by the phenotypic characterization of *Saccharomyces* as well as the use of selective media. Lastly, proteomic approaches are considered.

1.4.1 Genetic and non-genetic methods

Characterization of *Saccharomyces* yeast strains based on genetic methods and respective sorting to application types were done over the years. Some experiments included the karyotyping of chromosomes by pulsed field gel electrophoresis to describe brewing yeast strains, new lager strains or hybrids (Kopecka et al., 2016, Krogerus et al., 2015, Masneuf et al., 1998, Sheehan et al., 1991). Amplified fragment-length polymorphism (AFLP) was used to investigate genetic variations of *Saccharomyces* and non-*Saccharomyces* yeasts (Azumi and Goto-Yamamoto, 2001, de Barros Lopes et al., 1999). Experiments based on random amplified polymorphic DNA (RAPD) could differentiate strains within the species *Saccharomyces cerevisiae* (Couto et al., 1996) and distinguish top-fermenting variants from other yeasts (Laidlaw et al., 1996). Timmins et al. (1998) have shown the discrimination of ale and lager yeasts by pyrolysis mass spectrometry and Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy was also used for the identification of yeasts like *S. cerevisiae* using a reference database (Wenning, 2004, Wenning et al., 2002) Considering the microsatellite loci analysis, strains of *S. cerevisiae* were matched to various origins like bread, beer, wine, sake or flor aging (Legras et al., 2014, Legras et al., 2007). The genetic diversity and population structure among *S. uvarum* strains was analysed likewise with microsatellite analysis and showed lower differences between strains from various origins (Masneuf-Pomarede et al., 2016). Goncalves et al. (2016) and Gallone et al. (2016) investigated the genomic background of *Saccharomyces* strains independently from each other and showed a wide distinction of them to ecotypes. Because of those analyses, mosaic genomes were found for example within wheat beer strains of *S. cerevisiae*. Gallone et al. (2016) described that those mosaic genomes of wheat beer strains are from *S. cerevisiae* wine and ale strains. Goncalves et al. (2016) divided his set of strains also into specific top-fermenting beer styles namely wheat beer, Ale and German Alt-Kölsch and showed genomic differences between those styles. On the other hand, Gallone et al. (2016) illustrated two different beer groups calling them Beer 1 and Beer 2, which distinguish form non-brewing applications. Furthermore, it was shown that Beer 1 contained

S. cerevisiae strains from different origins namely Germany/Belgium, US as well Britain and Beer 2 has more in common with the wine sub-population (Gallone et al., 2016).

1.4.2 Marker genes for classification

The current standard for yeast species identification are the fragments of the domains 1 and 2 (D1/D2) of the nuclear large subunit 26S rRNA gene and internal transcribed spacer regions (*ITS*) (Kurtzman, 2015, Schoch et al., 2009, White et al., 1990). Other genes like the subunits of RNA polymerase II (*RBP1* and *RPB2*), transcription elongation factor (*TEF1*), histidine requiring (*HIS4*) or the mitochondrial subunit 2 of cytochrome oxidase (*mtCOX2*) can be used for identification purposes as well (Weiss et al., 2013). The *ITS* region was used i.e. for a rapid identification of wine yeast (Guillamon et al., 1998) and in another scientific work for the detection of a new *Saccharomyces* (*S.*) species namely *S. jurei* (Naseeb et al., 2017).

A diagnostic marker gene (DMG) allows to differentiate microorganisms on species / strain levels or groups for example with respect to beer spoilage ability (Behr et al., 2016, Geissler, 2016, Suzuki et al., 2005). An example for DMGs for the classification of yeasts of the genus *Saccharomyces* is the gene *FSY1*, a fructose/H^+ symporter was successfully used to differentiate *S. eubayanus* and its hybrids containing this gene (Gonçalves et al., 2000, Pengelly and Wheals, 2013). For *S. cerevisiae* wine strains potential DMGs were described, which are involved in desiccation stress tolerance, e.g. *HSP12* (heat shock protein), *SSA3* (stress-seventy subfamily A) or *TPS*1 (trehalose-6-phosphate synthase) (Capece et al., 2016, Zambuto et al., 2017). These DMGs are supposed to help to select wine yeast strains, which survive the process of air-drying to get active dry yeasts for winemaking.

Several DMGs are used to distinguish between brewing and non-brewing *Saccharomyces* strains as well to discriminate brewing yeast with respect to their species level. One of the flocculation genes, *FLO1*, was used to differentiate brewing and non-brewing yeasts from each other (Yamagishi et al., 1999). In another scientific work, the hybrids of *S. pastorianus* (group I and group II) were described to have eight “lager-specific” genes, which have their origins from the parental yeast species of *S. cerevisiae* and *S. eubayanus* (Monerawela et al., 2015, Nakao et al., 2009). Because of those genes, it was possible to identify two classes of top-fermenting *S. cerevisiae* strains (ale and stout), which showed that the group I and group II *S. pastorianus* strains originate from separate hybridization events. Using the gene sequence of *COX2*, it is possible to distinguish top-fermenting *S. cerevisiae* and bottom-fermenting *S. pastorianus* from each other (Hutzler, 2009). Furthermore, it is possible to differentiate

S. cerevisiae from all other species of *Saccharomyces* to 100% because of the 100% presence of *COX2* within *S. cerevisiae* (Hutzler, 2009). On the other hand, the sequence of *LRE1* or BF-300 is used to detect the bottom-fermenting *S. pastorianus* strains, which could be achieved with a 100% sensitivity (Brandl, 2006, Hutzler, 2009, Rainieri et al., 2006). Another DMG, namely *STA1*, detects the high attenuating yeasts of *S. cerevisiae* var. *diastaticus* and distinguishes them from top-fermenting *S. cerevisiae* strains (Bayly et al., 2005, Brandl, 2006, Yamashita et al., 1984).

1.4.3 Phenotypic characterization

On phenotypic characteristics species of *Saccharomyces* were analysed with brewing experiments or the usage of several compounds. Gibson et al. (2013) performed physiology and fermentation experiments to analyse *S. pastorianus* (Saaz / Frohberg) and *S. eubayanus* strains. It was found out that beers produced with Saaz strains have less aroma-active compounds than those made with Frohberg or *S. eubayanus* strains and high differences within the fermentation performances were detected likewise (Gibson et al., 2013). Similar to Gibson et al. (2013), Walther et al. (2014) realized fermentation trials and also showed the cold adaptation of Saaz strains and good fermentation performance of Frohberg strains. Meier-Dörnberg et al. (2017a), (2017c) applied genetic and phenotypic methods to characterize brewing yeast strains of *S. cerevisiae* and *S. pastorianus.* Genetic differences within all yeast strains were demonstrated based on *IGS2*-314 loci and fermentation dynamics, flocculation behaviour as well as beer flavour showed considerable variations (Meier-Dörnberg et al., 2017a, Meier-Dörnberg et al., 2017c). The flavour of the beers ranged from floral to fruity to spicy (Meier-Dörnberg et al., 2017a, Meier-Dörnberg et al., 2017c).

In the time of craft brewing, it is interesting to find novel yeast strains, which are able to produce different metabolic compounds. The detection of one decisive metabolic activity can be shown by the production of POF to differentiate within the species of *S. cerevisiae.* Thereby, different approaches are applied either with chromatographic analysis (McMurrough et al., 1996, Vanbeneden et al., 2008), plating tests combined with sniffing (Goncalves et al., 2016, Meier-Dörnberg et al., 2017c), and finally a novel high-throughput absorbance-based screening method that allows quick determination of the POF production capacity of yeasts (Mertens et al., 2017).

1.4.4 Selective media

Different analyses can differentiate *Saccharomyces* from non-*Saccharomyces* wild yeasts based on growth behavior. A selective medium containing the organic dye crystal violet enables the detection of *Saccharomyces* wild yeast (Lin and Fung, 1985, Lin, 1975). However, it has been shown, that a wide range of sensitive *Saccharomyces* strains exist, which are able to grow on crystal violet agar plates (van der Aa Kuhle and Jespersen, 1998). Lin (1975) developed a differential medium namely Lin's medium, which contains crystal violet and fuschsin-sulphite. Lin's wild yeast medium (LWYM) suppressed the growth of brewery culture yeasts while supporting the growth of many wild yeasts (Beuchat, 1993, Lin, 1975). The utilization of the amino acid lysine made it possible to detect foreign yeasts in brewery pitching yeasts on lysine medium (Walters and Thiselton, 1953). *S. cerevisiae* and *S. pastorianus* are incapable to utilize lysine and are thus separated from yeast contaminations (Jespersen and Jakobsen, 1996, Walters and Thiselton, 1953). CLEN (cadaverine, lysine, ethylamine, and nitrate as the sole nitrogen source) was implemented by Martin and Siebert (1992) and is useful for a rapid growth as well detection of wild yeast in brewery samples. Dextrin applied as main carbon source, detects wild yeast causing super-attenuation like *S. cerevisiae* var. *diastaticus* (Bayly et al., 2005, Jespersen and Jakobsen, 1996). The fermentation of the trisaccharide raffinose differentiates bottom-fermenting *S. pastorianus* strains from top-fermenting *S. cerevisiae* strains (Gilliland, 1969, Van Uden, 1956). An agar-test containing melibiose is able to differentiate between *S. pastorianus* and *S. cerevisiae*, which is linked to *MEL1* (Hutzler, 2009). Tracer materials like copper are useful to support the growth of a wide range of wild yeasts (Lin, 1981, Taylor and Marsh, 1984).

In another scientific work, *S. cerevisiae* strains performed poorly in general stress conditions that are not usually encountered in the brewing environment (Gallone et al., 2016). A different stress tolerance of industrial *S. cerevisiae* strains was detected e.g. to the non-essential toxic heavy metal cadmium sulfate and showed a natural tolerance against high concentrations (Gallone et al., 2016, White and Munns, 1951).

1.4.5 Proteomic level

Proteome analysis were realized for example for a lager brewing yeast to obtain information on the identity of the parental strains (Joubert et al., 2000), the proteomic response to stress (Kobi et al., 2004, Trabalzini et al., 2003), or the comparison of transcriptomic and proteomic approaches between two commercial yeast strains (Zuzuarregui et al., 2006). Matrix-assisted

laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and two-dimensional gel electrophoresis were used to examine the proteome of a distillers yeast (Hansen et al., 2006). A closer look at the technology of MALDI-TOF MS and its application fields are provided within the next sub-chapter.

1.4.6 MALDI-TOF MS

The soft ionization mass spectrometry technique MALDI-TOF MS for the analysis of large, intact, and non-volatile biomolecules like amino acids or proteins was introduced in the late 1980's (Karas et al., 1985, Tanaka et al., 1988). Over the years, different reports were published, which explained the functionality of MALDI-TOF MS and its possible application fields (Demirev and Sandrin, 2016, Giebel et al., 2010, Hillenkamp and Peter-Katalinic, 2013).

Briefly, a short summary is given about the functionality of MALDI-TOF MS. First, biological samples have to be prepared for the MALDI-TOF MS analysis, which can be realized e.g. with a direct transfer of colonies or an ethanol/formic acid extraction either with colonies or a specific volume of incubated media. Figure 1.2 visualizes as an example the sample preparation of microorganisms in six steps: (I) Biological material of one colony is picked with a sterile toothpick and (II) transferred on one spot of the 96 steel target. (III) Subsequently, spots are overlaid with 1 µl of 70% formic acid, which improves the disruption of the cells (Bruker (2012) Bruker Biotyper 3.1 user manual). (IV) After a drying step, 1 µl of an organic compound, termed matrix (alpha-cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA)) is overlaid over the sample and forms a heterogeneous crystalline matrix that surrounds analytic molecules in the biological sample (Demirev and Sandrin, 2016). The matrix has to fulfill some criteria namely absorbance at the laser wavelength, solubility in a suitable solvent, and stability in a vacuum (McEwen and Larsen, 2014). (V) After another drying step the MALDI-target is finally prepared and (VI) is placed in the vacuum of the MALDI-TOF mass spectrometry.

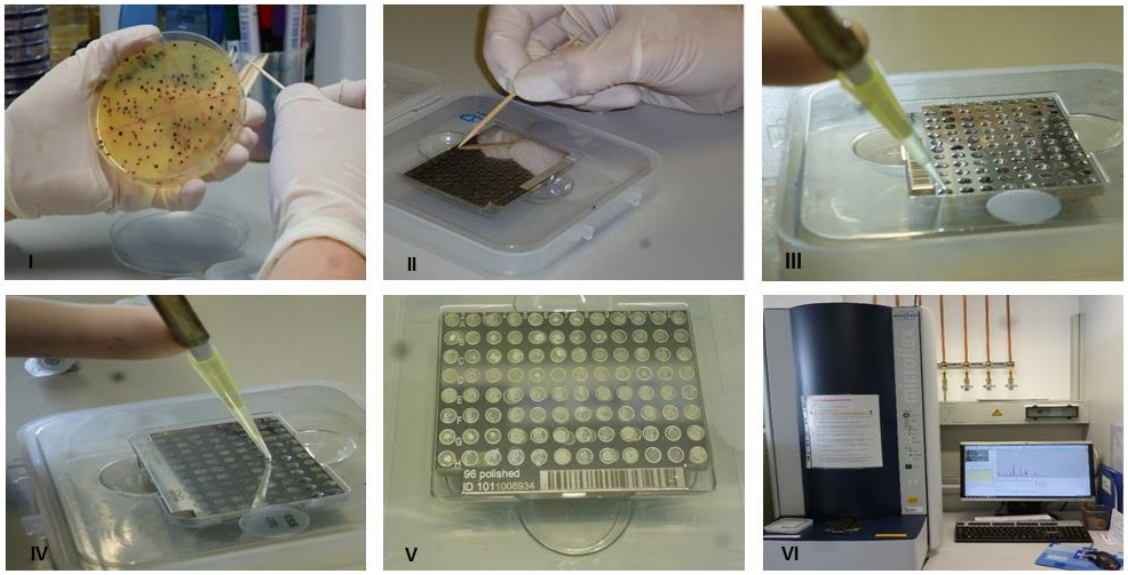

Figure 1.2: Overview about the general sample preparation for the MALDI-TOF MS analysis, which is displayed with the direct transfer method and combined with formic acid. The preparation is displayed in six steps: (I) picking biological material with a sterile toothpick; (II) transfer sample material on one spot of the 96 steel target; (III) overlay spots with 70% formic acid; (IV) after a drying step, overlay each sample material with 1 µl matrix solution; (V) final prepared MALDI target; (VI) target is placed in the vacuum of the MALDI-TOF MS (Microflex LT (Bruker Daltonics))

After the MALDI target is loaded into the MALDI-TOF MS, a vacuum is built up. A schematic representation of the MALDI-TOF MS functional principle is displayed in Figure 1.3 and is explained in four steps. An ultraviolet laser, typically a nitrogen laser with 337 nm, is used to desorb a small amount of the mixture of matrix and sample from one of the target spots (Figure 1.3 A). Thereby, the matrix absorbs the energy from the laser and the sample becomes vaporized, releasing ions of various sizes (Giebel et al., 2010). The formed ions pass through the accelerating grids (Figure 1.3 B) and fly through a flight tube (Figure 1.3 C), where smaller ions travel faster than larger ones (Demirev and Sandrin, 2016, Giebel et al., 2010). At the end of the flight tube the ions hit a detector, which calculates a mass spectrum for each measured spot (Figure 1.3 D). The principle of each calculation is based on the link between TOF (t_{TOF}) and mass to charge (m/z), which is illustrated in the following equation (1):

$$t_{TOF} = \frac{d}{\sqrt{2U}} * \sqrt{\frac{m}{z}} \qquad (1)$$

Besides the measured time of flight (t) for each ion, two other values are defined, namely the defined distance of travelling (d) as well as the accelerating voltage (U). In this case, for every measured ion the mass to charge value can be calculated resulting in a mass spectrum, an example of which is given in Figure 1.4.

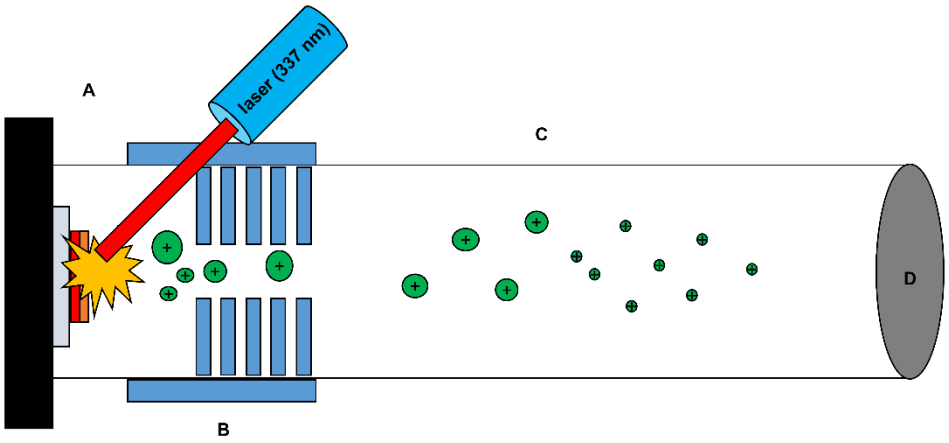

Figure 1.3: Schematic of MALDI-TOF MS according to Giebel et al. (2010). Though the areas are described: (A) an ultraviolet laser, typically a nitrogen laser with 337 nm, is used to desorb a small amount of the mixture of matrix and sample from one of the target spots. (B) The formed ions pas through the acceleration grids and (C) fly through a flight tube. (D) At the end of the flight tube the ions hit a detector, which calculates a mass spectrum for each measured spot.

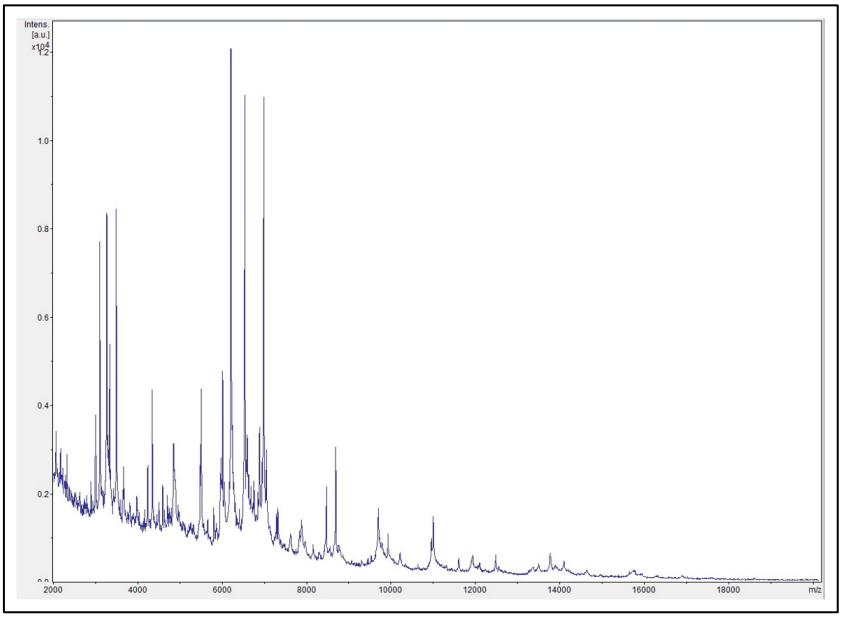

Figure 1.4: Example for a mass spectra generated with MALDI-TOF MS, which covers a mass to charge area (m/z; x-axes) from 2000 m/z to 20000 m/z. The y-axes illustrates the intensity of each peak.

Two different systems are available namely Microflex LT (Bruker Daltonics) or VITEK MS (bioMérieux), which showed to be a rapid and effective tool for a biotyping of microorganisms on genus, species or strain level (Guo et al., 2014, Kern et al., 2014a, Wieme et al., 2014). Thereby, the mass spectra of unknown microorganisms are compared to reference spectra of known microorganisms from various ecotypes (Demirev and Sandrin, 2016), which are implemented in a database by the provider Biotyper (Bruker Daltonics) and SARAMIS (bioMérieux). These databases can also be expanded with the user's own entries. Biotyper compares peak patterns with reference spectra on peak position, peak intensity, and peak

frequency (Demirev and Sandrin, 2016). Finally, a log score value is calculated and presented. SAMARI works similar to Biotyper, but it uses a confidence percentage for genus and species identification (Demirev and Sandrin, 2016). The application area of MALDI-TOF MS is normally the clinical sector (Croxatto et al., 2012), but the system is used as well within the food and beverage industy and science for the identification of contaminations (Kern et al., 2014b, Wieme et al., 2014) or starter cultures (Nacef et al., 2017, Pavlovic et al., 2014). Furthermore, it showed to be an effective tool for the separation of yeasts of the genus *Saccharomyces* (Blattel et al., 2013, Moothoo-Padayachie et al., 2013), the identification of wine yeast of *S. cerevisiae* and non-*Saccharomyces* yeasts (Gutierrez et al., 2017) and the classification of wine yeast strains based on the sub-proteomic fingerprint to their application potential (Usbeck et al., 2014).

2 Hypotheses and Objectives

If we look for the "golden standard" for the classification of *Saccharomyces* yeasts with respect to their application potential it is necessary to perform fermentation trials. This analysis matches a strain on phenotypic characteristics to wine, various beer styles, bread and so on (personal communication with Dr. Mathias Hutzler, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany). This approach is mainly based on trial and error and it is a time-consuming, elaborate and expensive process. Yeast strains are matched sometimes to their application potentials by the experience of employees. In this study, a simple method using a molecular characterization of yeasts of the genus *Saccharomyces* with the respect to their application potential is provided. The molecular characterization is realized on one side on sub-proteomic level by MALDI-TOF MS typing. Strains of *Saccharomyces cerevisiae* (var. *diastaticus*), *S. pastorianus* and wild isolates are investigated and their spectra are recorded by a standardized method with MALDI-TOF MS. Finally, a classification to various beer styles and the possible distinction between brewing and non-brewing yeast strains is analyzed. Along the sub-proteomic insights, a genomic analysis of a chosen number of yeast strains shall give insights into the identification of DMGs. Those DMGs enable the characterization of *Saccharomyces* strains according to their application potential based on genomic traits. In a proof of concept, a plating test is developed for the detection of one DMG.

Hypotheses:

The domestication of the genus *Saccharomyces* to industrial applications yielded in various strains, which have their own unique impact on the brewing and non-brewing sector. A general characterization to their application potential e.g. wine, beer styles, or bread is based on fermentation trials. Because of the adaptation of *Saccharomyces* yeast to industrial applications and a human-based domestication (Gallone et al., 2016) it shall be possible to characterize those strains with respect to their application potential by molecular methods. The adaptation and isolation to the stressful industrial environments resulted in specific sub-proteomic patterns, which enable a typing of *Saccharomyces* strains with respect to application potentials by MALDI-TOF MS. The domestication process or adaptation provided DMGs, which can be used to differentiate *Saccharomyces* strains according to their application potentials. In a proof of concept study, it shall be possible to correlate the growth on a selective media to one DMG.

These hypotheses resulted in the following objectives.

General objective:

The characterization of *Saccharomyces* strains with respect to their application potential will be realized with different molecular approaches: sub-proteomic (MALDI-TOF MS), genetics (DMGs) and phenotypic characteristics (selective media). Subsequently, the results of the molecular characterizations are compared with the "true industrial application" of the strains. Finally, a workflow shall be presented, which enables the rapid molecular characterization of *Saccharomyces* strains and classification with respect to their application potential.

The first approach is to optimize a sample preparation for the classification of yeasts by MALDI-TOF MS. This enables a standardized workflow and can be used for the establishment of a database of *Saccharomyces* strains. The optimized sample preparation is used to characterize a small set of reference *S. cerevisiae* strains (Usbeck, 2016) to major beer styles and those strains shall enable to classify new *S. cerevisiae* strains to their application potential. The expansion of the set with more *S. cerevisiae* as well as *S. pastorianus* and *S. cerevisiae* var. *diastaticus* strains shows the discriminant power of MALDI-TOF MS to beer styles, species and variety level. Besides the purpose to characterize yeast strains from the brewing environment, strains from the non-brewing sector can also be matched with their application potential. Because of the recorded sub-proteomic patterns, MALDI-TOF MS enables the user to distinguish yeast strains from the brewing and non-brewing sector from each other. In a proof of concept approach a set of wild yeast strains can be characterized to their species level as well as application.

Another approach is to use the software BIAst Diagnostic Gene findEr (BADGE) (Behr et al., 2016) to predict DMGs from the genome sequence of 25 top-fermenting *S. cerevisiae* strains (Goncalves et al., 2016). The designed primer pairs shall be tested with yeast strains from different application potentials. This shall demonstrate a genome derived possibility to characterize yeast strains of the genus *Saccharomyces* with the respect to application potentials by the use of novel DMGs. Subsequently, a selective media is used, which enables to assign one of the DMGs to a specific phenotypic characteristic.

3 Material and Methods

3.1 Strains

Table 3.1, Table 3.2, Table 3.3 and Table 3.4 list all strains with their particular information, referring to the different experimental sections. Some strains, which are used in single experiments, are mentioned individually in the corresponding section.

Table 3.1: Strains used for optimized sample preparation obtained by the BLQ. All strains are listed with their strain coding (TMW), alternative identifiers, genus as well species level and ecotype. TMW = Technische Mikrobiologie Weihenstephan; TUM = coding of Research Center Weihenstephan for Brewing and Food Quality; CBS = Central Bureau of Fungal Cultures; BLQ = Research Center Weihenstephan for Brewing and Food Quality

TMW	Alternative ID	Genus	Species	ecotype
3.0250	TUM 68	*Saccharomyces*	*cerevisiae*	Wheat beer
3.0275	TUM 34/70	*Saccharomyces*	*pastorianus*	Lager
3.0409	TUM SL17	*Saccharomycodes*	*ludwigii*	Low-alcohol beer
3.0600	CBS 2797	*Dekkera*	*bruxellensis*	Bordeaux wine

Table 3.2: Yeast strains of the genus *Saccharomyces* (*S.*) from the brewing environment obtained by BLQ. All strains are listed with their species, strain coding (TMW), alternative identifier, ecotype and isolation or origin (if available). The accession numbers for raw data (reads) is given 25 cases where genomes were from Goncalves et al. (2016) (Bioproject "PRJEB13332" found in https://www.ncbi.nlm.nih.gov/bioproject/PRJEB13332). Flocculation behavior is given for all *S. pastorianus* strains. TMW = Technische Mikrobiologie Weihenstephan; TUM = coding of Research Center Weihenstephan for Brewing and Food Quality; DSMZ = Deutsche Sammlung von Mikroorgansimen und Zellkulturen; BLQ = Research Center Weihenstephan for Brewing and Food Quality; WB = wheat beer

Species	TMW	Alternative ID	Ecotype / Application	Isolation source / Origin	Sequence read archive (NCBI)*
S. cerevisiae	3.0250	TUM 68	WB	Freising-Weihenstephan, Germany	ERR1352875
S. cerevisiae	3.0251	TUM 127	WB	Freising-Weihenstephan, Germany	ERR1352876
S. cerevisiae	3.0252	TUM 148	Alt	Dusseldorf, Germany	ERR1352854
S. cerevisiae	3.0253	TUM 149	WB	Munich, Germany	ERR1352845
S. cerevisiae	3.0254	TUM 165	Kölsch	Burton-upon-Trent, Great Britain	ERR1352860
S. cerevisiae	3.0255	TUM 175	WB	Freising-Weihenstephan, Germany	ERR1352847
S. cerevisiae	3.0256	TUM 177	Kölsch	Krefeld, Germany	ERR1352852
S. cerevisiae	3.0257	TUM 184	Alt	Düsseldorf, Germany	ERR1352872
S. cerevisiae	3.0258	TUM 205	WB	Würzburg, Germany	ERR1352846
S. cerevisiae	3.0259	TUM 308	Alt	Rhineland-Palatinate, Germany	ERR1352849
S. cerevisiae	3.0260	TUM 210	Ale / Stout	Great Britain	ERR1352863
S. cerevisiae	3.0261	TUM 211	Ale / Stout	Great Britain	ERR1352864
S. cerevisiae	3.0262	TUM 213	Ale	Great Britain	ERR1352856
S. cerevisiae	3.0332 a	TUM 998	Kölsch	Cologne, Germany	-
S. cerevisiae	3.0332 n	TUM 552	Kölsch	Cologne, Germany	-
S. cerevisiae	3.0336	TUM 192	Alt	Dusseldorf, Germany	-

S. cerevisiae	3.0337	TUM 338	Alt	Dusseldorf, Germany	ERR1352855
S. cerevisiae	3.0338	TUM 503	Ale	USA	ERR1352858
S. cerevisiae	3.0339	TUM 506	Ale	Great Britain	ERR1352866
S. cerevisiae	3.0343	TUM 505	WB	Bavaria, Germany	-
S. cerevisiae	3.0634	TUM 341	Alt	North Rhine-Westphalia, Germany	-
S. cerevisiae	3.0635	TUM 431	Alt	North Rhine-Westphalia, Germany	-
S. cerevisiae	3.0636	TUM 508	Ale	Ireland	ERR1352862
S. cerevisiae	3.0637	TUM 510	Ale	Great Britain	ERR1352857
S. cerevisiae	3.0666	TUM 220	WB	Bavaria, Germany	-
S. cerevisiae	3.0667	TUM 214	WB	Bavaria, Germany	-
S. cerevisiae	3.0668	TUM 513	Ale	USA	ERR1352850
S. cerevisiae	3.0669	TUM 454	WB	Bavaria, Germany	-
S. cerevisiae	3.0672	TUM 478	Ale	USA	-
S. cerevisiae	3.0673	TUM 511	Ale	USA	ERR1352842
S. cerevisiae	3.0674	TUM 457	WB	Bavaria, Germany	-
S. cerevisiae	3.0675	TUM 174	Alt	North Rhine-Westphalia, Germany	ERR1352853
S. cerevisiae	3.0864	TUM 380	Lambic	Belgium	ERR1352874
S. cerevisiae	3.0865	TUM 381	Belgian Beer	Germany	ERR1352844
S. cerevisiae	3.0866	TUM 507	Ale	Ale from wheatmalt, unknown	ERR1352848
S. cerevisiae	3.0867	TUM 480	Opaque beer	South Africa	ERR1352868
S. cerevisiae	3.0937	TUM 378	Wit beer	Belgium	-

S. cerevisiae	3.0961	FK28	Kölsch	North Rhine-Westphalia, Germany	-

Species	TMW	Alternative ID	Ecotype	Isolation soure / Origin	Property
S. cerevisiae* var. *diastaticus	3.0273	TUM 3-D-2	Spoilage	Northern Germany, Germany	High attenuation
S. cerevisiae* var. *diastaticus	3.0274	TUM 3-H-2	Spoilage	Northern Germany, Germany	High attenuation
S. cerevisiae* var. *diastaticus	3.0624	TUM PI BB 105	Spoilage	unknown	High attenuation
S. cerevisiae* var. *diastaticus	3.0625	TUM 71	Spoilage	North Rhine-Westphalia, Germany	High attenuation
S. cerevisiae* var. *diastaticus	3.0628	DSMZ 70487	Spoilage	Super-annutated beer	High attenuation
S. cerevisiae* var. *diastaticus	3.0811	TUM PI BB 121	Spoilage	unknown	High attenuation
S. cerevisiae* var. *diastaticus	3.0812	TUM 1-H-7	Spoilage	Bavaria, Germany	High attenuation

Species	TMW	Alternative ID	Ecotype	Isolation source / Origin	Flocculation behavior
S. pastorianus	3.0275	TUM 34/70	Lager	Freising-Weihenstephan, Bavaria, Germany	Flocculation
S. pastorianus	3.0276	TUM 34/78	Lager	Freising-Weihenstephan, Bavaria, Germany	Flocculation
S. pastorianus	3.0277	TUM 59	Lager	Nuremberg, Germany	Flocculation
S. pastorianus	3.0278	TUM 69	Lager	Nuremberg, Germany	Flocculation
S. pastorianus	3.0279	TUM 120	Lager	Fürth, Germany	Flocculation
S. pastorianus	3.0280	TUM 128	Lager	Region Vienna, Austria	Flocculation
S. pastorianus	3.0281	TUM 168	Lager	Hesse, Germany	Flocculation
S. pastorianus	3.0282	TUM 8-I-4	Lager	unknown	Flocculation
S. pastorianus	3.0283	TUM 8-J-4	Lager	unknown	Flocculation

S. pastorianus	3.0284	TUM 8-J-5	Lager	unknown	Flocculation
S. pastorianus	3.0813	TUM PI BA 124	Lager	North Rhine-Westphalia, Germany	Flocculation
S. pastorianus	3.0938	TUM 193	Lager	Freising-Weihenstephan, Bavaria, Germany	Flocculation
S. pastorianus	3.0285	TUM 66/70	Lager	Dortmund, Germany	Powdery
S. pastorianus	3.0286	TUM 204	Lager	Munich, Germany	Powdery
S. pastorianus	3.0351	TUM 92	Lager	Bavaria, Germany	Powdery
S. pastorianus	3.0352	TUM 106	Lager	North Rhine-Westphalia, Germany	Powdery
S. pastorianus	3.0354	TUM 145	Lager	Illinois, USA	Powdery
S. pastorianus	3.0356	TUM 167	Lager	Lower Saxony, Germany	Powdery
S. pastorianus	3.0357	TUM 170	Lager	North Rhine-Westphalia, Germany	Powdery
S. pastorianus	3.0358	TUM 182	Lager	North Rhine-Westphalia, Germany	Powdery
S. pastorianus	3.0359	TUM 183	Lager	North Rhine-Westphalia, Germany	Powdery

Table 3.3: Non-brewing yeast strains of *Saccharomyces* (*S.*) *cerevisiae* obtained by the BLQ. All strains are listed with their species, strain coding (TMW), alternative identifiers, ecotype / application as well isolation source / origin; BLQ = Research Center Weihenstephan for Brewing and Food Quality; TUM = coding of Research Center Weihenstephan for Brewing and Food Quality; ATCC = American Type Culture Collection

Species	**TMW**	**Alternative ID**	**Ecotype / application**	**Isolation source / origin**
S. cerevisiae	3.0308	S288c	Laboratory	ATCC (204508)
S. cerevisiae	3.0264	TUM V1	Wine	Bordeaux, France
S. cerevisiae	3.0333	TUM V2		Bingen, Germany
S. cerevisiae	3.0334	TUM V6		Willsbach, Germany

S. cerevisiae	3.0335	TUM V8		Loureiro, Portugal
S. cerevisiae	3.0929	TUM V9		Wädenswil, Switzerland
S. cerevisiae	3.0930	TUM V12		Stein, Germany
S. cerevisiae	3.0931	TUM V15		Épernay, France
S. cerevisiae	3.0929	TUM D2	Liquor	Distillery, unkown
S. cerevisiae	3.0265	TUM D4		Distillery, unkown
S. cerevisiae	3.0932	TUM S1		Unknown
S. cerevisiae	3.0266	TUM S2	Sparkling wine	Unknown
S. cerevisiae	3.0933	TUM S3		Unknown
S. cerevisiae	3.0923	-	Baking	Compressed fresh yeast, Germany
S. cerevisiae	3.0934	TUM 516	Rice wine	Rice wine, unknown
S. cerevisiae	3.0935	TUM 518	Banana wine	Banana wine, unknown
S. cerevisiae	3.0936	TUM 520	Corn wine	Corn wine, unknown

Table 3.4: Wild isolates of yeast strains. All strains are listed with their strain coding (TMW), isolation source, origin and source of supply. TMW = Technische Mikrobiologie Weihenstephan; BLQ = Research Center Weihenstephan for Brewing and Food Quality

TMW	Isolation source	Origin	Source of supply
3.0897	Fermented grape	California, USA	TMW
3.0909	Fermented raisin	Algeria	TMW
3.0924	Oak bark	Bavaria, Germany	BLQ
3.0925	Apple	Bavaria, Germany	BLQ
3.0926	Hop	Bavaria, Germany	BLQ
3.0927	Hop	Bavaria, Germany	BLQ

3.2 Media, buffer and solutions

The adjustment of the pH of all media, buffer and solutions was achieved with dilutions of HCl (Carl Roth GmbH & CO KG, Karlsruhe, Germany) or NaOH (Carl Roth GmbH & CO KG, Karlsruhe, Germany) (1 mol/l) , 2 mol/l, 4 mol/l or 6 mol/l). Unless otherwise noted, all media, buffer and solutions were autoclaved at 121 °C for 15 min. The sugar was autoclaved separately and added to the media under a sterile bench after cooling to below 50 °C.

3.2.1 Media

3.2.1.1 Yeast Peptone Dextrose (YPD) growth media

In general, yeast strains were cultivated using YPD growth media (liquid or agar plates). Table 3.5 shows the composition of YPD growth media and all compounds were solved in 1 l distilled water. The pH was usually adjusted to the pH of 6.5 ± 0.1.

For the optimal sample preparation (see chapter 3.4), YPD media with a pH of 5.0 ± 0.1 was used for *Dekkera bruxellensis* TMW 3.0600 (Table 3.1).

Table 3.5: Composition of YPD media; Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in g/l.

Compounds	Provider & Purity	Concentration [g/l]
Tryptone / Peptone ex casein	Carl Roth GmbH & Co KG, Karlsruhe, Germany	10
Yeast extract	Carl Roth GmbH & Co KG, Karlsruhe, Germany	5
Glucose	Merck, Darmstadt, Germany	20
Agar (for solid medium)	Carl Roth GmbH & Co KG, Karlsruhe, Germany	15

Cadmium sulfate hydrate (3 Cd_2SO_4 * 8 H_2O) (Carl Roth GmbH & Co KG, Karlsruhe, Germany) was added to YPD agar as an additive for the proof of concept study (chapter 4.4). A stock solution of 10.14 mM Cd_2SO_4 * H_2O was prepared. The additive was solved in ultrapure water (J.T. Baker, Deventer, the Netherlands) and was sterilized by filtration (pore size 0.2 µm; Syringe filters, RC; Sarstedt, Nürnbrecht, Germany). The YPD agar plates were prepared as described above. After cooling to 45 to 50 °C a volume of 493 µl of the sterile stock solution was added to the media for a final concentration of 5 µM Cd_2SO_4 under a sterile bench.

3.2.1.2 Malt extract (ME) agar plates

ME agar plates were used for the cultivation step within the preparation of cryogenic stocks (see chapter 3.3.1). Table 3.6 lists all compounds of this medium, which are dissolved in 1 l distilled water. The pH was adjusted to 5.6 ± 0.1.

Table 3.6: Composition of ME agar plates; Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in g/l.

Compounds	Provider & Purity	Concentration [g/l]
Malt extract	AppliChem GmbH, Darmstadt, Germany	10
Peptone ex soya	Carl Roth GmbH & Co KG, Karlsruhe, Germany	5
Agar	Carl Roth GmbH & Co KG, Karlsruhe, Germany	15

3.2.1.3 Glycerol-stock medium

Glycerol-stock medium was used for the preparation of cryogenic stocks (see chapter 3.3.1) for all yeast strains of chapter 3.1. All compounds, which are listed in Table 3.7, were dissolved together in 1 l tap water.

Table 3.7: Composition of glycerol-stock media. Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in g/l.

Compounds	Provider & Purity	Concentration [g/l]
Sodium glutamate monohydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany	11
Lactose monohydrate	Sigma Aldrich, Darmstadt, Germany	16
Agar	Carl Roth GmbH & Co KG, Karlsruhe, Germany	1
Ascorbic acid	Carl Roth GmbH & Co KG, Karlsruhe, Germany	0.1
Glycerol	Gerbu Biotechnik, Heidelberg, Germany; 99.5%	120

3.2.2 Buffer and solutions

3.2.2.1 Organic solvent (OS)

OS was prepared for the making of matrix solutions and the solution of the bacterial test standard. The composition of OS is listed in Table 3.8. The preparation is performed under a fume hood.

Table 3.8: Composition of OS. Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in µl/ml.

Compounds	Provider & Purity	Concentration [µl/ml]
Ultra-pure water	J.T. Baker, Deventer, the Netherlands; HPLC grade	475
Acetonitrile	Carl Roth GmbH & Co. KG, Karlsruhe, Germany; 100%	500
Trifluoracetic acid	Sigma-Aldrich, Darmstadt, Germany; >99%, HPLC	25

3.2.2.2 Bacterial test standard (BTS)

The preparation of BTS (Bruker Daltonics, Bremen, Germany) was done as the manufacture suggested. The BTS was resuspended in 100 µl OS (see chapter 3.2.2.1), stored at -20 °C and used for the calibration and validation of MALDI-TOF MS (see chapter 3.3.2).

3.2.2.3 Tris-(hydroxymethyl)-aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) (TE) buffer solution

TE-buffer solution was used for the yeast genomic DNA extraction (chapter 3.6.4) to dissolve the formed yeast pellet. After all ingredients of Table 3.9 were dissolved in 1 l distilled water, the pH was adjusted to 8.0 ± 0.1. Finally, the buffer solution was autoclaved.

Table 3.9: Composition of TE-buffer solution. Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in g/l or ml/l.

Compounds	Provider & Purity	Concentration [g/l] / [ml/l]
Tris-Base	Gerbu Biotechnik, Heidelberg, Germany; > 99%	1.21 g
0.5 M EDTA (pH 9.2)	Gerbu Biotechnik, Heidelberg, Germany; > 99%	2 ml

3.2.2.4 Lithium acetate-sodium dodecyl sulfate (SDS)-solution (LioAc-SDS)

LioAc-SDS was used for the yeast DNA extraction (chapter 3.6.4). Table 3.10 shows all compounds for the solution, which were solved in 1 l distilled water. After the preparation, the solution was sterile filtered (pore size 0.2 µm; CytoOne®; Bottle top filtration unit; Starlab Internationl GmbH, Hamburg, Germany) into a sterile 1 liter bottle.

Table 3.10: Composition of LioAc-SDS. Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound.

Compounds	Provider & Purity	Concentration
Lithium acetate dehydrate	Sigma-Aldrich, Darmstadt, Germany	200 mM
SDS	Serva Eletrophoresis GmbH, Heidelberg, Germany	1%

3.2.2.5 Ringer-solution

Two Ringer tablets (Merck KGaA, Darmstadt, Germany) were dissolved in 1 l distilled water. For subsequent dilution rows, 900 µl of non-sterile Ringer-solution were filled in 1.5 ml SafeSeal micro tubes (Sarstedet, Nürnbrecht, Germany) and autoclaved.

3.3 General

3.3.1 Preparation of cryogenic stocks

All yeasts (see chapter 3.1) were stored in glycerol-stock-media (chapter 3.2.1.3) at -80 °C. For the preparation of the yeast collection, 2 colonies from every strain were inoculated across the entire ME agar plates (chapter 3.2.1.2) and incubated at 30 °C for 2 to 4 days. Subsequently, plates were overgrown with yeast. These yeast cells were resuspended in 6 ml of glycerol-stock-media media by pipetting up and down. Finally, the suspended yeast cells were transferred in a 15-ml-tube (Sarstedt, Nürnbrecht, Germany) and stored over night at 4 °C. The next day, 1.8 ml of the suspension media was transferred in cryogenic tubes (Nunc^{TM}, 1.8 ml; Thermo Fisher Scientific, Munich, Germany). The tubes were stored at -80 °C.

3.3.2 Calibration and Validation of MALDI-TOF MS

The calibration and validation of MALDI-TOF MS was performed once a week with BTS (see chapter 3.2.2.2) based on a modified *Escherichia coli*. 1 µl of BTS was spotted in middle position of the MALDI 96 steel-target (Bruker Daltonics, Bremen, Germany) and dried under a fume hood. Afterwards, BTS was overlaid with 1 µl alpha-cyano-4-hydroxy-cinnamic acid (CHCA) (see chapter 3.4.3.1) and dried as well. The measurement was realized as suggested by the manufacture Bruker Daltonics. BTS was used till the score value of this standard was below 2.4.

3.4 Optimal sample preparation for MALDI-TOF MS

3.4.1 Strains

Four different yeast strains (Table 3.1) were used to optimize the sample preparation on a MALDI 96 steel-target for MALDI-TOF MS analysis, which belong either to the genus of *Saccharomyces* and to non-*Saccharomyces* yeasts.

3.4.2 Cultivation

The yeasts TMW 3.0250, TMW 3.0275 and TMW 3.0409 (Table 3.1) were taken from the cryogenic stocks and were grown on YPD (see chapter 3.2.1.1) at 30 °C for 2 days. A single colony from the agar plate was picked and inoculated on YPD agar plates for 2 days at 30 °C. From the second plate (working plate), a colony was used to inoculate 15 ml YPD media in a 50-ml Erlenmeyer flasks (Zefa, Harthausen, Germany) closed with cotton plugs (Zefa, Harthausen, Germany) and incubated aerobically at 30 °C for 18 h on a WisML02 rotary shaker at 180 rpm (Witeg Labortechnik GmbH, Wertheim, Germany).

Dekkera bruxellenis TMW 3.0600 (Table 3.1) was taken from the cryogenic stock and was grown on YPD at 30 °C for 4 days. A single colony from the agar plate was picked and inoculated on YPD agar plates for 4 days at 30 °C. From the working plate, one colony was used to inoculate 15 ml YPD media (pH 5.0; see chapter 3.2.1.1) in 50-ml Erlenmeyer flasks closed with cotton plugs and aerobic incubated at 30 °C for at least 36 h on a rotary shaker at 180 rpm.

After incubation, the samples were prepared as stated in chapter 3.4.3.

3.4.3 Sample preparation

A volume of 1 ml of each sample was centrifuged (2 min, 13.000 rpm) twice and supernatant removed. The yeast pellet was subsequently resuspended in 300 µl ultra-pure water (J.T. Baker, Denventer, the Netherlands) followed by 5 min mixing. Afterwards, 900 µl absolute ethanol (VWR, Fontenay-sous-Bois, France) was added to the suspension and mixed for the same time. After centrifugation, the supernatant was discarded and the pellet air dried for 30 min. Subsequently, proteins were extracted by addition of 50 µl 70% formic acid (Sigma Aldrich, Darmstadt, Germany) and 5 min mixing. 50 µl acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added and the sample likewise mixed.

After centrifugation (2 min, 13.000 rpm), the prepared samples were used step by step for different sample preparations on the MALDI 96 steel-target (Bruker Daltonics, Bremen, Germany). First, five different matrix solutions were tested (see chapter 3.4.3.1). Using the best of the five matrix solution, the optimal ratio of sample to matrix was identified (see chapter 3.4.3.2). Finally, four different overlay techniques were examined (see chapter 3.4.3.3).

After every preparation method, the MALDI steel-target was introduced to the MALDI-TOF MS and the sub-proteomic spectra were recorded. Chapter 3.4.4 describes the MALDI-TOF MS settings.

3.4.3.1 Matrix solutions

Commercially available matrix substances were tested with regard to their suitability to record sub-proteomic spectra of yeasts. All matrices used are listed in Table 3.11 and were prepared as described below Table 3.11.

Table 3.11: Matrices tested. Compounds (chemicals) are listed within the table, which includes the provider and purity (if available); last column shows the concentration of each compound in mg/ml.

Compounds	**Provider & Purity**	**Concentration [mg/ml]**
alpha-cyano-4-hydroxycinnamic acid (CHCA)	Sigma-Aldrich, Darmstadt, Germany; matrix solution for MALDI-TOF MS; >99% (HPLC)	10
Sinapinic acid (SA)	Bruker Daltonics, Bremen, Germany	10
2,5-Dihydroxybenzoic acid (2,5-DHB)	Bruker Daltonics, Bremen, Germany	10
2,5-Dihydroxyacetophenone (2,5-DHAP)	Sigma-Aldrich, Darmstadt, Germany	15.2
3-Hydroxypicolinic acid (3-HPA)	Sigma-Aldrich, Darmstadt, Germany; >99%	40

The matrices CHCA, SA and 2,5-DHB were prepared with a final concentration of 10 mg/ml and were solved in OS (see chapter 3.2.2.1).

2,5-DHAP was prepared with a final concentration of 15 mg/ml. The matrix was solved in absolute ethanol (VWR, Fontenay-sous-Bois, France) and 18 mg/ml di-Ammoniumhydrogencitrat (Carl Roth GmbH & Co. KG, Karlsruhe, Germany; >98%) at a ratio of 3:1.

40 mg of 3-HPA were mixed in 900 µl of 50% acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 1 min and shall not be diluted completely. This step was followed by adding 100 µl 100 g/l di-Ammoniumhydrogencitrat and a final mixing was done.

After centrifugation of the samples (see chapter 3.4.3), 1 µl of the supernatant was spotted on a MALDI 96 steel-target, dried in a fume hood and overlaid with 1 µl matrix solution (Table 3.11) and dried as well. Five biological replicates with technical triplicates were recorded to get 15 spectra per strain and per matrix substance.

3.4.3.2 Ratio of matrix to sample

Five different ratios of matrix to sample were tested with the final used matrix of chapter 3.4.3.1. Table 3.12 lists all ratios of matrix to sample.

Table 3.12: Ratios of matrix to sample in µl

Matrix (µl)	Sample (supernatant) (µl)
1	2
1	1.5
1	1
1.5	1
2	1

After the sample preparation, different volumes of the supernatant (Table 3.12) were spotted on the MALDI 96 steel-target and dried under the fume hood. The volume of matrix was laid over the corresponding spot of each sample and dried as well. Five biological replicates with technical triplicates were recorded to get 15 spectra per strain and per matrix / sample ratio.

3.4.3.3 Overlay techniques

With the tested matrix and ratio of matrix to sample four different overlay techniques were tested: a) a volume of sample was spotted on the target, dried in a fume hood and finally overlaid with matrix (sample-matrix); b) matrix was spotted on the target, dried in a fume hood and overlaid with sample material (matrix-sample); c) first matrix on the target, dried in a fume hood, overlaid it with sample material, dried in a fume hood and finally overlaid with matrix again (matrix-sample-matrix); d) sample and matrix are mixed on the same ratio before

application on the target (sample/matrix). Five biological replicates with technical triplicates were recorded to get 15 spectra per strain and per overlay technique.

3.4.4 MALDI-TOF MS configurations

Mass spectra were generated by a Microflex LT MALDI-TOF MS (Bruker Daltonics, Bremen, Germany), which was equipped with a nitrogen laser (λ = 337 nm) at a laser frequency of 60 Hz operating in linear positive ion detection mode under MALDI Biotyper 3.0 Realtime classification (RTC) (Bruker Daltonics, Bremen, Germany) and FlexControl 3.4 (Bruker Daltonics, Bremen, Germany), which is a package of Bruker Compass 1.4 (Bruker Daltonics, Bremen, Germany). The mass range covers an area from 2 kDa to 20 kDa at a voltage of 20.0 kV (ion source 1), 16.80 kV (ion source 2), 6.00 kV (lens) and 2939 kV (linear detector). The laser intensity was adjusted between 35 to 40% with an offset of 48%. For each spectrum, 240 single spectra, recorded by 40-shot steps from random positions of the target spot, were summarized to one main spectrum.

3.4.5 Data analysis

Each raw spectrum was converted using FlexAnalysis software (Version 3.4; Bruker Daltonics, Bremen, Germany) to a text file and an octave-software was applied to achieve a pre-processing, which was realized according to Usbeck et al. (2013). Based on a sharedroot computer cluster (ATIX; http://opensharedroot.org) using a self-tailored MASCAP (Mantini et al., 2007, Mantini et al., 2010), which was implemented in octave software, all exported mass spectra of each sample were pre-processed by substracting the baseline, smoothing and normalizing signal intensities (Usbeck et al., 2013). A maximum tolerance of 600 ppm of the mass to charge deviation was accepted for the comparison (Fushiki et al., 2006, Usbeck et al., 2013, Wang et al., 2006). The pre-processed mass spectra were used for peak detection by picking peaks which show the highest intensity among their nearest points.

After the pre-processing step, a peak detection rate (PDR) was performed, which expressed the ratio between number of spectra containing the considered peak and the total number of analyzed spectra (Mantini et al., 2007, Usbeck et al., 2013). By considering the number of spectra, it was possible to compare the different sample preparation to each other with respect to their efficiency and receive the maximal possible number of reproducible peaks in the mass spectrum as well assigned to five intervals according to their peak intensity (Kern et al., 2013, Usbeck et al., 2013). Furthermore, the mass spectra were visualized of the preparation

techniques illustrating differences regarding various applications of matrices and overlay techniques in the mass range of 2000 m/z to 13000 m/z similar to Schott et al. (2016).

3.5 Classification of yeasts of the genus *Saccharomyces* by MALDI-TOF MS

3.5.1 Strains

Yeast strains, which are used within the brewing environment for top-fermenting and bottom-fermenting beer styles as well as *S. cerevisiae* var. *diastaticus* (Table 3.2) were analyzed. Furthermore, non-brewing *S. cerevisiae* strains (Table 3.3) and wild isolates (Table 3.4) were classified likewise by MALDI-TOF MS.

3.5.2 Cultivation of yeasts for bioinformatic analysis

The inoculation of yeast strains was done and additionally modified according to Usbeck et al. (2014). Yeasts were taken from the cryogenic stocks (see chapter 3.3.1) and were grown on YPD agar plates (see chapter 3.2.1.1) at 30 °C for 2 to 3 days. A single colony from the agar plate was picked and inoculated on YPD agar plates at 30 °C for 2 to 3 days. From the second plate (working plate), a colony was used to inoculate 15 ml YPD media in 50-ml Erlenmeyer flasks (Zefa, Harthausen, Germany) closed with cotton plugs (Zefa, Harthausen, Germany) and aerobically incubated at 30 °C overnight on a WisML02 rotary shaker with 180 rpm (Witeg Labortechnik GmbH, Wertheim, Germany). After the incubation in YPD media, 1% of the pre-culture was propagated in another 50-ml Erlenmeyer flask containing 15 ml of YPD media and incubated at 30 °C for 18 h on a WisML02 rotary shaker at 180 rpm. The working plate was used for 4 to 5 days. After incubation, the samples were prepared according to the optimized sample preparation (see chapter 4.1) for MALDI-TOF MS analysis.

3.5.3 Cultivation of yeasts for database creation

Yeasts were taken from the cryogenic stocks and were grown on YPD agar plates at 30 °C for 2 to 3 days. A single colony from the agar plate was picked and inoculated on YPD agar plates for 2 to 3 days at 30 °C. From the second plate (working plate), a colony was used to inoculate 15 ml YPD media in 50-ml Erlenmeyer flasks closed with cotton plugs and aerobic incubated at 30 °C for 18 h on a WisML02 rotary shaker with 180 rpm. After incubation, the samples were prepared according to the optimized sample preparation for MALDI-TOF MS analysis (see chapter 4.1.1). Furthermore, database entries were recorded for every yeast strain for the establishment of a database of the genus *Saccharomyces* in MALDI-Biotyper 3.0 (Bruker Daltonics, Bremen, Germany).

3.5.4 Analyzing yeasts of the genus *Saccharomyces* by MALDI-TOF MS

Mass spectra were generated by a Microfelx LT MALDI-TOF MS (see chapter 3.4.4).

For each database entry, the extraction of a yeast strain was laid on vertical target columns, for example a specific volume of sample and matrix (see chapter 4.1.1) was spotted per position from A1 to H1 and measured 3 times to obtain 24 spectra per strain. As suggested by Bruker Daltonics the evaluation of the main spectra (MSP) was performed by FlexAnalysis 3.4 (Bruker Daltonics, Bremen, Germany). Subsequently, the evaluated spectra were loaded in the in-house database of the genus *Saccharomyces* in MALDI-Bioytyper 3.0

For comparison of tested strains to their database entries and bioinformatic analysis, ten biological replicates along with technical triplicates were recorded on ten different days to obtain 30 spectra per strain. The quantity of replicates covers the variety of peak intensities and mass to charge deviation (600 ppm).

3.5.5 Comparison of tested strains to their database entries

The 30 single spectra of each yeast strain were compared offline with the established database by the MALDI Biotyper 3.0 software. The first matches were taken into account to analyze strain or ecotype (application type) hits. These results were compared to the actual ecotype / application of each yeast (Table 3.2) based on the information of the yeast supplier.

3.5.6 Bioinformatic analysis

The exportation and pre-processing of the recorded spectra was realized as shown in chapter 3.4.5. The data analysis was performed based on similarity calculations like Euclidean distance or normalized dot-product for the comparison of recorded mass spectra. Eight different approaches were used to analyze the sub-proteome fingerprints of yeasts of the genus *Saccharomyces*.

The first one was to compare a small amount of brewing yeasts strains including 19 reference yeast strains of *S. cerevisiae* as well 15 strains, which were classified to a major beer style. The mass spectra of the 34 brewing yeast strains were compared to each other by a high-throughput multidimensional scaling (HiT-MDS) (http://dig.ipk-gatersleben.de/hitmds/hitmds.html) with Voronoi calculation.

The second approach was to expand the number and compare the mass spectra of 60 brewing yeast strains to each other by a HiT-MDS (http://dig.ipk-gatersleben.de/hitmds/hitmds.html)

and hierarchical cluster analysis including *S. cerevisiae*, *S. cerevisiae* var. *diastaticus* and *S. pastorianus*.

The third approach was to compare 39 of the top-fermenting and *S. cerevisiae* var. *diastaticus* strains by HiT-MDS with Voronoi calculation and a discriminant analysis of principal components (DAPC), because of the variety within this strains.

Afterwards, all brewing strains, which belong to the beer styles Alt beer or Kölsch were summarized to one beer style namely German Alt-Kölsch (Goncalves et al., 2016).

The fifth approach was to analyze six top-fermenting *S. cerevisiae* yeast strains, which are applied or isolated in different origins in Belgium, Africa or America: TMW 3.0673 (California Ale), TMW 3.0864 (Lambic, Belgium), TMW 3.0865 (Belgian beer), TMW 3.0866 (Ale from wheat malt), TMW 3.0867 (Opaque, South Africa) and TMW 3.0937 (Wit beer). 20 top-fermenting reference strains (five wheat beer strains, five Ale strains, five German Alt-Kölsch and five *S. cerevisiae* var. *diastaticus* strains) were taken and compared to the six strains by a HiT-MDS.

Afterwards, brewing and non-brewing yeast strains were compared to each other as stated in chapter 3.5.5.

Wild isolates were compared to 30 reference strains of top-fermenting, bottom-fermenting beer styles as well as *S. cerevisiae* var. *diastaticus* by hierarchical cluster analysis.

Finally, all 89 yeasts were visualized within a hierarchical cluster analysis showing the divergence to their application potential.

An MDS was used, which is a data processing method suitable for addressing several analytical purposes: (i) for dimension reduction of vector data, providing a nonlinear alternative to the projection to principal components; (ii) for the reconstruction of a data dissimilarity matrix of pairwise relationships in the Euclidean output space; (iii) for conversion of a given metric space, such as data compared by Manhattan distance, into Euclidean space and (iv) for dealing with missing data relationships using zero force assumption (Fester et al., 2009). It has been predominantly used as a tool for analyzing proximity data of all kinds. Most for all, MDS serves to visualize such data making them accessible to the eye of the researcher. For example, the distance between two points represent the correlation of the respective variables. As all variables are non-negatively intercorrelated, it is particularly easy to interpret this MDS configuration: The closer two points, the higher the correlation of the variables they represent (Borg et al., 2012). The visualization of relationships between different data records can be

obtained by reconstructing these relationships as pairwise distances in the usual Euclidean 2D plane or 3D space (Fester et al., 2009). A HiT-MDS is an optimized version for rapid distance reconstruction, based on correlations of distances between input and output space (http://dig.ipk-gatersleben.de/hitmds/hitmds.html). The HiT-MDS is mentioned within the dissertation work as MDS.

In order to decrease the complexity of the diagram from every brewing yeast strain, the 30 single spectra were summarized to one consensus spectrum for MDS. Summarized spectra were compared subsequently to each other for similarity and plotted in a 2D map. At the end of the calculation the reconstruction quality from 0 to 1 (1 is a prefect reconstruction) was displayed. This was performed within six approaches, but a Voronoi triangulation (Petřek et al., 2007) was performed for dividing the yeast strains in groups named to the beer styles only within the first, third, fourth and fifth classification. The Voronoi triangulation is based on a decomposition of metric space by distances between sets of points (Petřek et al., 2007), in this case beer styles, which are divided into cells each containing one focus, marked with the beer style name in capitals. It is included in octave (https://www.gnu.org/software/octave/).

Furthermore, the summarized spectra were evaluated by a hierarchical cluster analysis by an in-house software based on MASCAP (Mantini et al., 2010) within the second approach. The calculation of the cluster analysis was accomplished to weighted pair group method with averaging (WPGMA) (Gronau and Moran, 2007, Sneath et al., 1975) and a normalized dot-product, which determine the similarity between recorded mass spectra and is explained in Frank et al. (2007).

In addition, the 39 selected top-fermenting yeast strains were analyzed by DAPC using the adegenet package (2.0.1) for using RStudio software (Jombart and Collins, 2015). DAPC seeks synthetic variables, the discriminant functions, which show differences between groups as best as possible while minimizing variation within clusters (Thibaut Jombart, 2015). The raw data was transformed using principal component analysis, which is followed by k-means algorithm with increasing values of k to identify the optimal number of clusters. Different clustering solutions are compared using Bayesian Information Criterion (BIC). Ideally the optimal clustering solution should correspond to the lowest BIC and is visualized by an elbow in the curve. After choosing a number of clusters the discriminant analysis was performed to obtain a barplot of eigenvalues, and finally a scatterplot was obtained which represents the individuals as dots and the groups as inertia ellipses. Furthermore, it is possible to visualize groupings by a histogram and the main peaks responsible for the separation in a loading plot.

All single spectra of 39 yeast strains (n = 1170) were analyzed by this tool to obtain a scatterplot to visualize beer styles as inertia ellipses, histogram and loading plot.

Visualization of spectra from chosen strains were realized according to chapter 3.4.5.

For all bioinformatic analysis a mass to charge range from 2000 m/z to 20000 m/z is taken into account.

3.6 Genomic comparison

3.6.1 Strains

For the detection of DMGs, yeast strains from Table 3.2, Table 3.3 and Table 3.4 are used reflecting brewing and non-brewing applications as well as wild isolates.

3.6.2 Genome assembly, annotation and analysis

Genome data from 25 brewing yeast strains were obtained from the bioproject "PRJEB13332", generated within the scientific work of Goncalves et al. (2016) from NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB13332), and are highlighted with the respective sequence archive number (ERR) in Table 3.2. SRA data were assembled using ABySS 2.0.2 applying default settings (Simpson et al., 2009). The resulting scaffolds were size-filtered, retaining only scaffolds lager than 500 bp followed by a "reorderining process" of these scaffolds with respect to location on *S. cerevisiae* chromosomes using Mauve (v2.4.0) (Darling et al., 2004) and the complete S288c genome as a reference (Engel et al., 2014). The resulting genomes were annotated using MAKER (v2.31.9) (Cantarel et al., 2008) using ab initio gene predictions, EST (expressed sequence tags) alignment and protein alignment (https://downloads.yeastgenome.org/sequence/S288C_reference/). Annotation (functional assignment) was completed using blastp and the *Saccharomyces* Genome Database (SGD) ((Cherry et al., 2012), used database from December 2016).

3.6.3 Prediction of DMGs using BADGE

BADGE was used as described by Behr et al. (2016) and genome sequences of the 25 brewing yeasts were analyzed followed by DMG prediction. Default settings, described by Behr et al. (2016), were used applying the following modifications (Appendix 9.1), which allows the comparison on pan genome.

3.6.4 DNA extraction for PCR-screening

The yeast strains were transferred from the cryogenic storage (see chapter 3.3.1) on YPD agar plates (see chapter 3.2.1.1) and were grown at 30 °C for 2 to 3 days.

The extraction of genomic DNA was done according to Looke et al. (2011), for all yeasts mentioned above (chapter 3.6.1).

A single colony was picked and suspended in 100 µl 0.2 M lithium acetate (LiOAc) 1% sodium dodecyl sulfate (SDS) solution (see chapter 3.2.2.4). After an incubation for 10 min at 70 °C, 300 µl of absolute ethanol (VWR, Fontenay-sous-Bois, France) was added and mixed. The suspension of DNA including cell debris was spun down (3 min, 14800 rpm) and the supernatant was discarded. Subsequently, the pellet was washed with 70% ethanol. After a centrifugation for 15 s at 14800 rpm the supernatant was removed and the pellet air dried for 15 min. The pellet was dissolved in 100 µl TE-buffer (see chapter 3.2.2.3) and cell debris was spun down for 15 s at 14800 rpm.

The content of nucleic acid within the supernatant was analyzed with a NanoDrop 1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and genomic material was used for PCR-screening.

3.6.5 PCR-screening for DMGs

Primer sets (Appendix 9.2) were designed according to Behr et al. (2016) using FASTA files containing all sequences of a given DMG of the beer style wheat beer (WB). Polymerase chain reaction (PCR) was performed with the Taq DNA Core Kit 10 (MP Biomedicals) in a 25-µl master mix containing 1 µl of genomic DNA, 1x PCR buffer with MgCl, 200 µM of deoxynucleoside triphosphate, 0.5 µM of each primer (forward and reserve) and 1.25 U of Taq DNA polymerase. The PCR program compromised a denaturation at 94 °C for 2 min, followed by 32 cycles at 94 °C for 45 sec, an annealing step at 49 °C for 1 min and elongation at 72 °C for 1 min. A terminal extension was carried out at 72 °C for 5 min. PCR products were analyzed with 1% TBE (TRIS-Borat-EDTA) agarose gel electrophoresis and staining with dimidium bromide.

A DNA control was carried out on *ITS5* / *ITS4* (White et al., 1990) (Appendix 9.2) to check the presence of genomic DNA. Thereby, the preparation of the master mix was performed as described above. The PCR program was set to a denaturation at 94 °C for 5 min, followed by a 35 cycles at 94 °C for 1 min, an annealing step at 56 °C for 1 min and elongation at 72 °C for 1 min. A terminal extension was carried out at 72 °C for 5 min. The DNA control was analyzed with 1% TBE agarose gel electrophoresis and staining with dimidium bromide.

3.6.6 Data analysis

A statistical evaluation of PCR-screening results was carried out to check the quality of the evaluated DMGs according to Geissler (2016) with Spearman's rank correlation (Harrell, 2017, Wei et al., 2017) and Fisher's exact test (Fisher, 1925). Precision, sensitivity (recall), specifity, accuracy, f-measure, true positive, false positive, true negative, false positive rate and false negative rate as well as the total correct assignments by a specific DMG were calculated using a confusion matrix (Geissler, 2016).

3.7 Correlation of one DMG to a phenotypic characteristic by using a selective media

3.7.1 Strains

Six top-fermenting *S. cerevisiae* (TMW 3.0250, TMW 3.0251, TMW 3.0256, TMW 3.0257, TMW 3.0261 and TMW 3.0262) and two *S. cerevisiae* var. *diastaticus* (TMW 3.0273 and TMW 3.0274) strains were used within this study. Table 3.2 lists the information about these strains.

3.7.2 Pre-culture cultivation

Yeast strains were transferred from cryogenic stocks (see chapter 3.3) on YPD agar plates (see chapter 3.2.1.1) and were grown at 30 °C for 2 days. From the inoculated plates a single colony was picked to inoculate 15 ml YPD media in 50 ml Erlenmeyer flasks (Zefa, Harthausen, Germany) closed with cotton plugs (Zefa, Harthausen, Germany) and aerobic incubated at 30 °C for 18 h on a WisML02 rotary shaker with 180 rpm (Witeg Labortechnik GmbH, Wertheim, Germany). After incubation, the pre-cultures were prepared for the different cadmium analysis.

3.7.3 Cd_2SO_4 plating test

YPD agar plates containing 5 µM of the additive Cd_2SO_4 were prepared as mentioned in chapter 3.2.1.1.

Based on the pre-cultures, a dilution row was prepared from 10^0 to 10^{-5} with a Ringer-solution (see chapter 3.2.2.5) in 1.5-ml safe seal micro tubes (Sarstedt, Nürnbrecht, Germany). From every dilution 10 µl were spread on YPD agar plates (control) and YPD 5 µM cadmium agar plates as droplets. The droplets were spread as a pyramid, which is illustrated in Figure 3.1 and explains the preparation for every strain. After the plates were prepared, the droplets were air dried under the sterile bench and incubated for 2 days at 30 °C.

For every strain three biological replicates with technical triplicates were prepared to get nine plates for control and nine cadmium plates. After the incubation, pictures were recorded with the Doc-IT Imaging Station (UVP, LLC, Upland, Ca, USA) and the growth behavior was analyzed to achieve a visual evaluation.

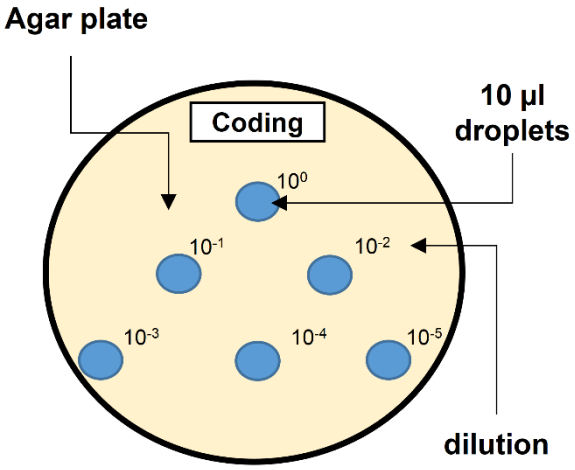

Figure 3.1: Preparation of the platting test with droplets. A coding was done for a simple labeling of the plates and assignment of the strains.

3.7.4 Tolerance of yeast strains to different Cd_2SO_4 concentration

Pre-cultures were prepared for every strain as described above (see chapter 3.7.2).

The tolerance to different Cd_2SO_4 concentration were performed in microtest 96 well plates (sterile; Sarstedt, Nürnbrecht, Germany) containing 270 µl/well YPD and a decreasing concentration of Cd_2SO_4 (maximum concentration 5 mM and lowest 0.005 µM). A 115 mM stock solution Cd_2SO_4 was used to prepare YPD media with a concentration of 5 mM Cd_2SO_4. 300 µl of the solution was transferred in a well (A1) and B1 to G1 contained all 270 µl of YPD. The next step was to perform a 1:10 dilution from A1 to B1 to get the concentration of 500 µM. 30 µl of A1 were transferred to B1 and mixed well. This was carried out for all wells except the last row (H1 to H12), which served as positive control (only YPD).

Three microtest plates were prepared and to two of them were inoculated with 1% of the pre-cultures. Finally, all wells were overlaid with 30 µl sterile paraffin oil (Sigma-Aldrich, Darmstadt, Germany) to avoid dehydration and condensation. Microtest plates were incubated statically for four days at 30 °C. Pictures were taken and the optical density was measured with a plate reader (Sunrise remote, Tecan GmbH, Crailsheim, Germany) at 24 h-intervals. The following settings were used for the plate reader: optical density (OD) 590 nm; shaking for 40 s before plate reading; double orbital. The test was performed with three biological replicates per strain, as well as the third plate that was used as negative control.

3.8 Terms and definitions

The following term and definition will be used continuously within the results and discussion chapters.

"German Alt-Kölsch": Goncalves et al. (2016) proposed this name for a top-fermenting beer style. At this, beer styles of Alt and Kölsch are summarized to one term.

"Diagnostic marker gene (DMG)": Geissler (2016) explained this term as a gene, capable of differentiating two strains or groups

"Ale": In the current study, the term "Ale" defines one top-fermenting beer style and not in general the top-fermenting group, which is common e.g. in North America.

"Lager": Lager defines the beer style produced by bottom-fermenting beer production.

4 Results

4.1 Optimal sample preparation

Different sample preparations on the MALDI 96 steel target were tested for an optimized recording of spectra by MALDI-TOF MS. The optimized preparation was used for the further characterization of yeast of the genus *Saccharomyces* and as well the establishment of a MALDI-TOF MS database. This involved a variation of matrices (see chapter 3.4.3.1), ratios of matrix to sample (see chapter 3.4.3.2) and overlay techniques (see chapter 3.4.3.3). To acquire reproducible and reliable spectra four different yeasts (Table 3.1) were used, which were cultured as described in chapter 3.4.2: *Saccharomyces cerevisiae* (TMW 3.0250), *Saccharomyces pastorianus* (TMW 3.0275), *Saccharomycodes ludwigii* (TMW 3.0409) and *Dekkera bruxellensis* (TMW 3.0600). Five biological replicates with technical triplicates were recorded by MALDI-TOF MS for every strain and method.

Five variations of matrices compounds were tested for the optimal sample preparation (see chapter 3.4.3.1) and the evaluation of the recording of spectra for the reference strains is observed in Table 4.1.

Table 4.1: Evaluation of matrices variation. TMW = Technischen Mikrobiologie Weihenstephan; CHCA = alpha-cyano-4-hydroxycinaminic acid; SA = sinapinic acid; 2,5-DHB = 2,5-Dihydroxybenzoeic acid; 2,5-DHAP = 2,5-Dihydroxyaceto phenon; 3-HPA = 3-Hydroxy picolinic acid; ++ = very good recording of spectra; + = good recording of spectra; - = no reliable and reproducible recording of spectra

TMW	CHCA	SA	2,5-DHB	2,5-DHAP	3-HPA
3.0250	++	+	-	-	-
3.0275	++	+	-	-	-
3.0409	++	+	-	-	-
3.0600	++	+	-	-	-

Table 4.1visualizes the automated and manual recording of raw spectra by MALDI-TOF MS with the general configurations (see chapter 3.4.4). The application of the matrix CHCA and SA produced reliable and reproducible spectra, but there were no measurements with the matrix 2,5-DHB, 2,5-DHAP and 3-HPA. For further comparisons the mass spectra of CHCA and SA are illustrated in Figure 4.1.

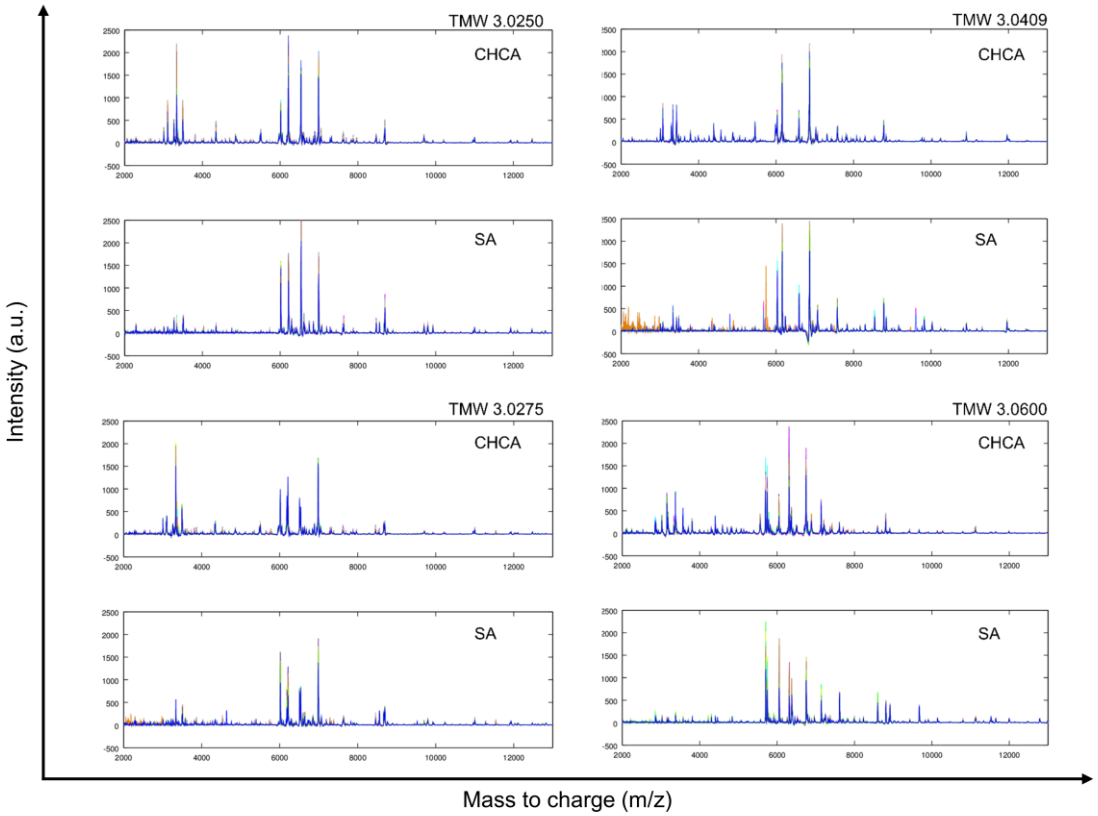

Figure 4.1: Mass spectra of all reference strains to the variation of the reliable and reproducible matrices. Y-axis represent the intensity of the recorded peaks; x-axis show the mass to charges from 2000 m/z to 13000 m/z; TMW = Technische Mikrobiologie Weihenstephan; CHCA = alpha-cyano-4-hydroxycinaminic acid; SA = Sinapinic acid; Da = Dalton; n = 15

Figure 4.1 shows that between the mass to charge areas of 2000 m/z to 5000 m/z are the highest differences. Using the matrix CHCA there are more numerous and more obvious peaks, which are not measured with SA. In the case of strain TMW 3.0409 it is possible to record more reproducible spectra with the matrix CHCA than using SA. This observation was detected in all four strains.

Because of the reproducible recording of spectra and the visible recording of more spectra the matrix alpha-Cyano-4-hydroxycinnamic acid (CHCA) was chosen for further experiments.

Using CHCA a variation of five different ratios of matrix to sample volume (Table 3.12) were tested and the results are displayed in a bar chart including the number of peaks (Figure 4.2).

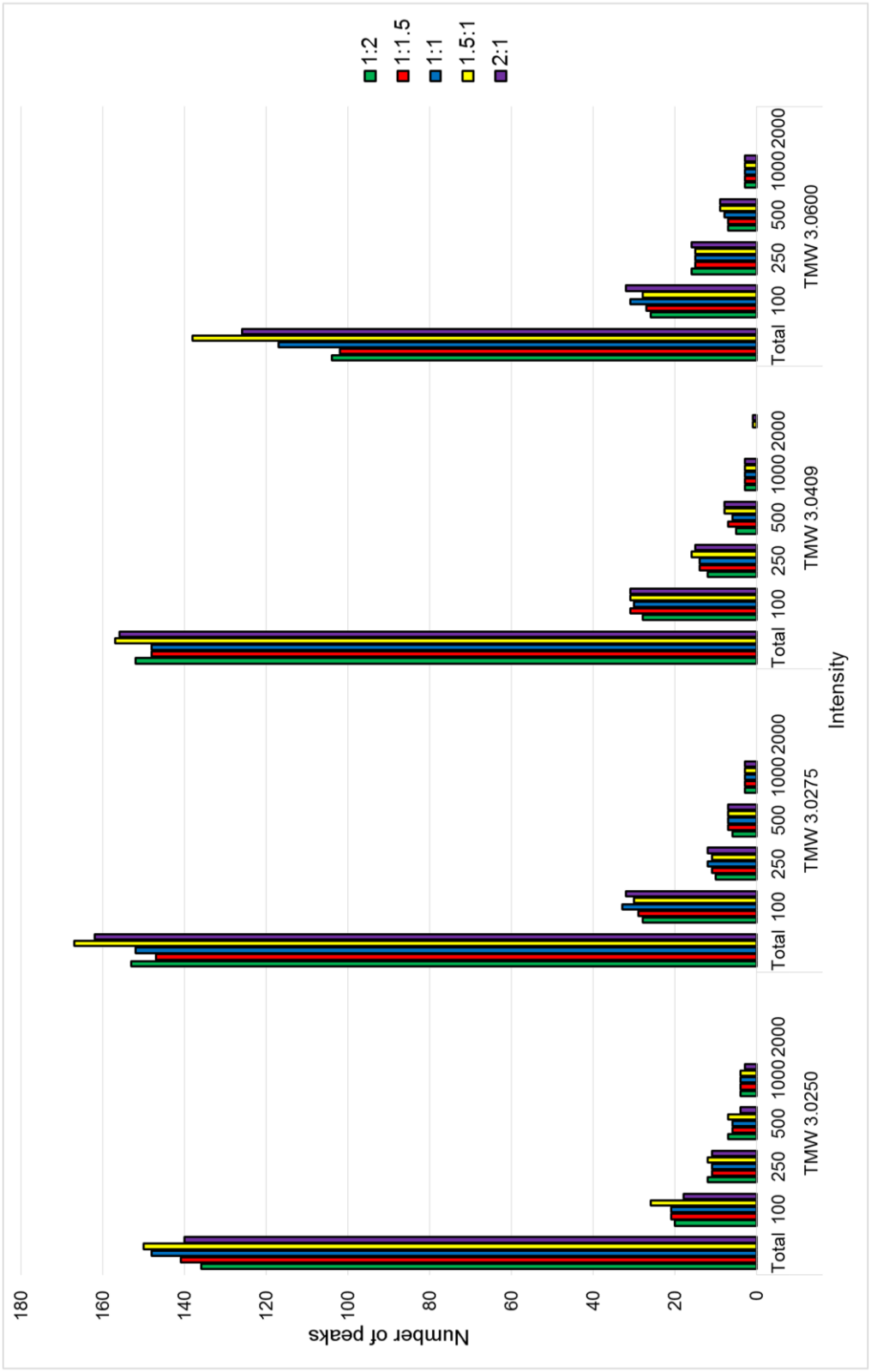

Figure 4.2: Number of peaks for the variation of ratios between matrix and sample volume for four yeast strains. X-axis shows the different peak intensities from 100 to 2000, including the total count of peaks. Y-axis presents the number of peaks for each ratio and strain. Different colors symbolize the ratios tested within the sample preparation: green = 1 µl matrix to 2 µl sample; red = 1 µl matrix to 1.5 µl sample; blue = 1 µl matrix to 1 µl sample; yellow = 1.5 µl matrix to 1 µl sample; purple = 2 µl matrix to 1 µl sample

The influence of the ratios between matrices to sample is shown in Figure 4.2 as well as the legend representing the varieties of the ratios. For every reference strain, the ratio of 1.5 µl matrix solution to 1 µl sample material resulted in the highest number of peaks in total. However, in case of this proportion a high signal-to-noise ratio was recorded and therefore, the area of 250 to 2000 was observed to make any differences between those ratios. No significant differences are found in all reference strains, because of this result a matrix to sample ratio of 1:1 was chosen as optimal sample preparation.

The next step was to analyze four different overlay techniques with the current matrix and ratio of matrix to sample. Figure 4.3 represent the mass spectra of all reference strains prepared with the overlay techniques described in chapter 3.4.3.3.

The mass spectra comparison illustrates that the use of different overlay techniques has a high impact on the quality and reproducibility of recorded spectra by MALDI-TOF MS. If the matrix was overlaid first on the target followed by the sample material, the recording of spectra could not be achieved or wasn't reproducible. The same is true for the case of a mix of matrix and sample that is laid on the target. These results are very well observed within the spectra of TMW 3.0600 in b) and d). In return, the overlaying of sample material and then matrix solution or the sandwich method (matrix-sample-matrix) resulted in reproducible spectra.

For the optimal sample preparation the overlay technique of sample-Matrix was selected, because it allowed for reproducible spectra while being time saving as well as a matrix solution saving compared to the sandwich method.

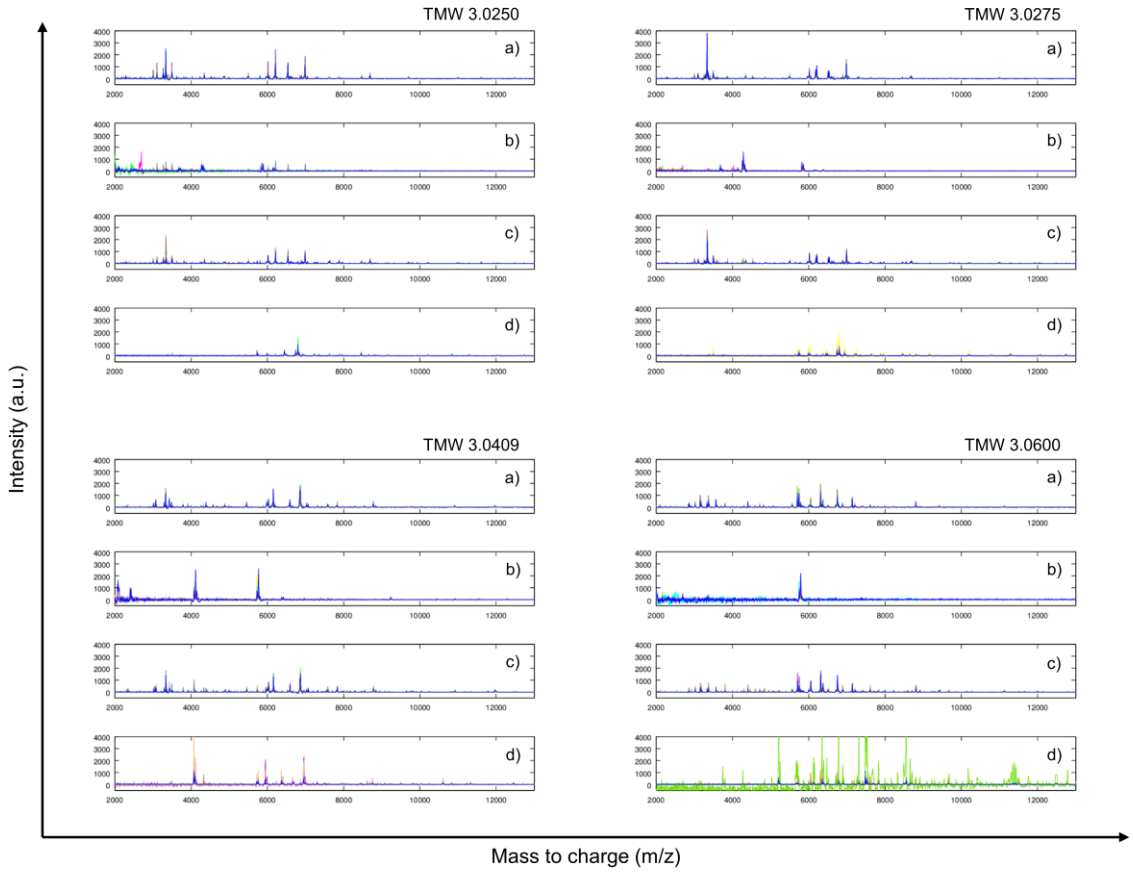

Figure 4.3: Mass spectra of four different overlay techniques. For every method and strain the recorded 15 single spectra were summarized to one mass spectra; **a)** = sample / matrix; **b)** = matrix / sample; **c)** matrix / sample / matrix; **d)** Mixing of sample and matrix; y-axis represent the intensity of the recorded peaks; x-axis show the mass to charges from 2000 m/z to 13000 m/z; TMW = Technische Mikrobiologie Weihenstephan;

4.1.1 Protocol for sample preparation of yeasts for MALDI-TOF MS analysis-

After the testing of different preparation methods, the sample preparation of chapter 3.4.3 was expanded with the corresponding results of this chapter. For all MALDI-TOF MS measurements applied in various studies, the samples were prepared as follows.

After liquid incubation, a volume of 1 ml of each sample was centrifuged (2 min, 13.000 rpm) twice and the supernatant removed. The yeast pellet was subsequently resuspended in 300 µl ultra-pure water (J.T. Baker, Denventer, the Netherlands) by pipetting, which was followed by 5 min of mixing. Afterwards, 900 µl absolute ethanol (VWR, Fontenay-sous-Bois, France) was added to the suspension and mixed for the same time. After centrifugation (2 min, 13.000 rpm), the supernatant was discarded and the pellet air dried for 30 min. Subsequently, proteins were extracted with 50 µl 70% formic acid (Sigma Aldrich, Darmstadt, Germany) and 5 min mixing. 50 µl of acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added and the sample mixed again for the same time. After centrifugation (2 min, 13.000 rpm), 1 µl of the

supernatant was spotted on a MALDI 96 steel-target, dried in a fume hood and overlaid with 1 µl of CHCA-solution (Sigma Aldrich, Darmstadt, Germany; see chapter 3.4.3.1) and dried as well.

Finally, the mass spectra were recorded using a Microflex LT MALDI-TOF MS (see chapter 3.4.4).

4.2 Classification of yeast of the genus *Saccharomyces* with respect to their application by MALDI-TOF MS

In total, 2670 single spectra were recorded for the classification of yeasts with respect to their application potential using the optimized sample preparation (see chapter 4.1.1) by MALDI-TOF MS. The following application types were considered: top-fermenting beer styles, wine, sparkling wine, special wine, liquor, bakery, laboratory, bottom-fermenting (flocculent and powdery) and *S. cerevisiae* var. *diastaticus*.

Eight approaches were realized for the classification of the recorded spectra: I) Classification of brewing yeast strains *S. cerevisiae*; II) Classification of 60 brewing yeast strains of the genus *Saccharomyces*; III) Consideration of 39 top-fermenting *S. cerevisiae* strains including *S. cerevisiae* var. *diastaticus*; IV) Summarizing Alt beer and Kölsch strains to the beer style German Alt-Kölsch; V) Classification of six brewing yeast strains; VI) Differentiation between brewing and non-brewing yeasts; VII) Classification of wild yeasts; VIII) Divergence of *Saccharomyces* with respect to their application potential

In addition, database entries were created for the establishment of an in-house MALDI Biotyper database including yeasts of the genus *Saccharomyces* of different application types.

4.2.1 Classification of brewing yeast strains of *S. cerevisiae*

19 top-fermenting brewing yeast strains of *S. cerevisiae* (Usbeck, 2016) were considered as reference strains of various beer styles: five wheat beer strains, five Ale strains, five Alt beer strains, two Kölsch strains and two strains of *S. cerevisiae* var. *diastaticus*. The tested yeast strains were assigned to different beer styles after the comparison to their own database entries (Figure 4.4). The comparison revealed a correct classification of all wheat beer strains to the wheat beer style, which are used according to practical experience for the wheat beer production. A differentiation between Alt beer and Kölsch strains couldn’t be achieved and is displayed to the total hit rates on beer styles in Table 4.2 as well within the mix up between those strains in Figure 4.4. A correct classification on strain level has only been partly achieved and is seen for three strains of the Ale style and for both *S. cerevisiae* var. *diastaticus* strains to a 100% hit rate. In total a 49% strain level identification could be achieved, whereas a correct match of 85% compared with the practical experience was attained on beer style level (Table 4.2).

	Wheat beer					Ale					Alt beer					Kölsch		Diastaticus	
	250	251	253	255	258	260	261	262	338	339	252	257	259	336	337	256	332a	273	274
250	**37**	20		40	3														
251		**37**	27	33	3														
253	3	37	**50**	7	3														
255	20	7	33	**30**	10														
258	20	40	20	20															
260						**53**				3		10	13	10	3	7			
261						80	**7**					7		3		3			
262								**100**											
338									**100**										
339										**100**									
252											**17**	63	10	3	3		3		
257												**33**	10	27	27	3			
259											3	17	**30**	17		23	10		
336											10	3		**43**		37	7		
337								3	3			20	3	20	**43**				
256		10									3	13		30		**37**	13		
332a												47	7	10	13	7	**17**		
273																		**100**	
274																			**100**

Figure 4.4: Comparison of recorded reference strains to their own database entries. The hit rates (%) of the tested strains are displayed to the database entries and show whether hits on strain (green squares) or ecotype-level (all yeast strains of an appointed beer style); a hit rate of 100% displays a total strain identification; database entries are displayed on the top of the table with ID of the Technischen Mikrobiologie Weihenstephan (TMW) and organized by beer styles, as indicated above; all recorded strains with the ID of TMW are shown on the left side; 30 spectra of each strain were compared to the database entries; 250 = TMW 3.0250

Table 4.2: Tabular list of the hit rates on the different beer styles and the total strain level identification. Sum displays all recorded spectra of a specific beer style or the total count of recorded spectra for the 19 strains; the third column represent all correct assignments; fourth column all mismatches; last column displays the hit rate (%) on the different beer styles, total hit rate on all beer styles and the hit rate on strain level identification

Beer style	Sum	Correct	Wrong	Hit rate (%)
Wheat beer	150	150	0	100
Ale	150	133	17	89
Alt beer	150	121	29	81
Kölsch	60	22	38	37
S. cerevisiae* var. *diastaticus	60	60	0	100
All beer styles	570	486	84	85
Strain	570	280	290	49

The findings of Figure 4.4 and Table 4.2 are displayed in Figure 4.5 by a multidimensional scaling (MDS) with Voronoi triangulation (blue line) containing the 19 brewing yeasts. The mean spectra of each strain was compared and ordered according to their similarity. The overlap between Alt beer and Kölsch strains from Figure 4.4 is observed in the center of the MDS. Furthermore, the groups of wheat beer (left part of the MDS), Ale (upper part of the MDS) and *S. cerevisiae* var. *diastaticus* (right part of the MDS) distinguish from each other.

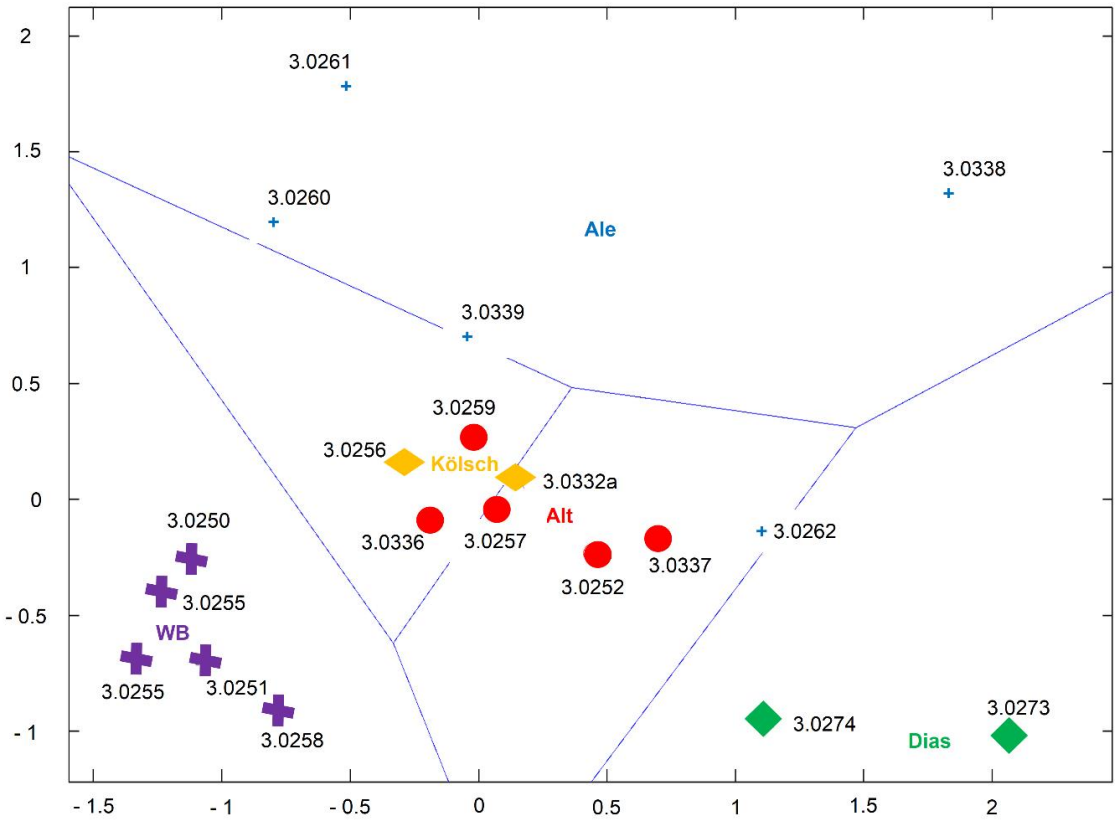

Figure 4.5: MDS of 19 reference strains of different beer styles including a Voronoi triangulation. Every mark represents the mean spectrum of 30 single spectra of each strain; Coding according to Technsiche Mikrobiologie Weihenstephan (TMW); x- and y-axes present the distances between each point; WB = wheat beer, purple crosses ; Alt = Alt beer, red circle; Kölsch, yellow rhombus; Ale, blue crosses, Dias = *S. cerevisiae* var. *diastaticus*, green diamond; ans = 0.94220

Reference strains were used as a first database to match new brewing yeast strains to their application potential. As a result of that, Figure 4.6 displays the comparison of the 15 yeast strains to the 19 reference strains. One strain is counted to one beer style if more than 50% of the recorded spectra are matched to one group. A first comparison revealed a correct assignment of all strains applied in the industry for wheat beer as well to *S. cerevisiae* var. *diastaticus* (Figure 4.6). Two out of four Ale strains were correctly matched to their application type. TMW 3.0254 was mismatched to the Ale style and is actually applied for the production of the Kölsch beer style. Furthermore, one strain was classified wrongly to Alt beer style instead to the Ale style according to the brewer's experience, namely TMW 3.0668.

	Wheat beer					Ale					Alt beer					Kölsch		Diastaticus	
	250	251	253	255	258	260	261	262	338	339	252	257	259	336	337	256	332a	273	274
254						17	83												
332n											23	7	10		23	3	33		
343	3	23	17	13	13					3	3	13	7	3					
624																		20	80
625																		100	
634											47	3		3	3		43		
635		10										23	7	20	3	33	3		
636						87	13												
637						83	17												
666	3	7		90															
667		57	37	3			3												
668											10	10	7	3	27	7			
669		17	63	10	10														
674	23	33	27	17															
675											27	3		10	23		37		

Figure 4.6: Matching of 15 unknown brewing yeast strains to 19 reference strains. Left side displays all yeast strains of an unknown beer style and on the top are 19 reference brewing yeast strains of different top-fermenting beer styles; results are displayed in percent and 30 recorded single spectra per strain are compared to the database; TMW = Technische Mikrobiologie Weihenstephan; the used coding of yeast strains is a shortened coding based on the TMW coding, e.g. 250 ≙ 3.0250; n = 1020

A multidimensional scaling (MDS) of the 34 top-fermenting *S. cerevisiae* is shown in Figure 4.7 and highlighted those strains which are matched wrongly on the basis of brewer's experience. TMW 3.0254 is clearly assigned to the Ale group on the left part of the MDS and TMW 3.0668 is placed in the center of the Alt beer cluster. A differentiation between Alt beer and Kölsch strains was hardly achieved and is displayed within the Figure 4.6 as well Figure 4.7. Wheat beer strains still distinguish from all other top-fermenting beer styles as well the four strains of *S. cerevisiae* var. *diastaticus* formed an own group on the right side of the MDS (Figure 4.7). The eight Ale strains show a high degree of dissimilarity with regard to their sub-proteome. In total, 11 out of 15 (approx. 73%) brewing yeast strains could be classified correctly to the practical experience.

Considering all 34 *S. cerevisiae* (var. *diastaticus*) strains, 28 out 34 yeasts (approx. 82%) could be classified correctly to the practical experience.

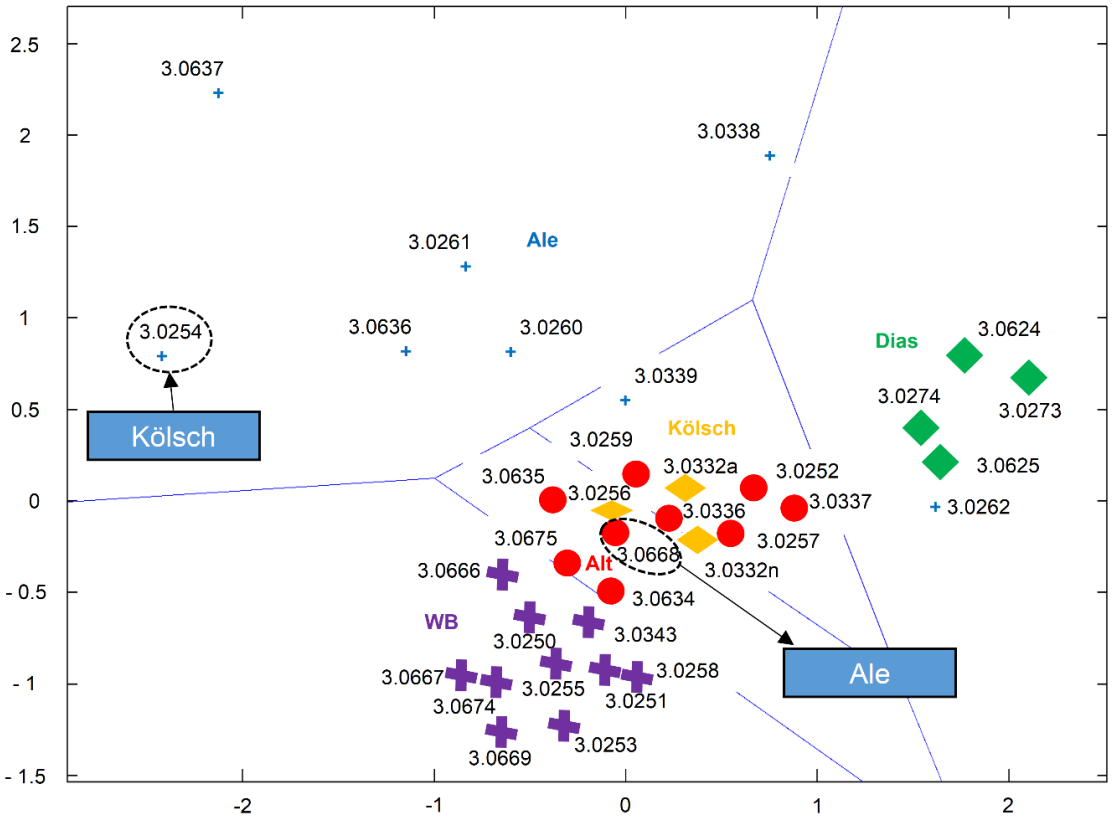

Figure 4.7: Multidimensional scaling of 34 brewing yeast strains of major beer styles to the MALDI-TOF MS classification including highlighted strains, because of mismatch. Coding to Technsiche Mikrobiologie Weihenstephan (TMW); x- and y-axis present the distances between each point; WB = wheat beer, purple crosses; Alt = Alt beer, red circle; Kölsch, yellow rhombus; Ale, blue crosses, Dias = *S. cerevisiae* var. *diastaticus*, green diamond; ans = 0.96201

15 new MALDI Biotyper database entries were created and added to the 19 reference strains of major beer styles as well used for the common analysis.

4.2.2 Expanded classification of brewing yeast strains of the genus *Saccharomyces*

For further differentiation of the brewing yeasts among each other 21 bottom-fermenting *S. pastorianus* strains (divided by flocculation behavior) were recorded by MALDI-TOF MS. The 19 top-fermenting *S. cerevisiae* and *S. cerevisiae* var. *diastaticus* strains were expanded with the unknown samples of chapter 4.2.1 and matched to beer styles according to the MALDI classification. Furthermore, three strains of *S. cerevisiae* var. *diastaticus* were added to achieve a range within the variety level. The same was done for *S. cerevisiae* when one Ale style strain and one Kölsch style strain was added. In total, 60 brewing yeast strains from the brewing environment were classified by MALDI-TOF MS and are listed separately in Appendix 9.3. Strains added to the classification are highlighted with the bold typeface. Overall, 1800 single spectra were used for the comparison to database entries and bioinformatic analysis. Figure 4.8

displays the complete classification of all 60 brewing yeast strains and shows the fully separation of *S. pastorianus* and *S. cerevisiae* (100%) as well *S. cerevisiae* var. *diastaticus* (100%). A closer at for the 32 top-fermenting *S. cerevisiae* and seven *S. cerevisiae* var. *diastaticus* strains is given with the percentage hit rate in Table 4.3. Furthermore, nearly all wheat beer strains (300 single spectra) were classified as such and showed an average hit rate to this beer type of 99%. 94% of brewing yeasts belonging to the Ale type formed the Ale group with one exception. TMW 3.0668 (TUM 513) is used as an Ale strain by brewers (Table 3.2). Nevertheless, it was classified by MALDI-TOF MS to the Alt beer / Kölsch group within the classification of unknown yeast strains to beer styles (chapter 4.2.1) as well after the comparison to the expanded database (Figure 4.8). As a result of that, TMW 3.0668 was labeled for further analysis as an Alt beer strain. Similarity, TMW 3.0254, which was originally classified as a Kölsch strain (Table 3.2), was matched by MALDI-TOF MS as an Ale strain. This strain was re-labeled for additional analysis as an Ale-strain. Regarding the strains of Alt beer and Kölsch and considering the average hits of 86% (Alt beer) and 31% (Kölsch), there is no clear separation of these beer types observed in Figure 4.8 and Table 4.3. The seven strains of *S. cerevisiae* var. *diastaticus* formed a single group and showed a match of 100% (Table 4.3) to the variety level. The classification for flocculation behavior and hit-rates for bottom-fermenting yeast strains are outlined in Table 4.3. A match of 74% (flocculent) and 94% (powdery) is displayed (Table 4.3). Moreover, a mix up between those two flocculation properties is observed for most of the strains (Figure 4.8). An overall average hit rate of 35% to strain level was achieved for 1800 recorded spectra and is presented in Table 4.3. Only the Ale strains TMW 3.0262 and TMW 3.0339 matched 100% to their database entries ((Figure 4.8). In case of a total classification to an application type, 86% were assigned correctly to their application type (Table 4.3). Considering the amount of yeasts, 52 out 60 (approx. 87%) brewing strains were classified correctly to their true application potential (Table 3.2).

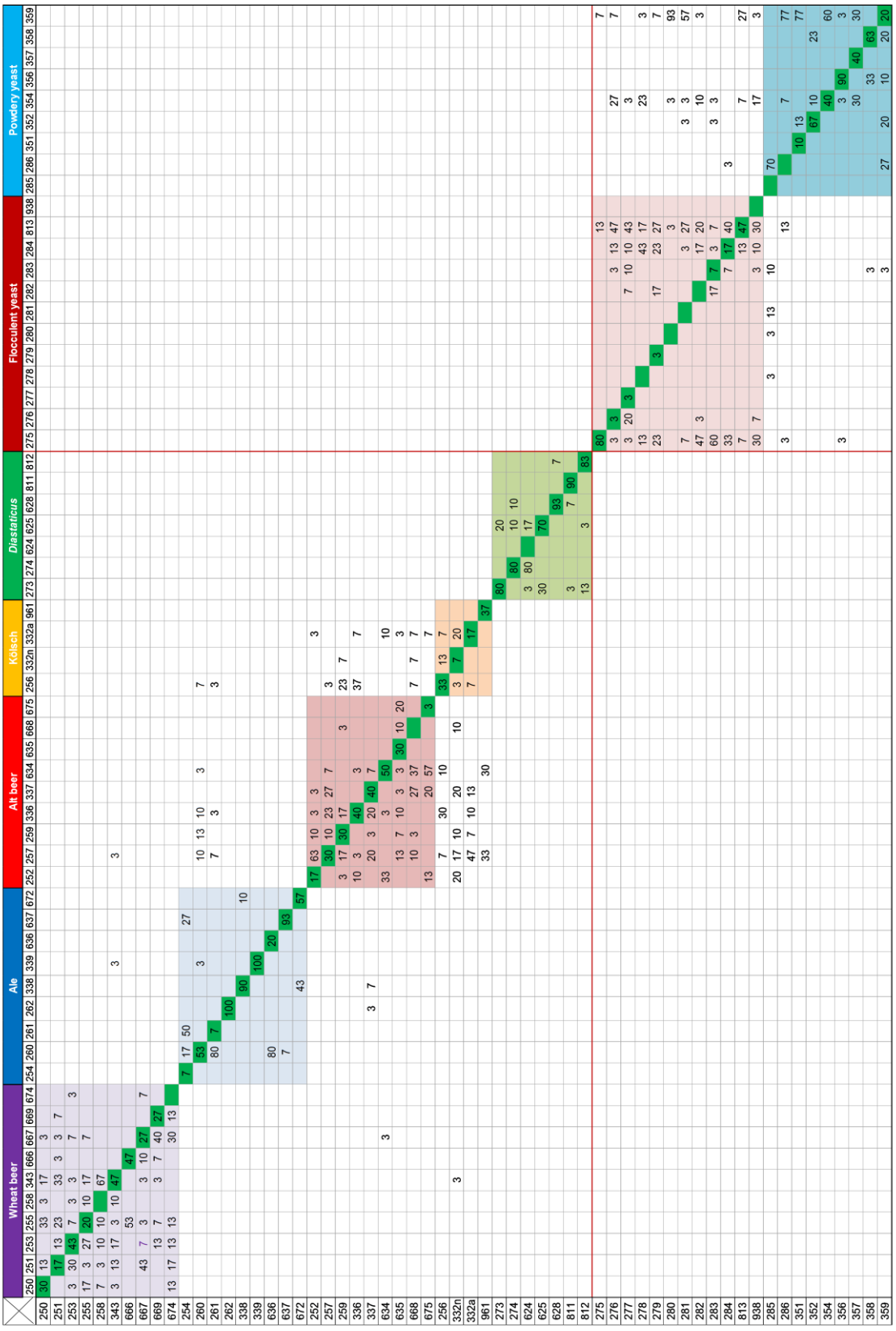

Figure 4.8: Classification of 1800 recorded spectra, which are compared to 60 brewing yeast database entries of the genus Saccharomyces. The hit rates (%) to the database entries of the tested strains are displayed. Hits on strain (green squares) or ecotype-level (all yeast strains of an appointed application) are indicated; a hit rate of 100% displays a total strain level identification; database entries are displayed on the top of the table with the abbreviated ID of the Technische Mikrobiologie Weihenstephan (TMW) and organized to beer styles, variety and flocculation behavior, which are displayed above. On the left side are all recorded strains with the ID of TMW; 30 spectra of each strain were compared to the database; the used coding of yeast strains is a shortened coding based on the TMW coding, e.g. 250 ≙ 3.0250

Table 4.3: Tabular list of the hit rates on the different beer styles / variety, flocculation behavior and the total strain level as well application type / ecotype classification for 60 brewing yeasts. The first section displays the results for all *S. cerevisiae* (var. *diastaticus*) strains to a specific beer style and variety. The middle section lists the results of S. pastorianus strains, which are divided by their flocculation behavior. The last section lists the sum of all recorded spectra of 60 strains and show the classification on total strain level as well application type. The column sum displays all recorded spectra of a specific beer styles, variety, flocculation behavior; the third column represent all correct assignments; fourth column all mismatches; last column displays the hit rate (%) to the specific group

Species	Beer style / Variety	Sum	Correct	Wrong	Hit rate (%)
S. cerevisiae	Wheat beer	300	298	2	99
	Ale	270	253	17	94
	Alt beer	270	233	37	86
	Kölsch	120	37	83	31
	S. cerevisiae var. *diastaticus*	210	210	0	100
	All ecotypes	1170	1031	139	88
	Strain	1170	484	686	41

Species	Flocculation behavior	Sum	Correct	Wrong	Hit rate (%)
S. pastorianus	Flocculent yeast	360	266	94	74
	Powdery yeast	270	257	13	95
	All flocculation behavior	630	523	107	83
	Strain	630	146	484	23

		Sum	Correct	Wrong	Hit rate (%)
All	Strain	1800	630	1170	35
	Application type	1800	1554	246	86

The findings of the database comparison are reflected in the bioinformatic analysis of all 60 brewing yeast strains. The separation of top-, bottom-fermenting brewing yeasts and *S. cerevisiae* var. *diastaticus* strains by MDS is shown in Figure 4.9. The analyzed mass spectra were separated in three groups (A, B, C). The data was labeled with the strain ID and the fermentation behavior. Group A harbored spectra of top-fermenting *S. cerevisiae* strains and formed the biggest section. Spectra of bottom-fermenting *S. pastorianus* strains were included in group B and placed on the right side of the MDS. The seven strains of the variety *S. cerevisiae*

var. *diastaticus* formed group C and are found below group A. A separation between group "B" to "A and C" is recognized as well as a good differentiation of "A" to "C" in Figure 4.9. An outlier was found inside the 21 *S. pastorianus* strains in group B namely TMW 3.0357, which distinguishes from all other strains and is placed on the right side of Figure 4.9.

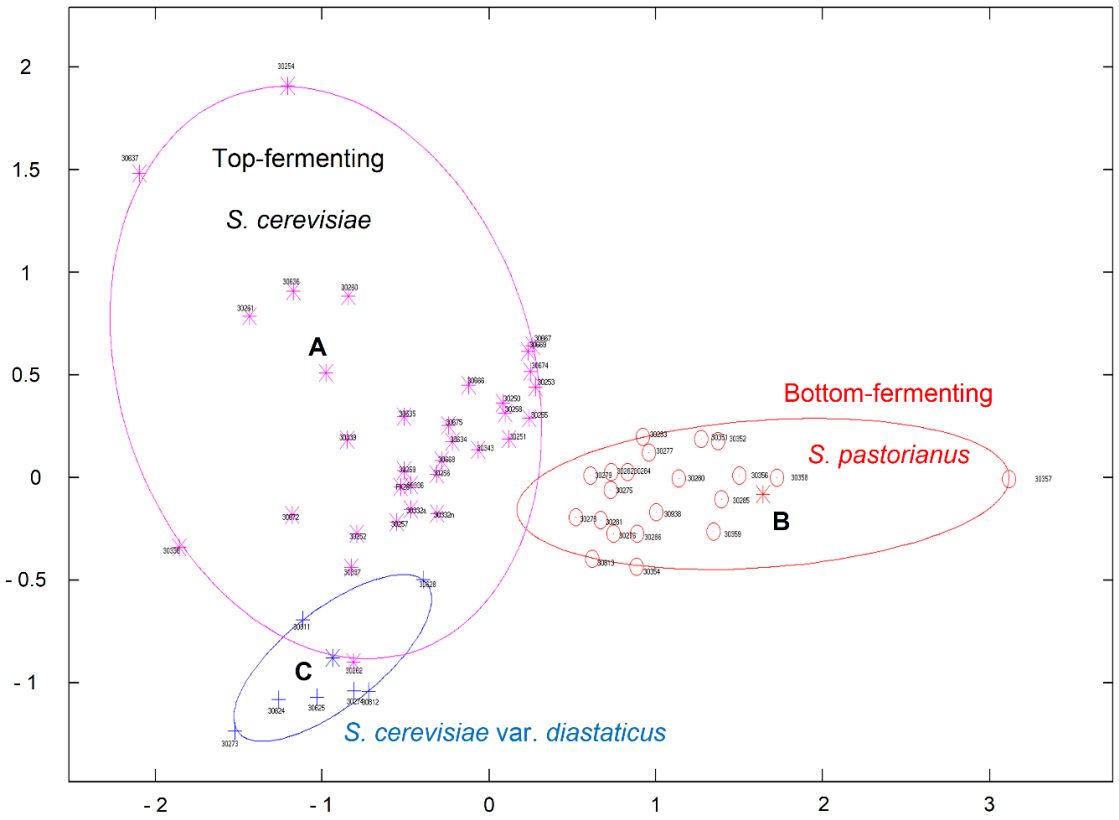

Figure 4.9: Multidimensional scaling (MDS) of 60 brewing yeast strains separated in top- (A) and bottom-fermenting (B) as well as *S. cerevisiae* var. *diastaticus* (C). All labels represent the mean spectra of 30 single spectrums of each strain. Strains are presented by their IDs according to Technische Mikrobiologie Weihenstephan (TMW). Top-fermenting strains are depicted in purple colored stars, a purple ellipse symbolized the group and the letter A is the center; bottom-fermenting are depicted in red colored circles, a red ellipse symbolized the group and the letter B is the center; *S. cerevisiae* var. *diastaticus* are depicted in blue colored crosses, a blue ellipse symbolized the group and the letter C is the center; The x- and y-axis represent the distances from every label to each other; ans = 0.95179; n = 60

Spectra of four brewing yeasts (of the top- and bottom-fermenting as well as *S. cerevisiae* var. *diastaticus* yeast) were stacked and visually compared (Figure 4.10). This illustration highlights major peak differences and the dissimilarity within the species. The dotted bars in the peak spectrum display main differences within the mass to charge ratio of 2000 m/z to 13000 m/z. In the area of 6000 to 7000 m/z major peak differences are visualized. Furthermore, TMW 3.0273 has a unique sub-proteomic peak around 11800 m/z, which did not occur in any other species.

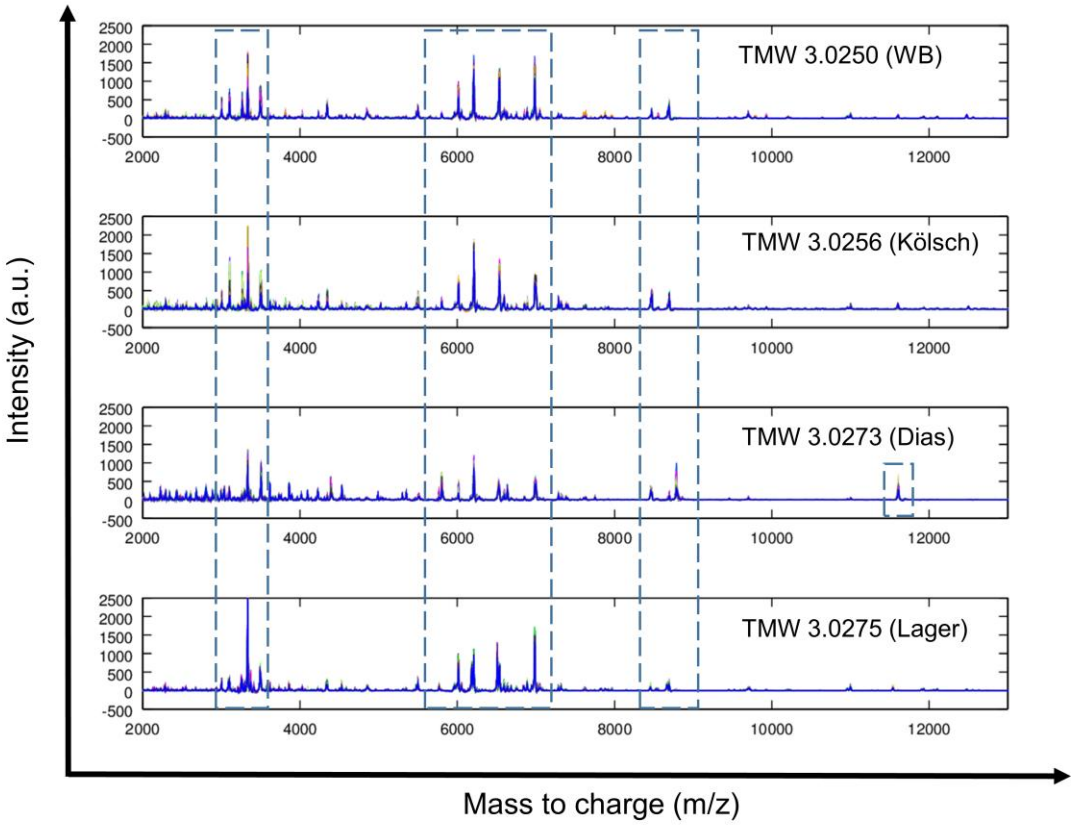

Figure 4.10: Stacked spectra of four different strains. 30 single spectra of each strain were summarized to one mean spectra; y-axis represents the intensity of the recorded peaks; x-axis show the mass to charge ratios from 2000 m/z to 12000 m/z; the ID of each strain is indicated on the right side of every spectrum by its TMW (Technische Mikrobiologie Weihenstephan) code; beer styles or variety are shown in brackets (WB = wheat beer; Dias = *S. cerevisiae* var. *diastaticus*); blue boxes with dotted lines highlight peak differences

A hierarchical cluster analysis was performed to separate the brewing yeasts in a dendrogram (Figure 4.11). 60 yeast strains, identified with their TMW-number, were clustered and additionally labelled according to the fermentation type and beer style / variety. Considering the fermentation type, three different labels are present: bottom-fermenting (BF), *S. cerevisiae* var. *diastaticus* and top-fermenting (TF) whereby TF is separated in three parts. The labeling according to the beer style is similar to the labeling according to the fermentation type. However, the main difference is that top-fermenting brewing yeast strains are separated with respect to the current beer style. The wheat beer (WB; purple) strains are separated in a single cluster apart from all other top-fermenting strains. The separation of Kölsch / Alt beer strains from each other is not possible (mix of red and orange) and therefore this group is called the German Alt-Kölsch group. Ale strains are more heterogeneous and are separated in six groups. The first cluster is parted in two sub-clusters containing the four strains TMW 3.0637, TMW 3.0254, TMW 3.0672 and TMW 3.0338. Four strains as well are represented in the second cluster that is divided into three sub-clusters including TMW 3.0636, TMW 3.0260,

TMW 3.0261 and TMW 3.0262, which is an outlier to the *S. cerevisiae* var. *diastaticus* strains. Furthermore, the Ale strain TMW 3.0339 is related to the German Alt-Kölsch strains.

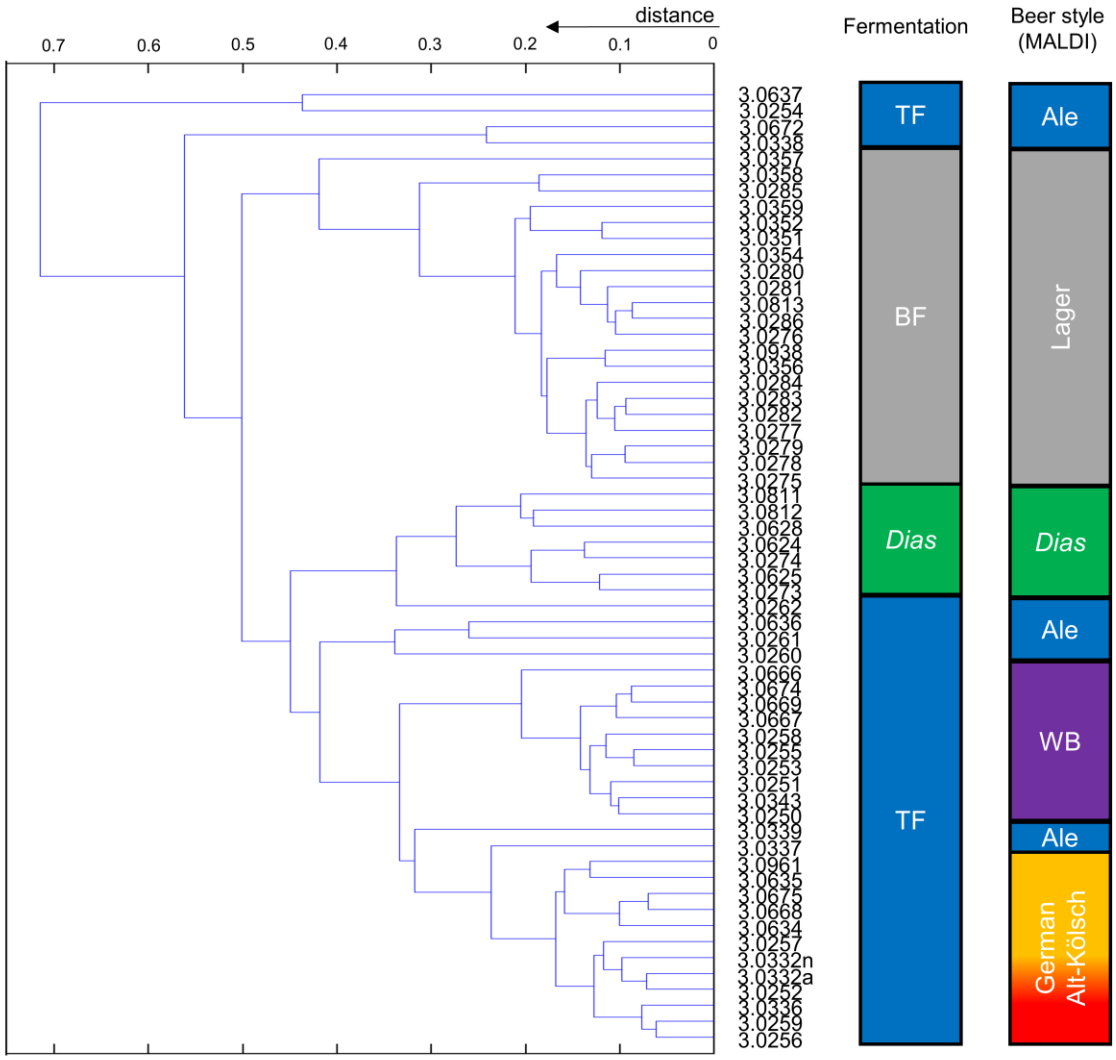

Figure 4.11: Hierarchical cluster analysis of 60 yeast strains which are displayed in a dendrogram and labeled to fermentation and beer style. Every ID represents the mean spectra of 30 single spectra per strain; the fermentation type is labeled to top-fermenting (= TF; blue), bottom-fermenting (= BF; grey) and *S. cerevisiae* var. *diastaticus* (= Dias; green); beer styles are illustrated to Ale (blue), *S. cerevisiae* var. *diastaticus* (= Dias; green), Lager (grey), wheat beer (= WB; purble), German Alt-Kölsch (mix up between red and orange); the distance is instructed from 0.0 (high similarity) to 0.7 (large distinction).

4.2.3 Characterization of top-fermenting and *S. cerevisiae* var. *diastaticus* strains by bioinformatic methods

For 32 top-fermenting and seven *S. cerevisiae* var. *diastaticus* strains are considered more closely. A similarity computation was done to visualize differences between the strains in a 2D map by MDS with Voronoi triangulation (blue line) in Figure 4.12. If the distances between the labels are big, the more different the MALDI patterns (based on one mean spectrum summarized 30 spectra per strain) will be.

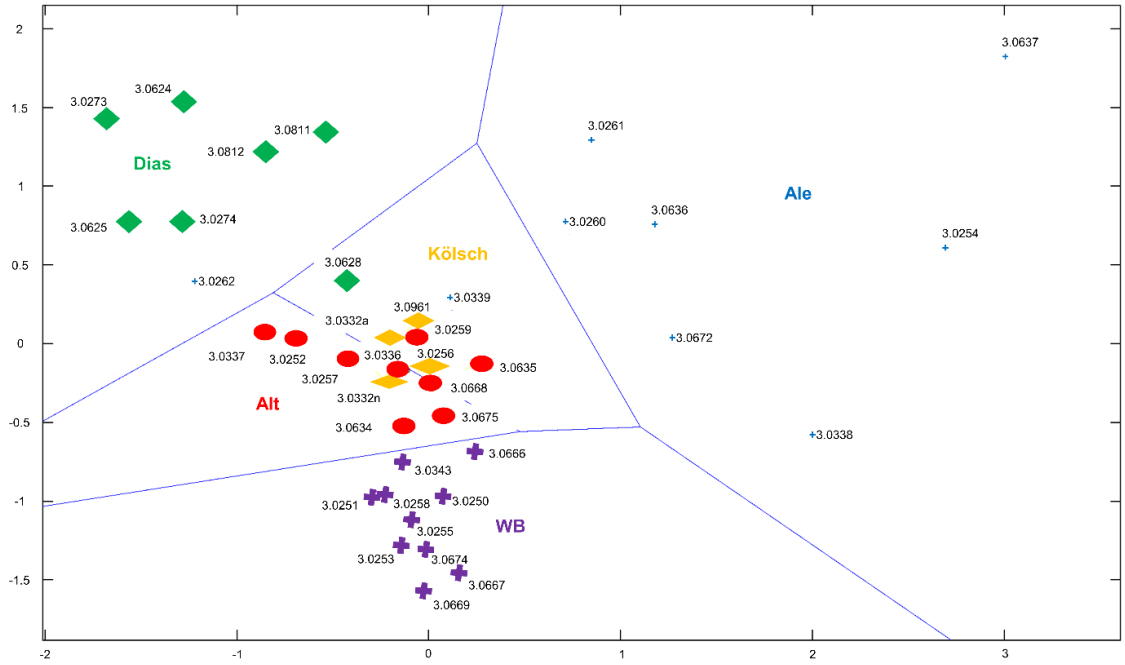

Figure 4.12: Multidimensional scaling (MDS) containing 32 top-fermenting brewing yeast and seven *S. cerevisiae* var. *diastaticus* strains divided in different beer styles by MALDI-TOF MS. Every mark represents the mean spectra of 30 single spectra of each strain; Coding according to Technsiche Mikrobiologie Weihenstephan (TMW); x- and y-axis present the distances between each point; WB = wheat beer, purple crosses ; Alt = Alt beer, red circle; Kölsch, yellow rhombus; Ale, blue crosses, Dias = *S. cerevisiae* var. *diastaticus*, green diamond; ans = 0.94807; n = 39

The 10 wheat beer strains (purple cross) were distinguished from the *S. cerevisiae* var. *diastaticus* group (Dias; green diamond), which is located on left top side of the 2D map. The differentiation of Alt (red circles) and Kölsch (yellow rhombus) was not achieved like it was observed in Figure 4.8. The Ale strains show a high degree of dissimilarity (blue cross) which is displayed in a wide spread group on the right side of the MDS. Furthermore, they clearly separate from the other beer styles. Nevertheless, strain TMW 3.0262 shows similarities to the variety *diastaticus*. The *S. cerevisiae* var. *diastaticus* strain TMW 3.0628 and the Ale strain TMW 3.0339 are both placed to the region of Kölsch.

The calculation of discriminant analysis of principal components (DAPC) with a loading plot and histogram of all single spectra is shown in Figure 4.13 and is supported by the cluster analysis (Figure 4.11) and MDS (Figure 4.12).

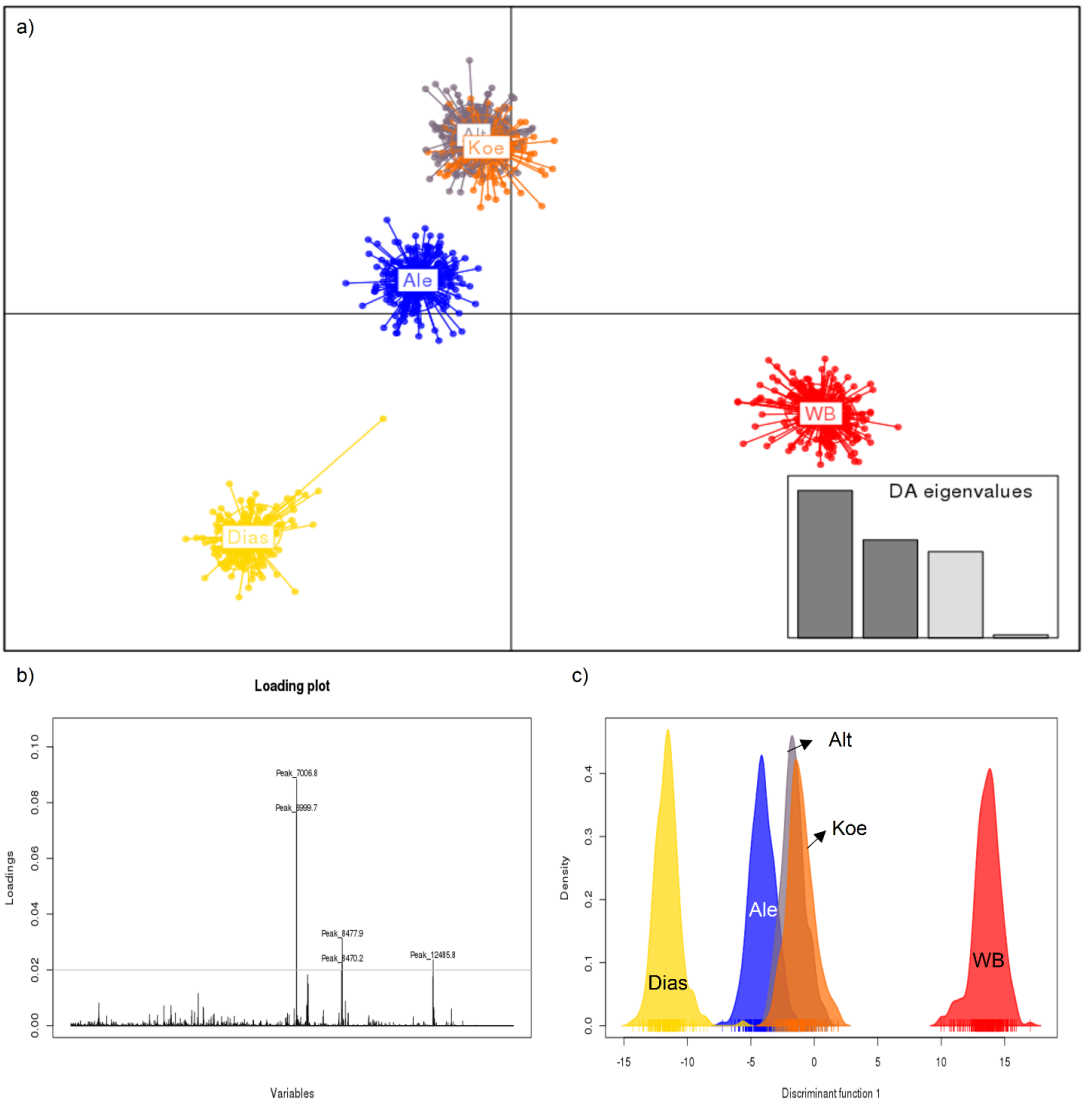

Figure 4.13: a) Discriminant analysis of principal components (DAPC) of top-fermenting brewing yeasts and *S. cerevisiae* var. *diastaticus*. 1170 single spectra are illustrated and labeled by dots; WB = wheat beer (red), Koe = Kölsch (orange), Alt = Alt-beer (grey), Ale (blue), Dias = *S. cerevisiae* var. *diastaticus* (yellow) b) Visualization of major peaks which are responsible for the separation by a loading plot c) Histogram of the recorded spectra and labeled to different beer types. WB = wheat beer (red), Koe = Kölsch (orange), Alt = Alt beer (grey), Ale (blue) and Dias = *S. cerevisiae* var. *diastaticus* (yellow)

All spectra are clustered in groups as ellipses and labeled according to the beer styles. Within five clusters of beer types that were distinguished, the wheat beer cluster (WB; red) and the *S. cerevisiae* var. *diastaticus* (Dias; yellow) display a clear separation from other groups (Figure 4.13a). The loading plot that is shown in Figure 4.13b summarizes all single spectra of yeast strains and represents those peaks, which are responsible for the separation. The highest loadings are achieved at 6999.2 m/z and 7006.8 m/z. The separation is supported by the histogram for discriminant axis/function (Figure 4.13c). The histogram displays the similarity of Alt (grey) and Kölsch (orange) and also shows the isolation of wheat beer (red) and *S. cerevisiae* var. *diastaticus* (yellow).

The visualization of eight single spectra overlays of TMW 3.0250 (WB), TMW 3.0252 (Alt), TMW 3.0256 (Kölsch), TMW 3.0273 (*S. cerevisiae* var. *diastaticus*), TMW 3.0254 (Ale), TMW 3.0261 (Ale), TMW 3.0262 (Ale) and TMW 3.0668 (Alt beer) is illustrated in Figure 4.14 and demonstrates the differences of MALDI fingerprints of various beer styles as well as varieties. The sub-proteome of TMW 3.0252 and TMW 3.0256 are similar to each other but slightly differ in the intensity of some peaks. The protein profile of TMW 3.0250 shows a single peak with a high intensity around 7000 m/z which was found in all WB-strains. The sub-proteome of the variety *S. cerevisiae* var. *diastaticus* represented by TMW 3.0273 displays several peaks between 3000 m/z to 5000 m/z. Furthermore, a single peak around 9000 m/z and 11800 m/z was detected, respectively. The sub-proteome of TMW 3.0668 visually shows more similarities to TMW 3.0252 and TMW 3.0256 than to the other Ale-strains. Nevertheless, TMW 3.0254 a Kölsch strain to brewer's experience and an Ale-strain to MALDI-TOF MS showed more similarities to TMW 3.0261 as to TMW 3.0256 strains. Those finding are reflected in Figure 4.8, Figure 4.11 and Figure 4.12.

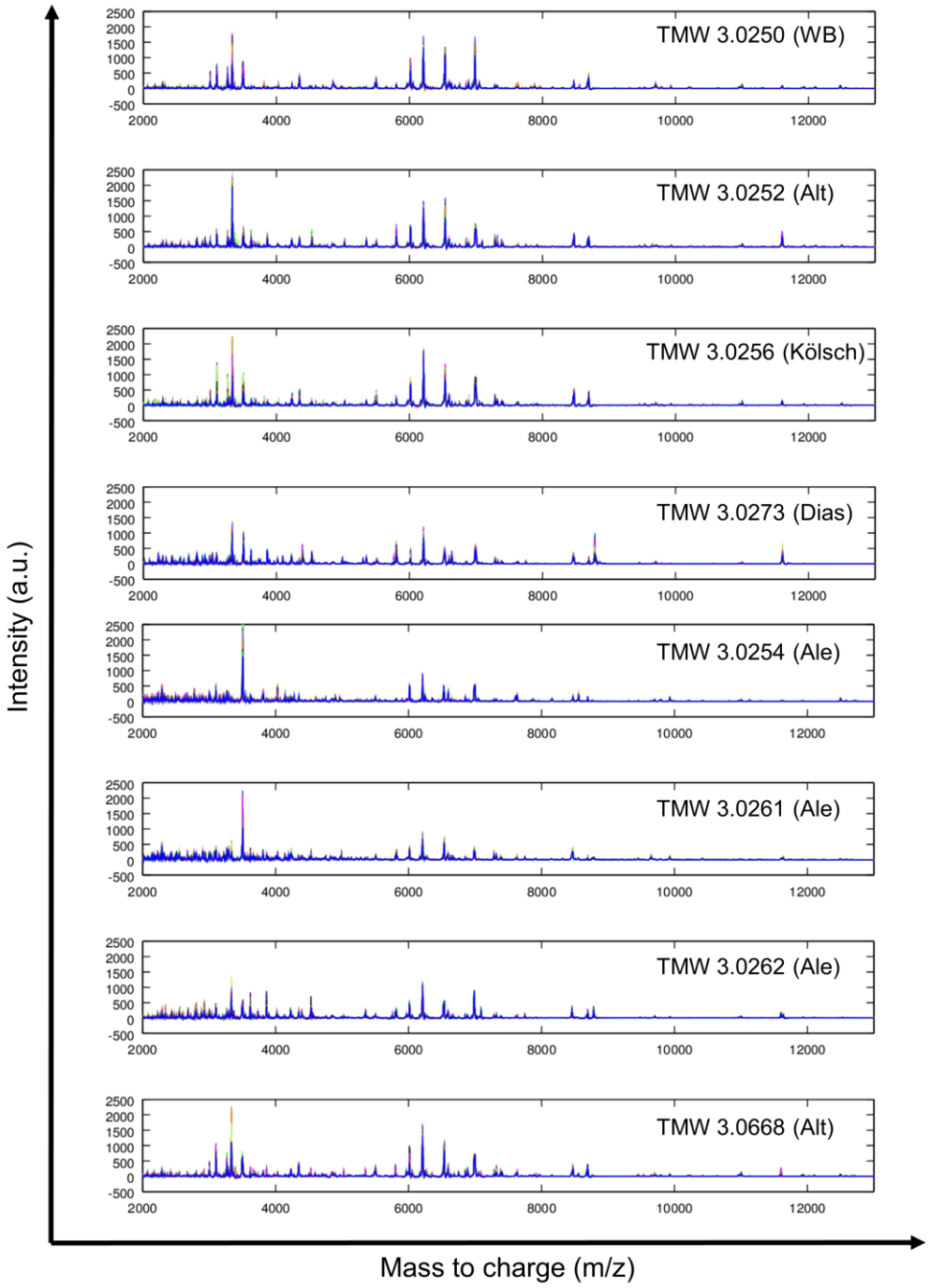

Figure 4.14: Stacked spectra of eight different S. cerevisiae strains. 30 single spectra of each strain were summarized to one mean spectrum; y-axis represent the intensity (=Int) of the recorded peaks; x-axis show the mass to charges from 2000 m/z to 12000 m/z; the ID of each strain is placed on the right side of every spectrum with the TMW (Technische Mikrobiologie Weihenstephan) code; beer styles and variety are written in brackets (WB = wheat beer; Alt = Alt beer; Dias = *S. cerevisiae* var. *diastaticus*)

4.2.4 Summarizing Alt beer and Kölsch strains to the beer style German Alt-Kölsch

The high similarity of the recorded sub-proteomic spectra of Alt beer and Kölsch strains lead to no clear separation of these two beer styles in chapter 4.2.1, 4.2.2 and 4.2.3. Because of that, a new database comparison was done based on the 39 *S. cerevisiae* yeast strains, which involved the fusion of the Alt beer and Kölsch beer styles to a new group namely German Alt-Kölsch like it was done in Figure 4.11. This group contains 13 *S. cerevisiae* strains.

Figure 4.15 reflects the new comparison of the recorded yeast strains to their own database entries, which includes the German Alt-Kölsch beer style (orange). It is observed that most of the 390 single spectra of German Alt-Kölsch strain were matched to this beer style and a 99% hit rate could be achieved (Table 4.4). The correct classification of the *S. cerevisiae* strains to application types was raised from 88% (Table 4.3) to 98% (Table 4.4) based on the combination of both beer styles. Moreover, the total ecotype matching of 1800 single spectra including bottom- and top-fermenting as well as *S. cerevisiae* var. *diastaticus* increased from 86% (Table 4.3) to 93% (Table 4.4).

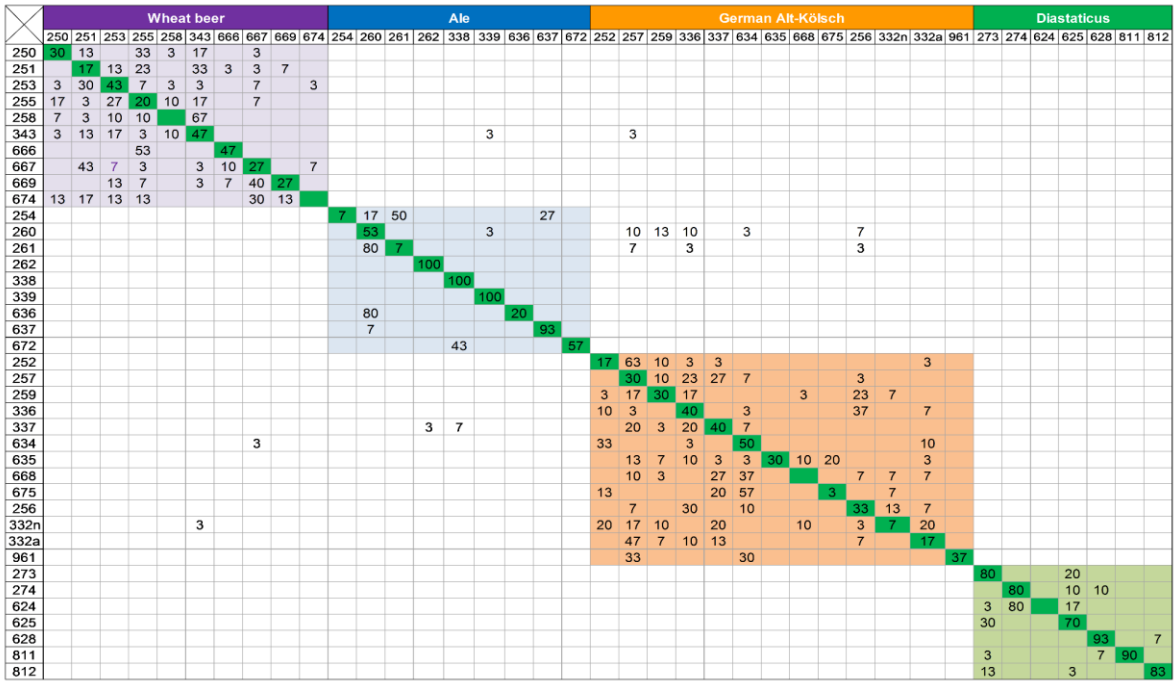

Figure 4.15: Comparison of 32 top-fermenting and seven S. cerevisiae var. diastaticus strains against their own database entries including the fusion of German Alt-Kölsch . The hit rates (%) of the tested strains are displayed to the database entries and show whether hits on strain (green squares) or ecotype-level (all yeast strains of an appointed beer style); a hit rate of 100% displays a total strain identification; database entries are displayed on the top of the table with the abbreviated ID of the Technischen Mikrobiologie Weihenstephan (TMW) and organized by beer style, which are shown above; on the left side are all recorded strains with the ID of TMW; 30 spectra of each strain were compared to the database entries; ; the used coding of yeast strains is a shortened coding based on the TMW coding, e.g. 250 ≙ 3.0250; n = 1170

Table 4.4: Tabular list of the hit rates on the different beer styles and the total strain level identification for 39 top-fermenting brewing yeast strains including the fusion of German Alt-Kölsch. Sum displays all recorded spectra of a specific beer style or the total count of recorded spectra for the 39 strains; the third column represents all correct assignments; fourth column all mismatches; last column displays the hit rate (%) on the different beer styles, total hit rate on all beer styles and the hit rate on strain level identification; BF = bottom-fermenting

Beer style	Sum	Correct	Wrong	Hit rate (%)
Wheat beer	300	298	2	99
Ale	270	253	17	94
German Alt-Kölsch	390	385	5	99
S. cerevisiae* var. *diastaticus	210	210	0	100
All *S. cerevisiae*	1170	1146	24	98
Application type	1800	1669	131	93

4.2.5 Classification of more brewing strains

After the classification of 60 brewing yeast strains with respect to major beer styles, six *S. cerevisiae* strains of beer styles were compared to 20 top-fermenting reference strains including *S. cerevisiae var. diastaticus*. Those six strains are TMW 3.0673 (California Ale), TMW 3.0864 (Lambic, Belgium), TMW 3.0865 (Belgium), TMW 3.0866 (Ale from wheat malt), TMW 3.0867 (Opaque, South Africa) and TMW 3.0937 (Wit beer, Belgium). A MDS was performed for 26 yeast strains to visualize differences of the six strains to reference strains of major beer styles, which is visualized in Figure 4.16.

The six strains (IB, purple multiplication sign) distinguish from the group of wheat beer strains (WB, black cross) and *S. cerevisiae* var. *diastaticus* (Dias, green star), which are located on the lower part and on the right side of Figure 4.16. The strains TMW 3.0864 and TMW 3.0865, two Belgian isolates, showed similarities and are placed next to the ellipses of German Alt-Kölsch (AK, blue cross) in the center of the MDS. In parallel, the Ale strain TMW 3.0866 as well as the Opaque strain TMW 3.0867 are located within the Ale clade (red circle). The last two strains, TMW 3.0673 and TMW 3.0937, are placed next to each other and distinguish from the German Alt-Kölsch sector. Furthermore, these strains are between the Ale and *S. cerevisiae* var. *diastaticus* groups.

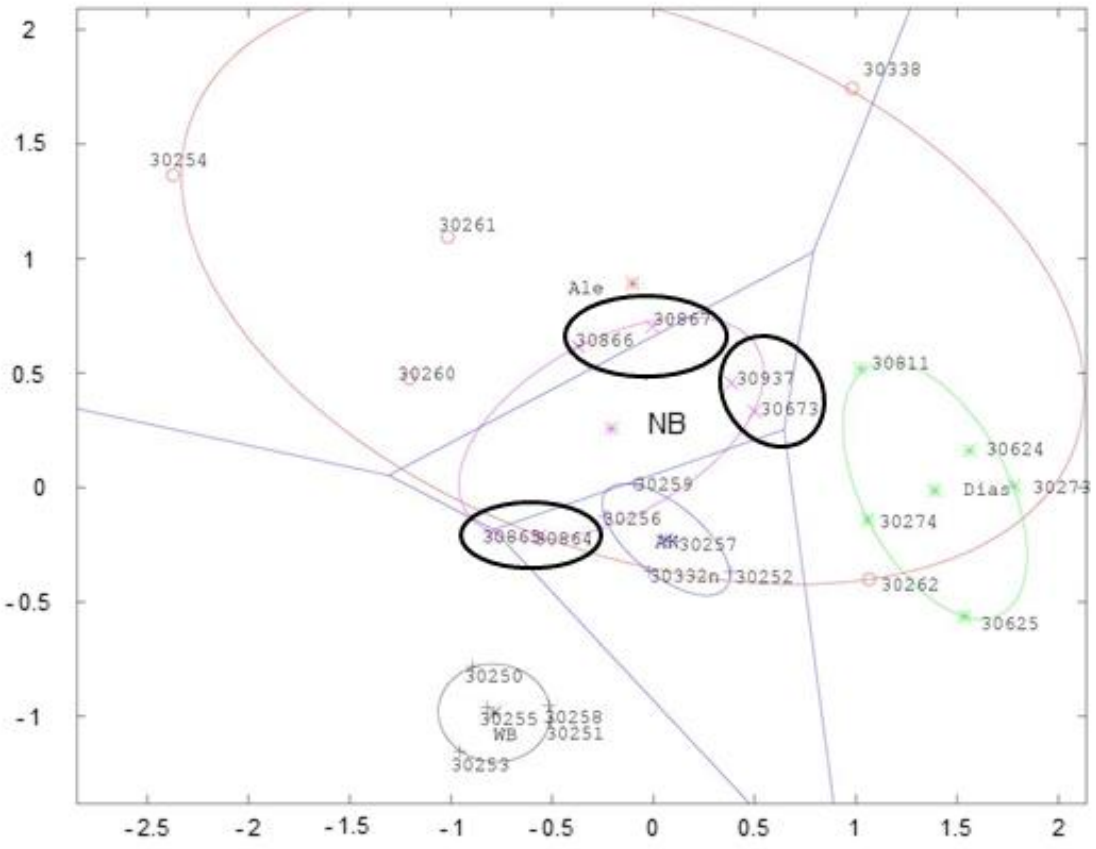

Figure 4.16: Multidimensional scaling (MDS) containing 20 reference strains divided in different beer styles including 6 strains of new brewing yeast strains, which are highlighted with black circles. Every mark represents the mean spectra of 30 single spectra of each strain; Coding according to Lehrstuhl für Technsiche Mikrobiologie (TMW); x- and y-axis present the distances between each point; NB = new brewing yeasts, purple; WB = wheat beer, black ; AK = German Alt-Kölsch, blue; Ale, red, Dias = *S. cerevisiae* var. *diastaticus*, green; ans = 0.94488; n = 26

The database of 60 *Saccharomyces* yeast strains was expanded with those strains. Two strains were added to the Ale group namely TMW 3.0866 and TMW 3.0673. Subsequently, a new group was established called “Special” including all Belgian strains TMW 3.0864, TMW 3.0865 and TMW 3.0937 as well as the Opaque strain TMW 3.0867.

In total, 66 brewing yeast strains of the genus *Saccharomyces* are reflected in the database, including the following application types: 38 top-fermenting *S. cerevisiae* strains (10 wheat beer, 12 Ale, 13 German Alt-Kölsch and four special), seven *S. cerevisiae* var. *diastaticus* strains and 21 bottom-fermenting *S. pastorianus* strains (12 flocculation and 9 powdery yeast). Those database entries from the brewing environment are used for the identification of blind-coded strains in chapter 4.2.7. The next sub-chapter shows the comparison of all brewing yeast strains including *S. pastorianus* to non-brewing yeast strains of *S. cerevisiae*.

4.2.6 Differentiation between brewing and non-brewing yeasts

After the primary classification of brewing yeasts, 17 non-brewing yeast strains of *S. cerevisiae* were added from different applications: wine, sparkling wine, special wine, laboratory, distillery, and bakery. 30 spectra per strain as well as database entries of all non-brewing strains were recorded by MALDI-TOF MS. Afterwards, 2490 single spectra (83 yeast strains) were compared to database entries to achieve a differentiation between brewing and non-brewing yeast strains. Figure 4.17 shows the new comparison of the recorded yeast strains to their own database entries, which includes non-brewing yeast strains. From Table 4.5 to Table 4.7, hit rates present the comparison on species level (Table 4.5), strain level (Table 4.5), differentiation between brewing and non-brewing ecotype of *S. cerevisiae* (Table 4.6), and a zoom in on all application styles (Table 4.7). A differentiation on species level could be achieved to 99.88% (Table 4.5), but a strain level identification was obtained 44% of times (Table 4.5). Furthermore, Figure 4.17 shows that within the non-brewing strains a higher level of strain level identification is achieved, which is especially observed within the wine group. Considering the match of all *S. cerevisiae* to main groups, Table 4.6 shows that 94% of all top-fermenting brewing yeast were matched to the brewing environment. Furthermore, an almost completely correct classification was achieved for non-brewing yeast (approx. 99%) and *S. cerevisiae* var. *diastaticus* (approx. 99%) (Table 4.6). In total, Table 4.6 lists that approx. 95% off all *S. cerevisiae* were correctly classified as one of the main ecotypes. Figure 4.17 visualizes the good differentiation between the ecotypes as well as that all *S. cerevisiae* strains distinguish to *S. pastorianus* and vice versa. A zoom in to the hit rates of various application potentials is presented in Table 4.7. 87% of the recorded spectra including *S. pastorianus* were matched correctly to their application type. Most *S. cerevisiae* strains were classified correctly with respect to their application potential like wheat beer, German Alt-Kölsch or wine (Figure 4.17 and Table 4.7). This is the case with 77 out 83 (approx. 93%) yeast strains.

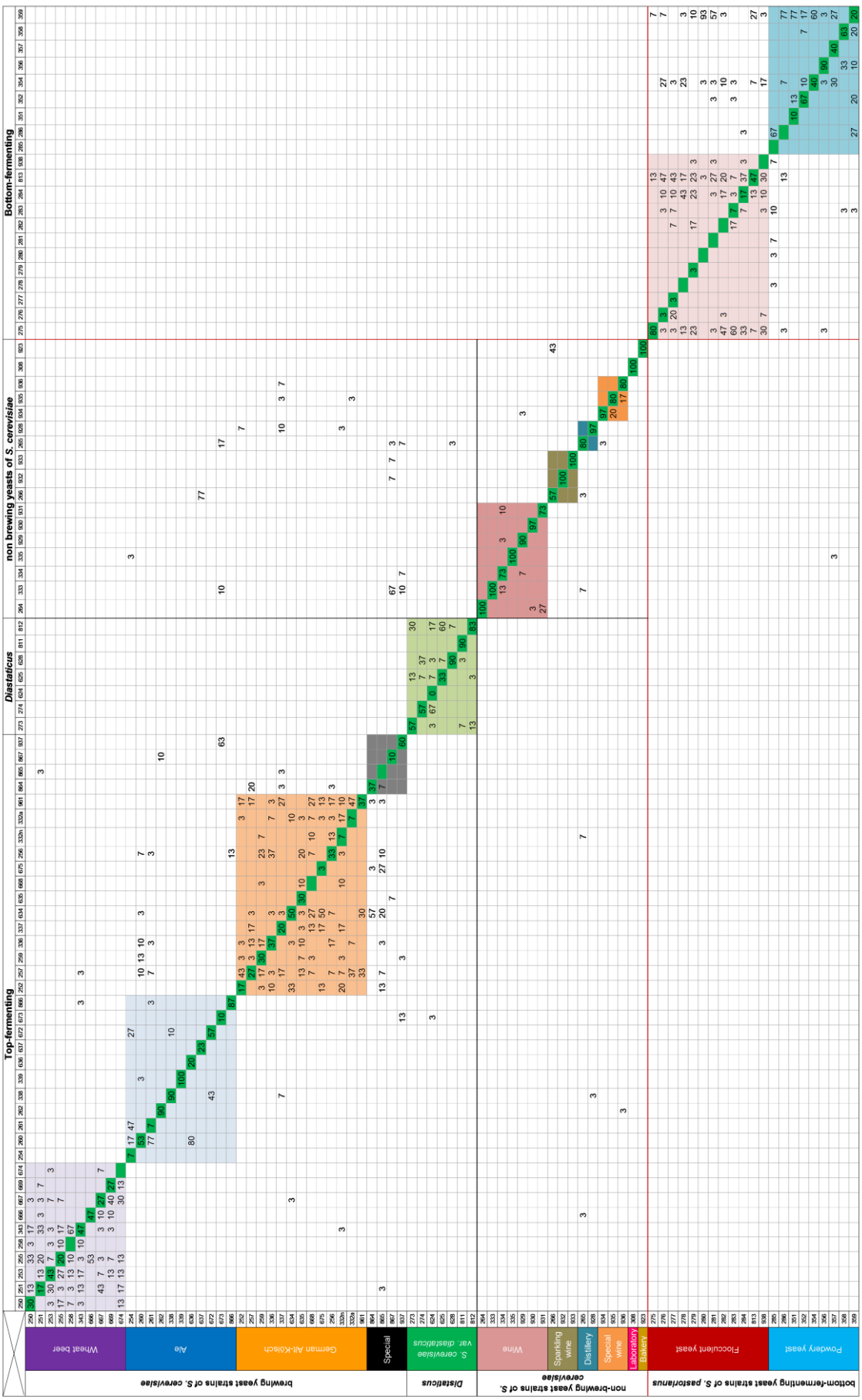
Top-fermenting
Diastaticus
non brewing yeasts of S. cerevisiae
Bottom-fermenting
brewing yeast strains of S. cerevisiae
Distaticus
non-brewing yeast strains of S. cerevisiae
bottom-fermenting yeast strains of S. pastorianus
Wheat beer
Ale
German Alt-Kölsch
Special
S. cerevisiae var. diastaticus
Wine
Sparkling wine
Distillery
Special wine
Laboratory
Bakery
Flocculent yeast
Powdery yeast

Figure 4.17: Comparison of 83 yeast strains of *S. cerevisiae*, *S. cerevisiae* var. *diastaticus* and *S. pastorianus* with their own database entries including brewing and non-brewing strains (page 77). The hit rates (%) of the tested strains are displayed and show hits on strain (green squares) or ecotype-levels (all yeast strains of an appointed beer style); a hit rate of 100% displays a total strain identification; database entries are displayed on the top of the table with the abbreviated ID of the Technischen Mikrobiologie Weihenstephan (TMW) and are organized by application styles, which are shown on the left side; on the left side are all recorded strains with the abbreviated ID of TMW; 30 spectra of each strain were compared to the database entries; the used coding of yeast strains is a shortened coding based on the TMW coding, e.g. 250 ≙ 3.0250; n = 2470

Table 4.5: Tabular list of the hit rates on species levels, total species level identification and strain level identification for 83 yeast strains of the genus *Saccharomyces*.

Species / strain level	Sum	Correct	Wrong	Hit rate (%)
S. cerevisiae	1650	1650	0	100
S. cerevisiae* var. *diastaticus	210	208	2	99.05
S. pastorianus	630	629	1	99.84
Species level	2490	2487	3	99.88
Strain level	2490	1089	1401	44

Table 4.6: Tabular list of hit rates for the general ecotype of 62 *S. cerevisiae* yeast strains.

S. cerevisiae main group	Sum	Correct	Wrong	Hit rate (%)
Brewing yeasts	1140	1066	74	94
Non-brewing yeasts	510	505	5	99.02
S. cerevisiae* var. *diastaticus	210	208	2	99.05
Total classification	1860	1779	81	95.64

Table 4.7: Tabular list of hit rates to their application potential

	Application potential	Sum	Correct	Wrong	Hit rate (%)
Top-fermenting	Wheat beer	300	297	3	99
	Ale	330	255	75	77
	German Alt-Kölsch	390	367	23	94
	Special	120	34	86	28
Variety	*S. cerevisiae* var. *diastaticus*	210	208	2	99.05
Non-brewing yeasts	Wine	210	209	1	99.52
	Sparkling wine	90	77	13	86
	Special wine	90	88	2	98
	Distillery	60	53	7	88
	Laboratory	30	30	0	100
	Bakery	30	30	0	100
Bottom-fermenting	Flocculation yeast	360	265	95	74
	Powdery yeast	270	252	18	93
	Total application type classification	2490	2168	322	87

For an upcoming test, all 83 strains were used as a database to identify and classify wild isolates.

4.2.7 Identification and Classification of wild isolates by MALDI-TOF MS

Six wild isolates from Table 3.4 were identified with the database of *Saccharomyces* by MALDI-TOF MS, which is implemented in chapter 4.2.6. Afterwards, the recorded spectra were classified to 30 reference yeast strains by a hierarchical cluster analysis that was visualized using a dendrogram.

The results of the identification, seen in Table 4.8, showed that all yeast strains belong to the genus of *Saccharomyces*. Two out of six strains could be matched to species level namely

TMW 3.0897, a *S. cerevisiae* strain, and TMW 3.0909, which belongs to *S. pastorianus*. Considering the *S. cerevisiae* strain, it was possible to predict the strain to the application type of wine. The classification of the other four strains wasn't successful, only an identification on genus level could be achieved.

Table 4.8: Identification of six wild isolates with the established database of *Saccharomyces*. The first column represents the coding (TMW) for each strain; the second and third column shows the identification on genus and possible species level; the fourth column visualize a prediction to a ecotype; the last column presents the average score value for each strain after the identification by MALDI-TOF MS

Strain	**Genus**	**Species**	**Predicted ecotype**	**Average score value**
3.0897	*Saccharomyces*	*cerevisiae*	wine	2.433
3.0909	*Saccharomyces*	*pastorianus*	Lager	2.608
3.0924	*Saccharomyces*	*cerevisiae* / *pastorianus*	-	2.020
3.0925	*Saccharomyces*	*cerevisiae* / *pastorianus*	-	2.012
3.0926	*Saccharomyces*	*cerevisiae* / *pastorianus*	-	2.077
3.0927	*Saccharomyces*	*cerevisiae* / *pastorianus*	-	1.923

The results of the identification (Table 4.8) are reflected within a hierarchical cluster analysis including all wild isolates (highlighted with WI) and 30 reference strains of *S. cerevisiae* (20 strains), *S. cerevisiae* var. *diastaticus* (5 strains) and *S. pastorianus* (5 strains) that is shown in Figure 4.18. As expected, strain TMW 3.0897 is matched within the wine clade of *S. cerevisiae* and *S. pastorianus* TMW 3.0909 showed high similarities to the clade of the Lager beer (Figure 4.18). The four strains of the genus *Saccharomyces* with no further identification on species level formed a group on top of the hierarchical tree and were separated from the other two wild isolates as well as from all reference strains (Figure 4.18).

Subsequently, in-house sequence analysis of the internal transcribed spacer (*ITS*) region as well *LT5* of TMW 3.0924, TMW 3.0925, TMW 3.0926 and TMW 3.0927 was carried out, showing that those yeast strains belong to the closest relative of *S. cerevisiae* namely *Saccharomyces paradoxus*.

In case of the classification of the wild isolates new database entries for MALDI-TOF MS were generated as well as a new group "*S. paradoxus*" was defined, which contains four strains.

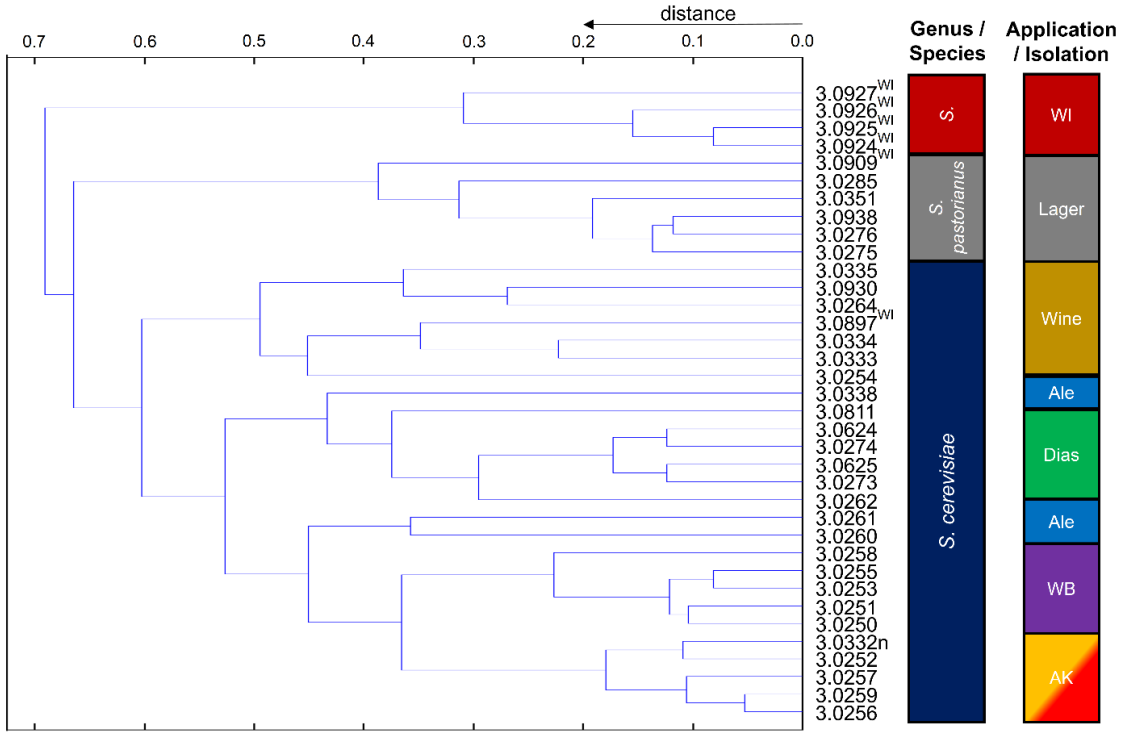

Figure 4.18: Hierarchical cluster analysis of wild isolates (superscripted with WI) including reference strains which are displayed in a dendrogram and labeled to genus / species level and application / isolation. Every ID represent the mean spectra of 30 single spectra per strain; the fermentation type is labeled to top-fermenting (= TF; blue), bottom-fermenting (= BF; grey) and *S. cerevisiae* var. *diastaticus* (= Dias; green); beer styles are illustrated to Ale (blue), S. cerevisiae var. diastaticus (= Dias; green), Lager (grey), wheat beer (= WB; purble), German Alt-Kölsch (=AK; mix up between red and orange); the distance is instructed from 0.0 (high similarity) to 0.7 (large distinction).

4.2.8 Divergence of *Saccharomyces* strains

All *Saccharomyces* yeast strains from chapter 4.2 were compared to each other by a hierarchical cluster analysis in Figure 4.19. This includes 38 top-fermenting *S. cerevisiae* strains of different beer styles: 10 wheat beer, 13 German Alt-Kölsch, 11 Ale and four special (Belgian and Opaque), seven *S. cerevisiae* var. *diastaticus*, 21 bottom-fermenting *S. pastorianus* and six wild isolates (one *S. cerevisiae*, one *S. pastorianus* and four *S. paradoxus*). This analysis is visualized in the dendrogram (Figure 4.19).

89 yeast strains, outlined with the TMW-number, were clustered and firstly labelled according to species level in Figure 4.19. Considering the species level, three different labels are present: *S. pastorianus* (*S. p.; grey*), *S. paradoxus* (*S. para.; dark red*) and *S. cerevisiae* (*S. c.; dark blue*) whereby *S. cerevisiae* is separated in two groups.

The second labeling was done according to the application potential of the yeast strains in Figure 4.19. This was done to achieve an overview of the application potentials especially for

all *S. cerevisiae* strains. Two major groups are shown in Figure 4.19 namely strains applied or isolated in the brewing environment (Beer) and non-brewing yeasts (NBY).

Wheat beer (WB; purple) strains distinguish from all other industrial applications of the brewing and non-brewing environment. Considering German Alt-Kölsch (AK; red-orange), two groups are observed, which are divided from each other, but distinguish from all other applications. Furthermore, two Belgian (B) strains are placed within the second AK group. A high degree of dissimilarity is still found for all Ale (blue) strains, which is visualized with three Ale groups (Ale^1, Ale^2 and Ale^3) and some outliers placed in the non-brewing section of wine ($Wine^1$ and $Wine^4$) and bread (brown). All seven *S. cerevisiae* var. *diastaticus* (Dias; green) strains formed an own cluster within the non-brewing group, but these distinguish from all brewing yeasts and non-brewing yeasts. A closer look at the non-brewing yeasts shows that those strains are more heterogeneous and divided in five groups containing outliers similar to the Ale strains. Especially the wine groups including wine yeasts, sparkling wine yeasts and special wine yeasts possess unique sub-proteomic patterns, which result in four different groups namely $Wine^1$, $Wine^2$, $Wine^3$ and $Wine^4$. $Wine^1$ and $Wine^2$ appear to be outliers within all *S. cerevisiae* strains, which is visualized in Figure 4.19. On the other hand is $Wine^3$, which contains *S. cerevisiae* strains of different wine application potentials as well the wild yeast TMW 3.0897 and one distillery (D) strain. $Wine^4$ includes all special wine yeast strains and three strains from other applications.

In summary, Figure 4.19 shows not only the separation for three different species, but also illustrates the divergence of industrial *S. cerevisiae* strains with respect to their application potential based on the recorded sub-proteomic spectra from MALDI-TOF MS.

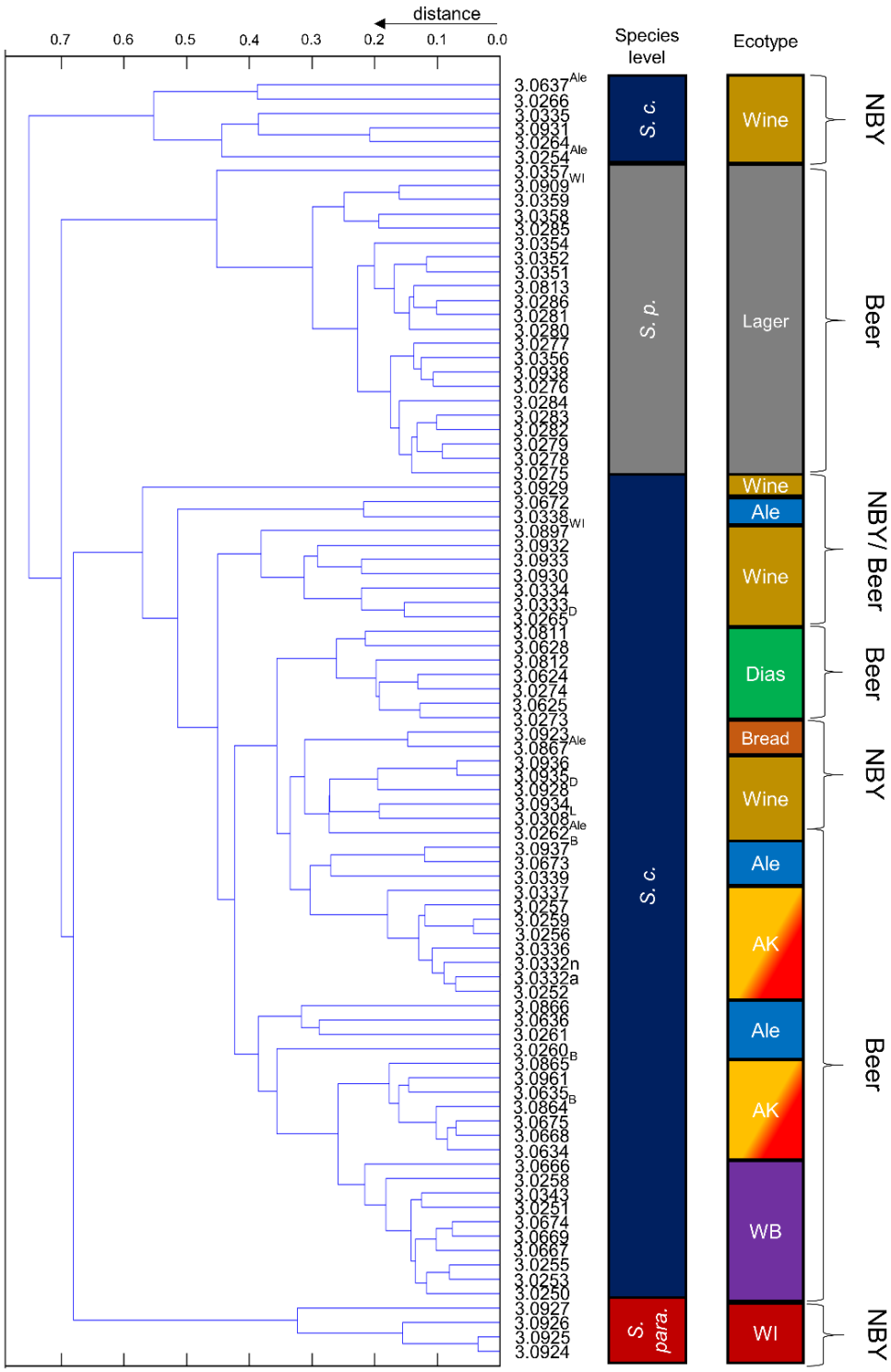

Figure 4.19: Cluster analysis of 89 Saccharomyces yeast strains which are displayed in a hierarchical dendrogram and labeled to species level and ecotype. Every ID (coding Technische Mikrobiologie Weihenstephan (TMW)) represent the mean spectra of 30 single spectra per strain; the species level is labeled to *S. cerevisiae* (= *S. c.*; blue), *S. pastorianus* (= *S. p.*.; grey) and *S. paradoxus* (= *S. para.*; dark red; the ecotype is labeled to Wine (gold), lager (grey), Ale (blue), *S. cerevisiae* var. *diastaticus* (=Dias; green), Bread (brown), German Alt-Kölsch (=AK, red / yellow), wheat beer (=WB; purple); wild isolate (=WI; dark red) ; strains isolated or used in other applications are highlighted with superscript letters: Ale, wild isolate (=WI), distillery (=D), laboratory (=L), Belgium (=B); brackets describes the general level of brewing yeast (=Beer), non-brewing yeast (=NBY) or spoilage the distance is instructed from 0.0 (high similarity) to 0.7 (large distinction).

4.3 Novel diagnostic marker genes for the differentiation of *Saccharomyces* with respect to their application potential

Besides the classification on sub-proteomic level, all yeast strains of chapter 4.2 were characterized on genetic level. A chosen number of yeast strains were taken and used for the prediction of novel diagnostic marker genes (DMGs) by BADGE (Behr et al., 2016). The detected DMGs were used for a possible classification of 89 yeasts of the genus *Saccharomyces* with respect to their application potential.

4.3.1 Prediction of DMGs for the classification of beer styles

BADGE was used to compare the genomic data of brewing yeasts in order to predict specific beer style DMGs. Considering wheat beer as one of the most interesting beer styles, we looked for specific DMGs for the wheat beer style. Therefore, we looked for genes which have at least a 60% presence within the wheat beer group and below 20% presence in the other beer styles of German Alt-Kölsch and Ale. Some wheat beer specific DMGs were obtained to differentiate wheat beer strains from strains of other beer styles. Two genes were chosen to test them as wheat beer specific DMGs and designed primer pairs (see Appendix 9.2). Table 4.9 shows the designation and predicted function of the selected genes and the sequence of both DMGs is presented in chapter 9.1.1. Furthermore, peer-reviewed papers about the two DMGs are presented in Table 4.9.

Table 4.9: Designation of BADGE DMGs and predicted function (annotation). The second column shows the systematic name and the last column shows peer-reviewed papers. WB = wheat beer

DMG designation	Systematic name	Annotation (RAST / blasp)	Peer-reviewed papers
WB-marker-I	YDR242W	Amidase (putative amidase)	(Gromadka et al., 1996, Matsuyama et al., 2006),
WB-marker-II	YCL073C	Glutathione exchanger	(Dhaoui et al., 2011, Engel et al., 2014, Gromadka et al., 1996, Oliver et al., 1992)

The WB_marker-I gene is predicted to encode for a putative amidase (EC 3.5.1.4) and is called *AMD2* (AMiDase2), which is predicted to encode for the hydrolysis of aliphatic acylamides to the corresponding carboxylic acids and ammonia. The WB_marker-II encodes a glutathione exchanger, *GEX1* (Glutathione EXchanger 1) and has a potential role in resistance to oxidative stress.

4.3.2 PCR screening on a small set of strains

The first approach was to test the DMGs on two strains from different beer styles, namely TMW 3.0250 (wheat beer) and TMW 3.0256 (German Alt-Kölsch) (Figure 4.20). The PCR screening of the small set confirmed our prediction that those genes were only present within TMW 3.0250. For further experiments, strain TMW 3.0250 (wheat beer) was used as positive control and TMW 3.0256 (German Alt-Kölsch) represented the negative control.

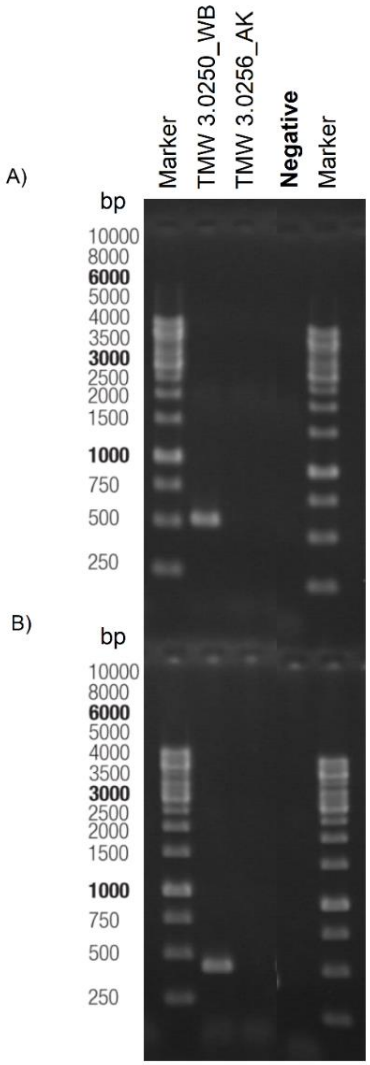

Figure 4.20: PCR-Screening of DMGs for two yeast strains of different beer styles including A) *AMD2* and B) *GEX1*. N = negative control; TMW = Technische Mikrobiologie Weihenstephan; WB = wheat beer; AK = German Alt-Kölsch; bp = base pair; Marker = molecular weight marker showing the range from 250 –10000 bp

Subsequently, the PCR screening was expanded to the 25 *S. cerevisiae* strains, where the genomes were used for the BADGE analysis. Figure 4.21 illustrates the results of the PCR screening.

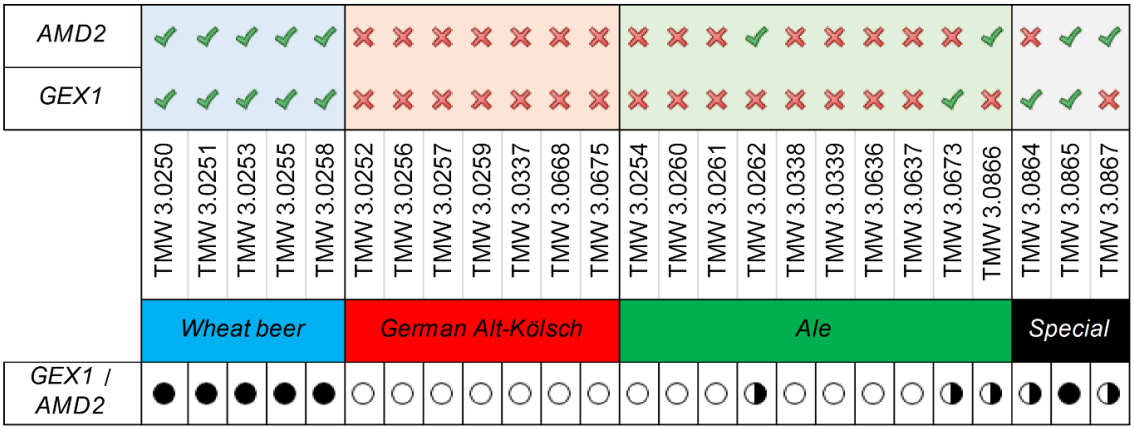

Figure 4.21: Overview of DMGs for 25 *S. cerevisiae* brewing yeast strains. A green check mark corresponds to the presence of a DMG and a red cross to absence; The special group contains two Belgium brewing strains and one strain from South Africa; circles describe the presence of DMGs: black circle = both genes are available; circle half black and white = one of the DMGs is present; white circle = no genes are found; TMW = Technische Mikrobiologie Weihenstephan

Both genes were found in all wheat beer strains as well as in one Belgian strain (TMW 3.0865). Moreover, only *AMD2* was found in two strains of the Ale style (TMW 3.0262 and TMW 3.0866) and in the African strain TMW 3.0867. The second gene, *GEX1*, was present in one Ale strain (TMW 3.0673) and one Belgian strain (TMW 3.0864). Both DMGs were absent in all German Alt-Kölsch strains and 70% of all Ale style strains.

Table 4.10 shows the results of Spearman's rank correlation and Fisher's exact test for the tested DMGs. *AMD2* and *GEX1* display a significant relation to all beer styles except German-Alt-Kölsch and Ale. Furthermore, the absence of both genes discriminates the German-Alt-Kölsch and Ale from wheat beer and special styles likewise.

Table 4.10: Statistical analysis of DMG screening of 25 brewing yeast strains of *S. cerevisiae*. Spearman's rank correlation of DMG presence to the wheat beer style. Fisher's P with respect to classification of wheat beer and special strains, are listed in order to illustrate the quality of the tested DMGs after the PCR-screening. The combination was used to check the potential of both DMGs for the discrimination to wheat beer and special strains. The percentage of correct identifications is listed for each DMG and the combination, based on the assumption that both DMGs are suited for the discrimination of beer styles. Further, fractions of false positive and false negative results are given, also regarding the classification of the beer styles.

DMG designation	**Fisher's P**	**Spearman's ρ**	**Correct discrimination (%)**	**False positive (x/8)**	**False negative (x/17)**
AMD2	0.00217	0.89	88	2/8	1/17
GEX1	0.00013	0.77	92	1/8	1/17
Combination of *AMD2* and *GEX1*	0.00016	1	92	0/8	2/17

4.3.3 Expansion of the PCR screening

The PCR screening was expanded to 83 yeast strains comprising different beer styles with 38 top-fermenting *S. cerevisiae* strains (10 wheat beer, 11 Ale, 13 German Alt-Kölsch, four Special), 21 bottom-fermenting *S. pastorianus* strains, seven *S. cerevisiae* var. *diastaticus* strains and 17 non-brewing yeast strains of *S. cerevisiae* (bakery, winery, distillery, sparkling wine, special wine, laboratory). Figure 4.22 illustrates the results of the expanded PCR screening. The percentage distribution of the genes within the single application types is shown in Figure 4.23. A closer look at the percentage distribution for non-brewing strains for both DMGs is given in Figure 4.24.

Considering the top-fermenting brewing yeast strains, all wheat beer strains possess both DMGs (Figure 4.22) similar to the small screening (Figure 4.21). Except for six strains, which were either positive for *AMD2* or *GEX1* (Figure 4.22), all strains of the German Alt-Kölsch and eight of the Ale style were negative for both genes (Figure 4.22). Regarding *S. cerevisiae* var. *diastaticus*, 86% were positive for both genes (Figure 4.22 and Figure 4.23). Furthermore, a variable distribution of both DMGs was detected within the non-brewing yeast strains of *S. cerevisiae*. 88 % non-brewing yeast strains show positive results for *GEX1*, which is displayed in Figure 4.23. It is noticeable that all *S. cerevisiae* applied in the winery section (wine, sparkling wine, special wine) possessed to 100% *GEX1* (Figure 4.22 and Figure 4.24) while *AMD2* is hardly detected within the different application types in the non-brewing area (Figure 4.24). 20 out of 21 bottom-fermenting *S. pastorianus* strains were found to be positive for *AMD2*. In total, 60 out of 83 yeast strains were positive for at least one DMG. The DNA control of the 23 negative detections on *ITS5* / *ITS4* shows the presence of genomic DNA (Appendix 9.4).

Table 4.11 shows the results of Fisher's exact test for the tested DMGs for all 62 *S. cerevisiae* strains without the bottom-fermenting *S. pastorianus*.

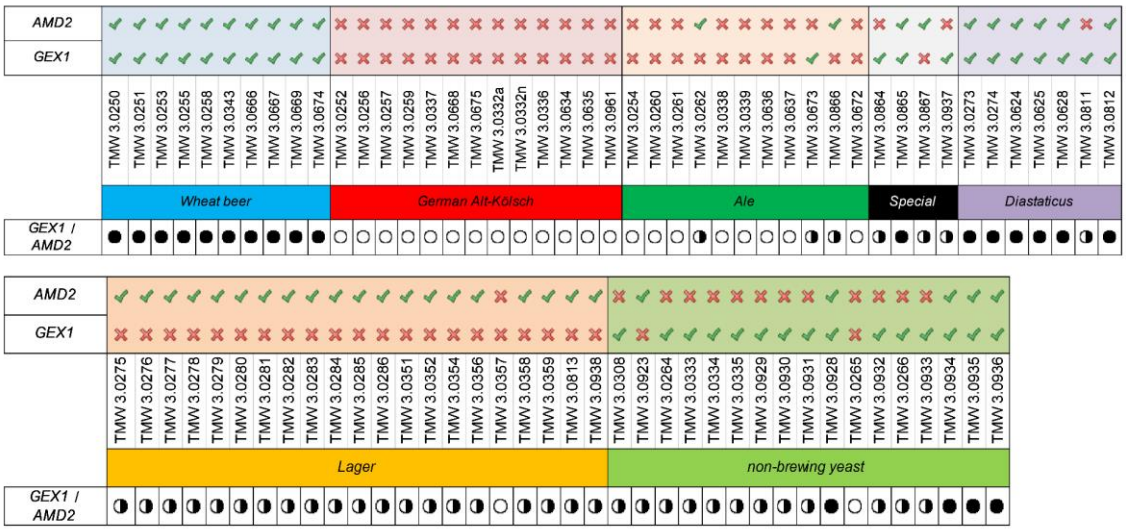

Figure 4.22: Overview of DMGs for 83 *S. cerevisiae* and *S. pastorianus* strains. A green check mark corresponds to the presence of a DMG and a red cross to absence; The part special contains three Belgian brewing strains and one strain from South Africa; circles describe the presence of DMGs: black circle = both genes are available; circle half black and white = one of the DMGs is present; white circle = no genes are found; TMW = Technische Mikrobiologie Weihenstephan

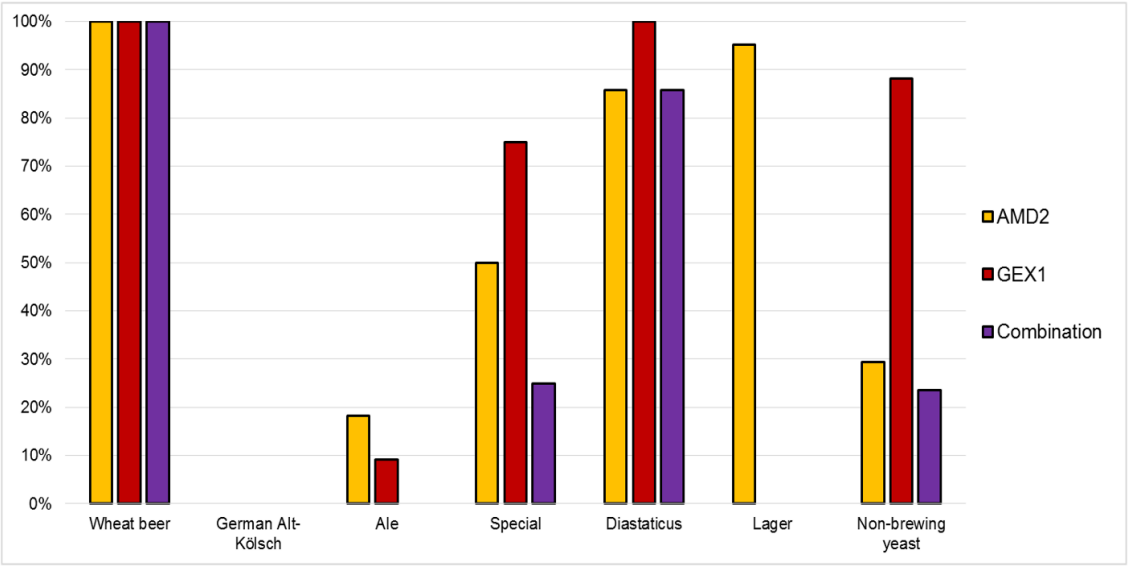

Figure 4.23: Percentage distribution of the diagnostic marker genes within the analyzed beer styles and non-brewing yeasts. The key on the right hand side displays the genes and the combination of both markers. Special includes four strains: three Belgian strains and one strain from South Africa

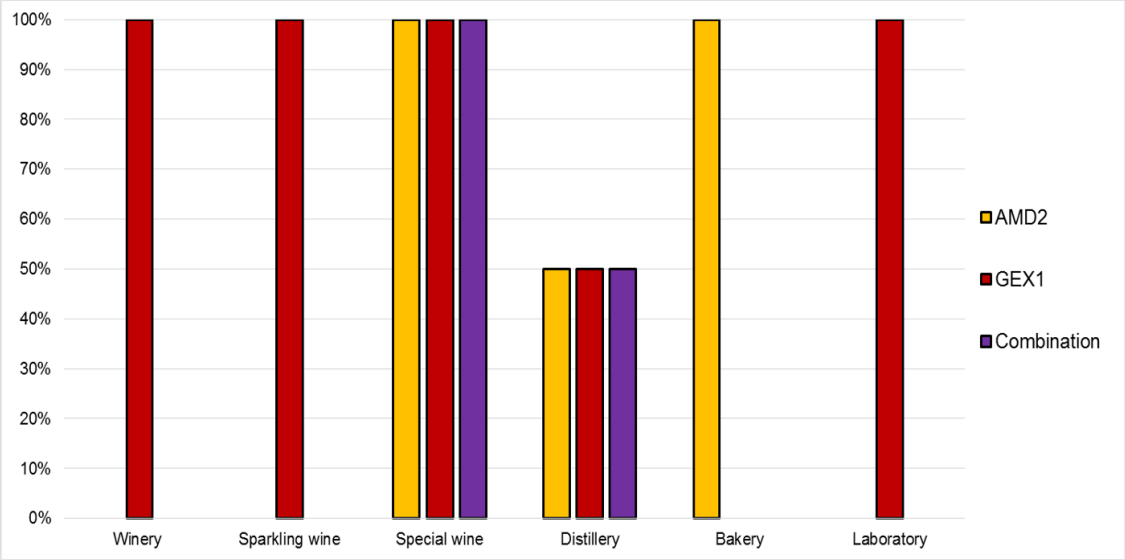

Figure 4.24: A zoom-in of the percentage distribution of DMGs within the 17 non-brewing yeast strains. The key on the right hand side displays the genes and the combination of both markers.

Table 4.11: Statistical analysis of DMG evaluation of all 62 *S. cerevisiae* strains. Fisher's P with respect to the classification of beer styles, if no genes are present: a) German Alt-Kölsch, Ale style and non-brewing yeast; b) German Alt-Kölsch and Ale style. The combination was used to check the prediction to German Alt-Kölsch, Ale style and non-brewing yeast strains. The percentage of correct identifications is listed for each DMG and combination, based on the assumption that each DMG is made for the discrimination of beer styles. Further fractions of false positive and false negative results are given, also regarding the classification of the beer styles.

DMG designation	**Fisher's P**	**Correct discrimination (%)**	**False positive (x/24 or 41)**	**False negative (x/21 or 38)**
***AMD2* (a)**	2.09E-07	84	7/41	3/21
***GEX1* (a)**	2.12E-05	73	16/41	1/21
***GEX1* (b)**	9.69E-13	94	1/24	3/38
Combination of *AMD2* and *GEX1*	3.37E-08	87	4/41	4/21

4.3.4 Overview of DMGs for wild yeasts

After the detection of DMGs within industrial strains, the PCR screening was expanded to the six wild yeasts (Table 3.4). Figure 4.25 illustrates the results of the PCR screening of wild yeasts.

Considering the total count, two out of six strains possess DMGs. *GEX1* was found in *S. cerevisiae* TMW 3.0897 and *S. pastorianus* TMW 3.0909 has *AMD2*. All four *S. paradoxus* are negative for both DMGs. The DNA control of the four negative detections on *ITS5* / *ITS4* shows the presence of genomic DNA (Appendix 9.4).

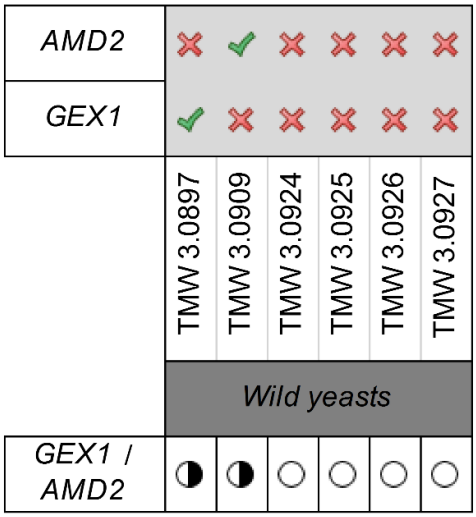

Figure 4.25: Overview of DMGs for six wild yeast strains. A green check mark corresponds to the presence of a DMG and a red cross to absence; circles describe the presence of DMGs: black circle = both genes are available; circle half black and white = one of the DMGs is present; white circle = no genes are found; TMW = Technische Mikrobiologie Weihenstephan

4.4 Correlation of one DMG to a phenotypic characteristic by using a selective media

This proof-of-concept study shows the possible correlation of *GEX1* to a phenotypic characteristic by using a selective media. The presence of *GEX1* seems to improve the tolerance of *S. cerevisiae* against the toxic heavy metal Cd_2SO_4 (Dhaoui et al., 2011). A plating test was implemented to check the tolerance against Cd_2SO_4 for eight *S. cerevisiae* strains from different ecotypes (wheat beer, German Alt-Kölsch, Ale, *S. cerevisiae* var. *diastaticus*), which are either positive or negative for *GEX1* (Figure 4.22). Furthermore, the tolerance of those eight strains to a concentration row of Cd_2SO_4 from 5 nM to 5 mM was considered as well.

4.4.1 Cd_2SO_4 tolerance test

The tolerance to a Cd_2SO_4 concentration of 5 µM of two *S. cerevisiae* strains is shown in Figure 4.26 by a preliminary tolerance test. The analyzed yeast strains were spread out with droplets on YPD (left side) and YPD inclusive 5 µM Cd_2SO_4 (right side) with different dilutions. As a result, a resistance to Cd_2SO_4 was detected for the German Alt-Kölsch strain TMW 3.0256 (Figure 4.26, top right). The wheat beer strain TMW 3.0250 showed no growth at any dilution (bottom right). Considering the control plate of this strain (bottom left part), a clear inhibitory effect of Cd_2SO_4 was determined on TMW 3.0250 (lower right part).

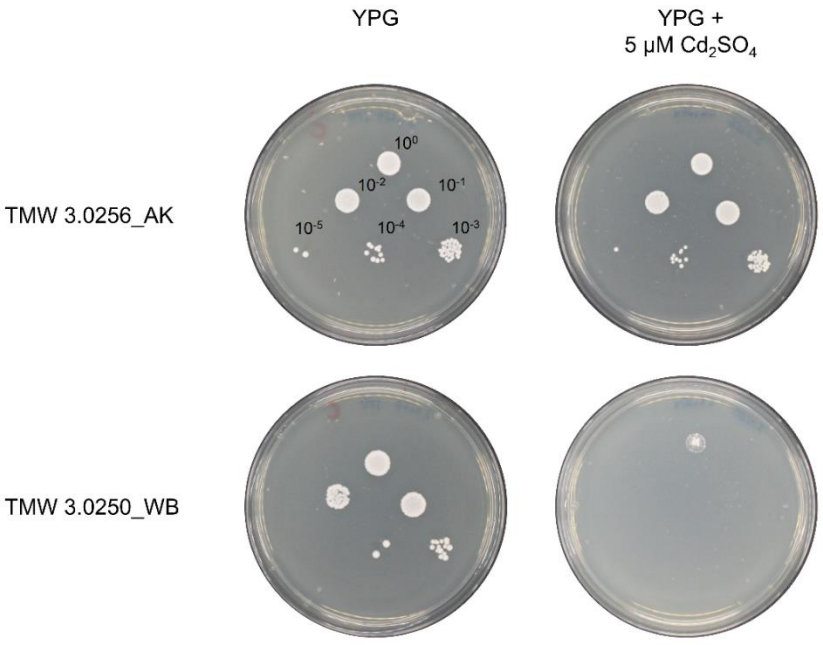

Figure 4.26: Preliminary tolerance test with two *S. cerevisiae* strains on YPD (control) and YPD containing 5 µM Cd_2SO_4. The plates were labeled with the strain ID (TMW = Technische Mikrobiologie Weihenstephan) as well beer style (WB = wheat beer; AK = German Alt-Kölsch)

Based on these findings, the tolerance test was expanded to eight strains from different beer styles and *S. cerevisiae* var. *diastaticus*. Thereby, three biological replicates with technical triplicates were done to get a total count of nine samples per strain as well as plates with or without the additive. After two days of incubation, the results were evaluated visually and compared to each other. Figure 4.27 represents the results of all eight strains on YPD (a) and YPD with Cd_2SO_4 (b). Considering the influence of Cd_2SO_4 for all strains, a negative effect is observed. The German Alt-Kölsch (TMW 3.0256 and TMW 3.0257), Ale (TMW 3.0261 and TMW 3.0262) and *S. cerevisiae* var. *diastaticus* (TMW 3.0273 and TMW 3.0274) strains have all smaller colonies at the dilution 10^{-4} compared to the control plates at the same dilution (Figure 4.27). However, the wheat beer strains (TMW 3.0250 and TMW 3.0251) were affected by Cd_2SO_4 at every dilution and showed no growth (Figure 4.27).

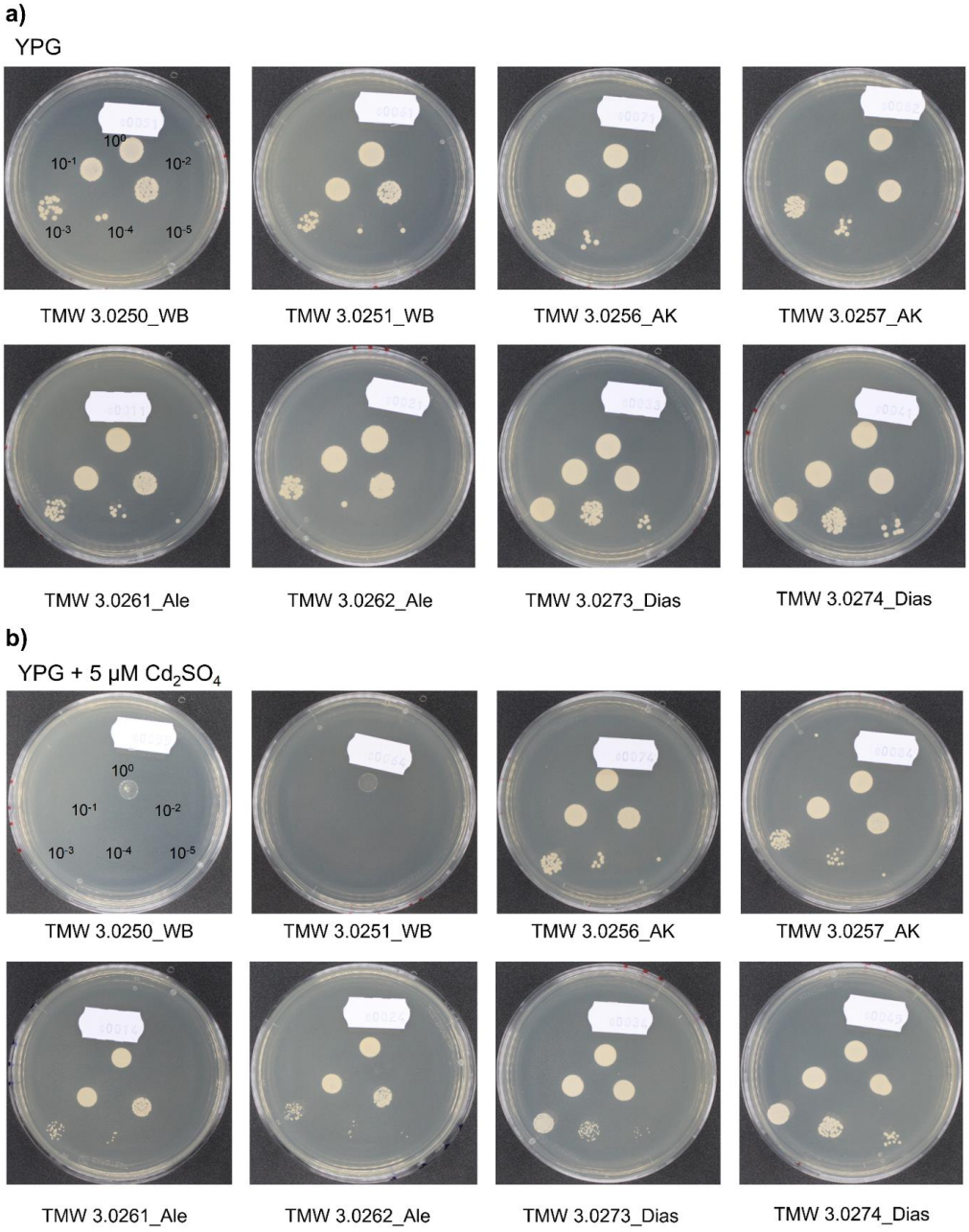

Figure 4.27: Plating test with eight *S. cerevisiae* (var. *diastaticus*) strains on **a)** YPD (control) and **b)** YPD containing 5 µM Cd_2SO_4. The plates were labeled with the strain ID (TMW = Technische Mikrobiologie Weihenstephan) as well ecotype (WB = wheat beer; AK = German Alt-Kölsch; Dias = *S. cerevisiae* var. *diastaticus*)

4.4.2 Tolerance to different concentrations of Cd_2SO_4

After the tolerance test on YPD plates, the tolerance to Cd_2SO_4 concentrations from 5 nM to 5 mM in liquid medium was tested using a microwell plate. Pictures were taken for a visual evaluation of the stress response every 24 h (Figure 4.28 and Appendix 9.5). Figure 4.28 shows the growth behavior of eight strains after 96 h, which includes three biological replicates per strain. The other incubation times from 0 h to 72 h are illustrated in Appendix 9.5. Furthermore,

the optical density was measured every 24 h checking the results of the visual evaluation. Figure 4.29 presents the results of the optical density for all strains (a to d) and highlights the growth behavior within the stressful environment with Cd_2SO_4 after 48 h (green) , 72 h (blue) and 96 h (orange).

Regarding Figure 4.28 and Figure 4.29, it is shown that all strains are negatively affected by Cd_2SO_4 at a concentration between 500 µM to 50 µM. The wheat beer strains (TMW 3.0250 and TMW 3.0251) possess the lowest tolerance to Cd_2SO_4, which is observed in Figure 4.28 and Figure 4.29a. At a concentration up to 5 µM no growth is observed visually (Figure 4.28) and by measurement of the optical density (Figure 4.29a). All other strains of German Alt-Kölsch, Ale and *S. cerevisiae* var. *diastaticus* grew at higher Cd_2SO_4 concentrations (Figure 4.28 and Figure 4.29).

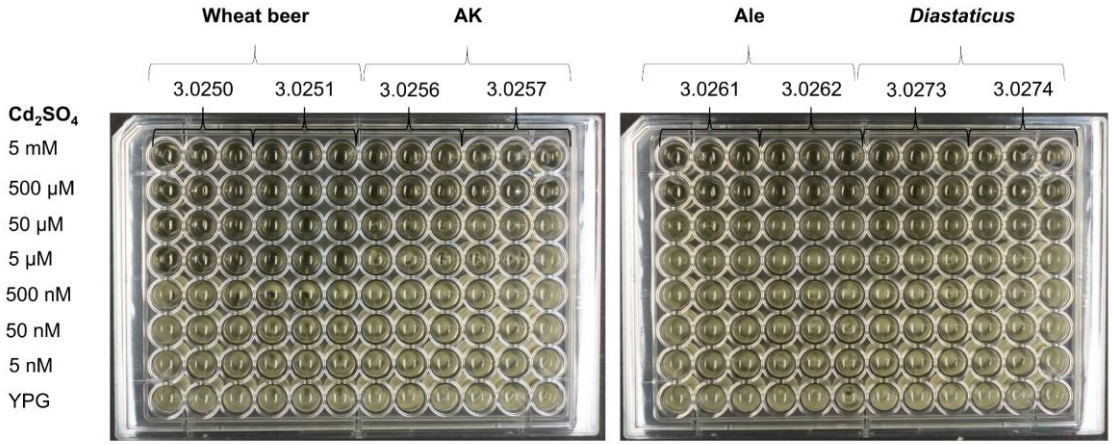

Figure 4.28: Visual evaluation of the growth behavior in a microwell plate after 96 h. All yeasts are labeled with the strain ID (TMW = Technische Mikrobiologie Weihenstephan) and their ecotype, which is indicated above (AK = German Alt-Kölsch; Diastaticus = S. cerevisiae var. diastaticus); on the left side the Cd_2SO_4 (cadmium sulfate) concentrations are listed including the positive control YPD without Cd_2SO_4; three biological replicates were analyzed per strain

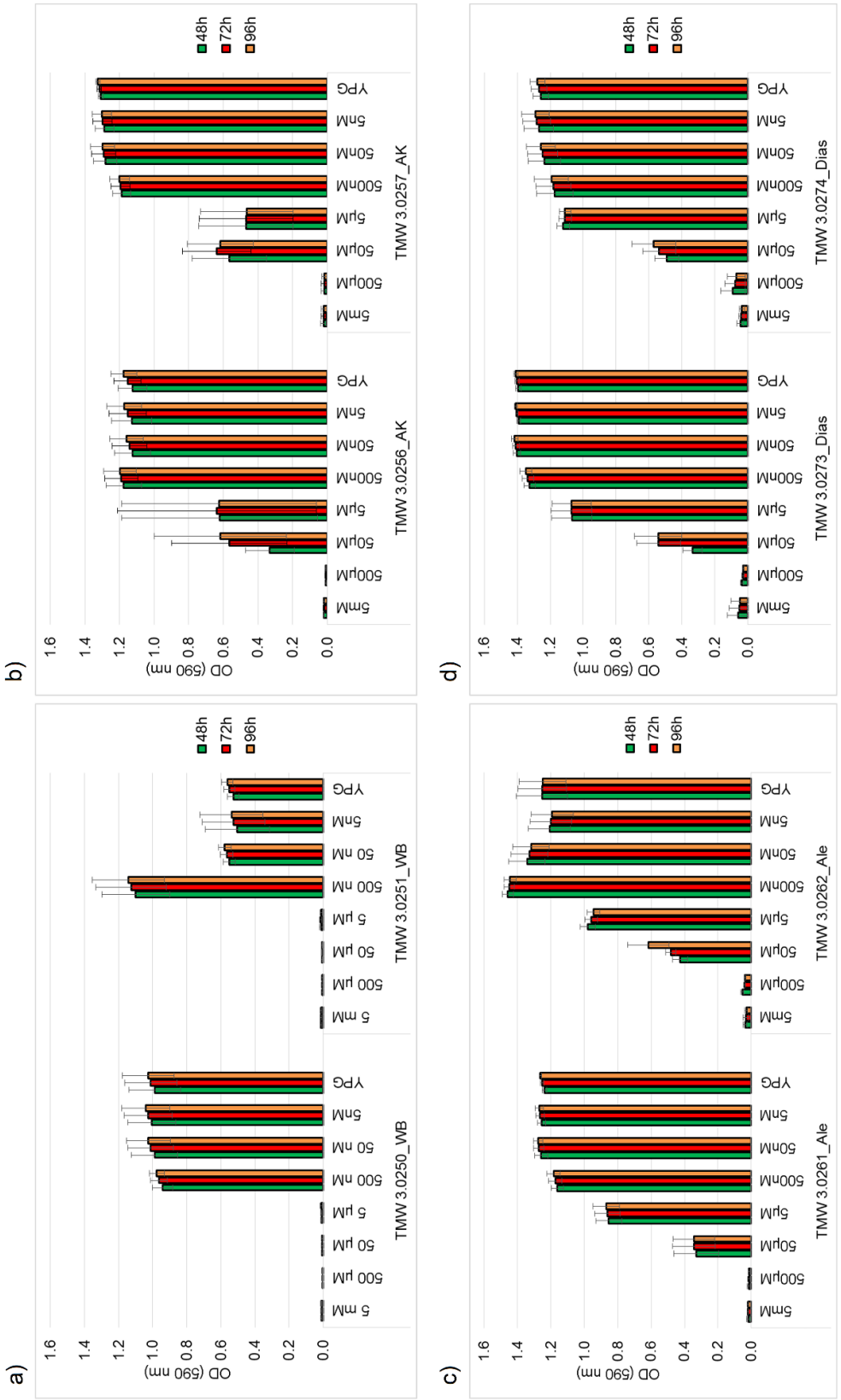

Figure 4.29: Optical density (OD) of eight yeast strains at different Cd_2SO_4 concentrations after 48 h (green), 72 h (blue) and 96 h (orange). **a)** wheat beer (WB) strains; **b)** German Alt-Kölsch (AK) strains; **c)** Ale strains; **d)** *S. cerevisiae* var. *diastaticus* (Dias) strains; all yeasts are labeled with the strain ID (TMW = Technische Mikrobiologie Weihenstephan)

4.5 Comparison of molecular classification to application potentials

Finally, all results from the MALDI-TOF MS classification (chapter 4.2), DMG classification (chapter 4.3) and phenotypic characteristics like phenolic off flavors (POF) based on the literature as well as the tolerance to Cd_2SO_4 (chapter 4.4) are listed within Table 4.12. Some results are reflected in this chapter. Later, the results are compared to the true application potential based on the information of the yeast supplier (personal communication to Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany) in chapter 5.5.

By consideration of all application potentials, the approach by MALDI-TOF MS enabled a classification of all *Saccharomyces* yeasts to 11 different application potentials including beer styles like wheat beer, German Alt-Kölsch, Ale, Lager and *S. cerevisiae* var. *diastaticus* (high attenuator) (Table 4.12). Furthermore, non-brewing *Saccharomyces cerevisiae* strains could be classified to wine, sparkling wine, special wine, schnapps, bread and laboratory (Table 4.12). The use of MALDI-TOF MS allows the identification of *S. cerevisiae*, *S. pastorianus*, *S. paradoxus* and the variety *S. cerevisiae* var. *diastaticus*. The DMGs *AMD2* and *GEX1* differentiate all *S. cerevisiae* (var. *diastaticus*) to four possible application potentials namely wheat beer, German Alt-Kölsch / Ale, wine and bread. The sup-proteomic data and DMGs are able to classify *S. cerevisiae* strains to the wheat beer style, but MALDI-TOF MS could differentiate between wheat beer and *S. cerevisiae* var. *diastaticus*. Considering the phenotypic characteristics, most wheat beer strains are POF positive, but are more sensitive to Cd_2SO_4 than *S. cerevisiae* var. *diastaticus* (Table 4.12). A similar finding was observed for German Alt-Kölsch and Ale strains with the sub-proteomic approach and the DMGs. Most of those strains produce no POF (Table 4.12). One exception is TMW 3.0262, an Ale strain. It is POF positive, classified as an Ale strain by MALDI-TOF MS and matched to the non-brewing application of bread using the DMGs (Table 4.12). This is also the case for TMW 3.0866 as well as TMW 3.0867.

Both methods, MALDI-TOF MS and DMGs were able to classify *S. cerevisiae* strains to non-brewing applications like wine, which is especially observed for the wild yeast strain TMW 3.0897 (Table 4.12). Furthermore, one wild strain could be classified as *S. pastorianus*, which possesses *AMD2* and was identified as this species by MALDI-TOF MS with a sufficiently high score. The four *S. paradoxus* strains were negative for both DMGs and distinguish from all other yeast strains on sub-proteomic level.

Table 4.12: Comparison of industrial classification to the results of molecular characterization. All strains are listed with their species or variety, strain coding (TMW), industrial classification (personal communication to BLQ), MALDI-TOF MS classification and classification by DMGs. Furthermore, physiological properties are included like phenolic off-flavors (POF) based on the literature Goncalves et al. (2016)[1], Meier-Dörnberg et al. (2017a)[2] as well as Meier-Dornberg et al. (2018)[3], which are visualized by + (positive) and – (negative). The tolerance to 5 µM Cd_2SO_4 is presented by a check mark (detection of growth) and X (no growth). All grey colored areas symbolize no further information. TMW = Technische Mikrobiologie Weihenstephan; WB = wheat beer; AK = German Alt-Kölsch (Goncalves et al., 2016); Dias = *S. cerevisiae* var. *diastaticus*; HT = high attenuator; LgS = lager-specific (Monerawela et al., 2015)[4]

Species or variety of *Saccharomyces*	TMW	Industrial classification	MALDI-TOF MS classification	DMGs *AMD2* / *GEX1*	POF	Tolerance to 5 µM Cd_2SO_4
S. cerevisiae	3.0250	WB	WB	WB	+ [1]	X
S. cerevisiae	3.0251	WB	WB	WB	+ [1]	X
S. cerevisiae	3.0252	Alt beer	AK	AK / Ale	- [1]	
S. cerevisiae	3.0253	WB	WB	WB	+ [1]	
S. cerevisiae	3.0254	Kölsch	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0255	WB	WB	WB	+ [1]	
S. cerevisiae	3.0256	Kölsch	AK	AK / Ale	- [1]	√
S. cerevisiae	3.0257	Alt beer	AK	AK / Ale	- [1]	√
S. cerevisiae	3.0258	WB	WB	WB	+ [1]	
S. cerevisiae	3.0259	Alt beer	AK	AK / Ale	- [1]	
S. cerevisiae	3.0260	Ale	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0261	Ale	Ale	AK / Ale	- [1]	√
S. cerevisiae	3.0262	Ale	Ale	Bread	+ [1]	√
S. cerevisiae	3.0332a	Kölsch	AK	AK / Ale		
S. cerevisiae	3.0332n	Kölsch	AK	AK / Ale		
S. cerevisiae	3.0336	Alt beer	AK	AK / Ale		
S. cerevisiae	3.0337	Alt beer	AK	AK / Ale	- [1]	
S. cerevisiae	3.0338	Ale	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0339	Ale	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0343	WB	WB	WB		
S. cerevisiae	3.0634	Alt beer	AK	AK / Ale		
S. cerevisiae	3.0635	Alt beer	AK	AK / Ale		

S. cerevisiae	3.0636	Ale	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0637	Ale	Ale	AK / Ale	- [1]	
S. cerevisiae	3.0666	WB	WB	WB		
S. cerevisiae	3.0667	WB	WB	WB		
S. cerevisiae	3.0668	Ale	AK	AK /Ale	- [1]	
S. cerevisiae	3.0669	WB	WB	WB		
S. cerevisiae	3.0672	Ale	Ale	AK / Ale		
S. cerevisiae	3.0673	Ale	Ale	Wine	+ [1]	
S. cerevisiae	3.0674	WB	WB	WB		
S. cerevisiae	3.0675	Alt beer	AK	AK / Ale		
S. cerevisiae	3.0961	Kölsch	AK	AK / Ale		
S. cerevisiae	3.0864	Lambic	AK	Wine	- [1]	
S. cerevisiae	3.0865	Belgian Beer	AK	WB	+ [1]	
S. cerevisiae	3.0866	WB	Ale	Bread	+ [1]	
S. cerevisiae	3.0867	Opaque	Ale	Bread	+ [1]	
S. cerevisiae	3.0937	Wit beer	AK	Wine		
S. cerevisiae* var. *diastaticus	3.0273	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	√
S. cerevisiae* var. *diastaticus	3.0274	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	√
S. cerevisiae* var. *diastaticus	3.0624	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	
S. cerevisiae* var. *diastaticus	3.0625	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	
S. cerevisiae* var. *diastaticus	3.0628	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	
S. cerevisiae* var. *diastaticus	3.0811	Spoilage	Dias / HT	Wine	+ [3]	
S. cerevisiae* var. *diastaticus	3.0812	Spoilage	Dias / HT	WB (*STA1*?)	+ [3]	
S. pastorianus	3.0275	Lager	Lager	LgS[4] (*AMD2*)	- [2]	
S. pastorianus	3.0276	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0277	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0278	Lager	Lager	LgS[4] (*AMD2*)		

S. pastorianus	3.0279	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0280	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0281	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0282	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0283	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0284	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0285	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0286	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0351	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0352	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0354	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0356	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0357	Lager	Lager	AK / Ale		
S. pastorianus	3.0358	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0359	Lager	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0813	Spoilage	Lager	LgS[4] (*AMD2*)		
S. pastorianus	3.0938	Lager	Lager	LgS[4] (*AMD2*)	- [2]	
S. cerevisiae	3.0264	Wine	Wine	Wine		
S. cerevisiae	3.0265	Distillery	Distillery	AK / Ale		
S. cerevisiae	3.0266	Sparkling wine	Sparkling wine	Wine		
S. cerevisiae	3.0308	Laboratory	Laboratory	Wine		
S. cerevisiae	3.0333	Wine	Wine	Wine		
S. cerevisiae	3.0334	Wine	Wine	Wine		
S. cerevisiae	3.0335	Wine	Wine	Wine		
S. cerevisiae	3.0923	Bakery	Bakery	Bread		
S. cerevisiae	3.0928	Distillery	Distillery	WB		
S. cerevisiae	3.0929	Wine	Wine	Wine		

S. cerevisiae	3.0930	Wine	Wine	Wine	
S. cerevisiae	3.0931	Wine	Wine	Wine	
S. cerevisiae	3.0932	Sparkling wine	Sparkling wine	Wine	
S. cerevisiae	3.0933	Sparkling wine	Sparkling wine	Wine	
S. cerevisiae	3.0934	Rice wine	Special wine	WB	
S. cerevisiae	3.0935	Banana wine	Special wine	WB	
S. cerevisiae	3.0936	Corn wine	Special wine	WB	
S. cerevisiae	3.0897	Fermented grape	Wine	Wine	
S. pastorianus	3.0909	Fermented raisin	Lager	LgS^4 (*AMD2*)	
S. paradoxus	3.0924	Oak bark	WI	AK / Ale	
S. paradoxus	3.0925	Apple	WI	AK / Ale	
S. paradoxus	3.0926	Hop	WI	AK / Ale	
S. paradoxus	3.0927	Hop	WI	AK / Ale	

5 Discussion

In this work, a molecular characterization of yeast strains with respect to their application potential was achieved. All methods could successfully be used for a classification to specific applications or variety level e.g. top-fermenting beer styles like wheat beer, German Alt-Kölsch, Ale and *S. cerevisiae* var. *diastaticus*. Furthermore, bottom-fermenting *S. pastorianus* strains and non-brewing *S. cerevisiae* strains could be distinguished from *S. cerevisiae* strains, which are applied in the brewing environment. Thereby, the approaches on sub-proteomic, genetic and phenotypic characteristic displayed different results, which are discussed separately. Subsequently, the results are taken together, compared to the true industrial application and shall show the importance of a complete characterization of yeasts of the genus *Saccharomyces*.

5.1 Optimal sample preparation

Even though a protocol for an ethanol / formic acid extraction method is given by Bruker Daltonics (Bruker (2012) Bruker Biotyper 3.1 user manual) it is always useful to optimize such instructions. Over the years, sample preparations as well as optimized conditions for yeasts were tested for an optimized and standardized record of sub-proteomic spectra by MALDI-TOF MS. Those tests were e.g. preparation methods (Moothoo-Padayachie et al., 2013, Usbeck et al., 2013), influence of laser energy (Usbeck et al., 2013) and different culturing conditions like various media (Moothoo-Padayachie et al., 2013, Usbeck et al., 2013).

In case of matrix substances, reliable and reproducible spectra were recorded by CHCA (Table 4.1 and Figure 4.1). SA matrix yielded in reliable and reproducible spectra equally to CHCA, however to achieve the recording of mass spectra the laser intensity was adjusted manually to 60%. The laser intensity for CHCA was instead adjusted between 35% and 40% (see chapter 3.4.4). All other matrices were unsuitable for the recording of spectra of yeasts. Furthermore, more sub-proteomics spectra were recorded in the mass to charge range of 2000 m/z to 5000 m/z (Figure 4.1) using CHCA then SA. Moothoo-Padayachie et al. (2013) also visualized spectra recorded with CHCA and SA matrix for *S. cerevisiae* strains. It was shown that sub-proteomic spectra were not as consistent with SA as those generated when employing CHCA (Moothoo-Padayachie et al., 2013). Within other approaches SA matrix was used to characterize *S. cerevisiae* (Amiri-Eliasi and Fenselau, 2001) or *Candida albicans* (Qian

et al., 2008). Because of the much more reliable recording of spectra, CHCA matrix was used for the upcoming studies.

No major differences were observed between different ratios of matrix to sample. A higher amount of matrix resulted in a higher number of total recorded peaks, but a bad signal to noise can influence the total count. Differences between peak intensities from 100 to 2000 were not detectable. Due to the high cost of matrix, the ratio of 1 µl matrix to 1 µl sample was chosen.

Overlay techniques like matrix / sample or the mix matrix / sample were unsuitable for a reliable recording of spectra (Figure 4.3). This might be due to insufficient ionization of the sample or the laser destroying the spotted sample during the measurement, both resulting in bad measurements. In contrast, sample / matrix and sandwich method yielded in reliable and reproducible spectra, which do not differ much in the recorded spectra. For the optimal sample preparation the overlay technique of Sample-Matrix was selected because it yielded reproducible spectra. Additionally it is a time saving as well as a more matrix saving method than the sandwich method.

A major optimization of the protocol of the ethanol / formic acid extraction from Bruker Daltonics and Usbeck et al. (2013) couldn't be achieved, however some minor modifications were realized, which enable a standardized preparation. Chapter 4.1.1 illustrates the modified protocol of the ethanol / formic acid extraction for yeasts incubated aerobically in liquid media. The modifications include the mixing time of ultra-pure water, ethanol, formic acid and acetonitrile, which was defined to 5 min to achieve a better resuspension of the yeast pellet. The volume of 70% formic acid and acetonitrile was specified to 50 µl, as described by Moothoo-Padayachie et al. (2013). Finally, 1 ml liquid culture was transferred into a 1.5 ml tube instead of 900 µl (Usbeck et al., 2013). This change was applied for of an easy handling of all samples. Because of those simple modification, a time table (Table 5.1) can be illustrated for the sample preparation and shows that only the preparation needs 60 min for one sample.

Table 5.1: Time table for sample preparation. The table lists the different steps of the sample preparation and the duration of each step.

Step of sample preparation	Duration in minute [min]
Discard of media supernatant by 2 times centrifugation	4
Resuspension of yeast pellet in ultra-pure water by vortexing	5
Addition of absolute ethanol and mixing	5
Discard of ethanol supernatant by 2 times centrifugation	4
Air drying of yeast pellet to remove ethanol	30
Resuspension of dried yeast pellet in 70% formic acid by vortexing	5
Addition of acetonitrile and vortexing	5
Centrifugation	2
Total time for sample preparation	60

The preparation of more parallel samples needs more time approx. e.g. 90 min for 24 samples. This timeline shows that a standardized sample preparation for database entries or typing of yeasts is fast, inexpensive and easy to learn. Subsequently, for typing experiments within this study all yeast samples were prepared using this standard procedure, further described in chapter 4.1.1.

5.2 Classification of yeasts by MALDI-TOF MS

In this section, the discriminatory power of MADLI-TOF MS is discussed with regard to the application potential of *Saccharomyces* yeast. It shall be shown that on the basis of sub-proteomic patterns yeasts of *Saccharomyces* can be characterized with respect to their brewing application and that a repeat of a previous classification of top-fermenting strains (Usbeck, 2016) is possible. *S. cerevisiae* is applied not only in the brewing sector but also in the non-brewing area e.g. of wine-making or bread-making. MALDI-TOF MS shall enable the differentiation of strains from these industrial sectors. Furthermore, these strains are also characterized to their application potential. Subsequently, it is shown that six wild yeasts provided by two institutes can be identified and characterized to a focused industrial application. Lastly, all yeast strains were illustrated in a dendrogram to display a clear separation of brewing and non-brewing strains as well as on species level.

To achieve these objectives, a sub-proteomic database was established for brewing and non-brewing yeast strains as well as for *S. cerevisiae* var. *diastaticus*. This database allows the assignment of *Saccharomyces* yeast strains not only to their respective species or variety, but also to a specific application potential. The use of MALDI-TOF MS as a tool to identify microorganisms on a specify level is common application by clinical samples (Yan et al., 2011). However, there are a lot approaches to apply this method for the classification of food fermentation microbiota and starter cultures (Nacef et al., 2017), food spoilage microbiota (Höll et al., 2016) or beverage spoiling strains (Wieme et al., 2014). This sub-proteomic method demonstrates the potential to investigate the influence of different stress responses (Schott et al., 2016, Schurr et al., 2015, Zuzuarregui et al., 2006). Furthermore, it can be used to classify microorganisms into different groups and assign strains to a specific ecotype: classification of *Lactobacillus brevis* strains according to their spoilage potential (Kern et al., 2014a), strain typing of shiga-toxigenic *Escherichia coli* or the sub-proteomic fingerprinting-based classification of wine strains to their application potential (Usbeck et al., 2014). Similar to these scientific works, a classification of industrial *S. cerevisiae* to specific application potentials were achieved, which is observed by the formation of various groups within this study.

5.2.1 Typing of yeasts of the genus *Saccharomyces*

As shown in chapter 4.2, the comparison of brewing yeast strains with the generated database showed a 100% separation on species level. Bottom-fermenting *S. pastorianus* strains such as TMW 3.0275 distinguished from all top-fermenting *S. cerevisiae* strains, because of the

different sub-proteomic patterns. Blattel et al. (2013) showed the discriminatory power of MALDI-TOF MS to different species of the genus *Saccharomyces*, but with another sample preparation. However, it was shown that among other species, *S. cerevisiae* and *S. pastorianus* were distinguishable by MALDI-TOF MS (Blattel et al., 2013). Nevertheless, it is important for the commercial usage in the brewing process to distinguish between those species, because of the different application potentials of top- and bottom-fermenting beer strains. MALDI-TOF MS enables a clear separation between those species. Though a strain level identification couldn't be attained, a formation of groups depending on the application potential was observed for all analyzed strains. Usbeck (2016) presented the separation of *S. cerevisiae* strains to beer styles, but on a smaller scale. The classification of those top-fermenting strains was confirmed and used for further studies. In this case, new top-fermenting yeast strains were characterized to their application potential. However, two strains were assigned to other beer styles than the ones they are currently used for. TMW 3.0668 clustered to German Alt-Kölsch, while it is classified by the experience of brewer as an Ale yeast. The spectra of TMW 3.0668 showed higher similarities to the spectra of German Alt-Kölsch strains than to the Ale yeasts. Goncalves et al. (2016) explained that some strains, which were used for fermenting Alt beer in Germany, are exported to countries like the USA where they are used for the fermentation of other beer styles and could subsequently be assigned to a new beer style such as Ale. This generally suggests that assignment to a beer style need not necessarily restrict the use of a specific yeast exclusively for that beer style. The same may apply to strain TMW 3.0254. This strain was classified as an ale strain, but is applied for the production of a Kölsch. On sub-proteomic level it shows a higher similarity to Ale strains. Besides those findings, the different groupings are also reflected.

At first, the classification of *S. pastorianus* is investigated. The impact of bottom-fermenting yeast strains on lager beer styles is considered in chapter 1.3.1. Besides metabolic differences, bottom-fermenting strains distinguish on one side based on their flocculation behavior. Brewers divide *S. pastorianus* flocculating and powdery (non-flocculation) strains (Verstrepen et al., 2003b). This behavior was used to classify bottom-fermenting strains. Even though different bottom-fermenting yeast strains were assigned to their flocculation behavior, a clear separation within this species could not be attained. In case of the *S. pastorianus* classification, the results visualized that it is not feasible to divide bottom-fermenting yeast strains based on their flocculation behavior. Another approach for the differentiation of *S. pastorianus* strains to a possible brewing application is the differentiation between Saaz and Frohberg-type strains. Those types are hybrids of *S. pastorianus*, which are a result of two separated hybridization

events of *S. cerevisiae* and *S. eubayanus* (Gallone et al., 2017). Furthermore, it was shown that Saaz strains have limited maltotriose utilization and produce less flavor compounds than Frohberg strains (Gibson et al., 2013). MALDI-TOF MS shall be enable a differentiation between those two hybrids, which would enable a classification based on the application potential of *S. pastorianus*. It shall be noted that all analyzed strains of *S. pastorianus* (Table 3.2) belong to the Frohberg-type, which is actually more distributed within the brewing environment worldwide then the Saaz-type (personal communication with Dr. Mathias Hutzler, Research center of Weihenstephan for brewing and food quality, Freising, Germany).

Considering top-fermenting *S. cerevisiae* strains as well as the variety *S. cerevisiae* var. *diastaticus*, a classification to various application potentials and variety level was achieved. Brewing yeasts of *S. cerevisiae* distinguish up to 94% from non-brewing yeasts. In this case, it is shown that within the non-brewing yeast strains a higher strain level identification was observed than in all brewing yeasts. Especially the wine yeasts and the sub-groups of special wine and sparkling wine showed a high strain level identification. Cappello et al. (2004) proposed that the variation of *S. cerevisiae* wine strains come from the representative area of isolation rather of the variety of grapes, because of the adaption i.e. to the specific climatic conditions of one area. All non-brewing yeast strains used within the winery section have different geographic origins (Table 3.3) and therefore other adaptations, which made each strain unique. Stressful industrial conditions as well as the fact of an extensive long-time breeding of a specific laboratory strain might explain the strain level identification of the commercial available bakery strain TMW 3.0923 and the laboratory strain TMW 3.0308 (S288c). However, that does not mean that non-brewing yeast strains can be classified specifically to one vineyard, distillery, bakery or laboratory, which needs to be demonstrated with more strains. This explains to some extent the reasons of the differentiation between non-brewing and brewing *S. cerevisiae* strains which were achieved.

A closer look at all *S. cerevisiae* strain from the brewing environment indicates the classification to various beer styles and variety level. The main differences within the brewing yeasts are observed within the wheat beer style, which is separated from all other beer styles. Besides the influence of wheat beer strains to the production of wheat beer explained in chapter 1.3.2, a differentiation is not only detected on sub-proteomic level to other brewing yeast strains. Goncalves et al. (2016) found that based on genomic analysis wheat beer strains could be differentiated from all other beer styles like English-Irish Ale and German Alt-Kölsch. Moreover, Gallone et al. (2016) described the group of wheat beer strains as well by genomic analysis and explained this population structure have highly mosaic genomes, which is a result

of a cross between ale and wine strain. The use of Polymerase Chain Reaction-Denaturing High Performance Liquid Chromatography (PRC-DHPLC) for the differentiation of brewing yeast strains showed that the profiles of wheat beer strains were similar to each other, but differentiate from other beer styles (Hutzler et al., 2010). The MALDI-TOF MS analysis resulted in a clear differentiation between wheat beer strains and other *S. cerevisiae* strains. This was in accordance to the other applied experiments.

A differentiation between Alt beer and Kölsch could not be achieved and was explained by recorded spectra. The mass spectra showed only a small deviation from each other and might be caused by the different geographical use of these strains (see chapter 1.3.3). Using genomic analysis (Goncalves et al., 2016) and PRC-DHPLC (Hutzler et al., 2010) no major differences were detected between these two beer styles as was the case for MALDI-TOF MS. In fact, brewing yeasts related to these beer styles can be used for Alt beer as well as Kölsch production and may be looked at as one group as proposed by Goncalves et al. (2016) with "German Alt-Kölsch". Because of the historic cultural "rivalry" between Cologne and Dusseldorf, the breweries of these cities actually use either a Kölsch or Alt beer-strain for their different beer styles (personal communication with Dr. Mathias Hutzler, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany). However, brewers started to create a new beer style, which combines the characteristics of Kölsch and Alt beer and called is "Költ" (Christiansen, 2018). However, the applied *S. cerevisiae* strain is unknown, but can be characterized to one specific application potential by MALDI-TOF MS.

The wide variety of Ale strains can also be explained by the sub-proteomic pattern. The investigated ale strains differ in having a high (e.g. TMW 3.0262) and a small (e.g. TMW 3.0261) amount of low molecular sub-proteins. Furthermore, these strains strongly distinguish from wheat beer strains. Several yeasts related to the Ale beer style cluster are outliers and are assigned to other beer styles. Considering DHPLC-chromatograms of IGS2-314 rDNA, Ale strains are heterogeneous and different profiles were measured (Hutzler et al., 2010), which is similar to the recorded sub-proteomic patterns by MALDI-TOF MS.

An incomplete separation based on application potentials is observed within the group of special strains that contains some Belgian strains and one Opaque strain. Most of the strains are matched within the German Alt-Kölsch cluster and one strain is classified to the non-brewing sector. A solution for this problem is to expand the amount of recorded spectra. This could be done with *S. cerevisiae* strains used for Lambic, Trappist and Wit beer production, which are

typical in Belgium. Furthermore, the amount of strains used for the production of Opaque beer should be increased likewise to achieve a classification of strains used for this beer style.

Within the brewing environment it is important to identify *S. cerevisiae* var. *diastaticus*, because it causes low specific gravities, an excessive pressure in bottled beer, and phenolic off-flavor (see chapter 1.2.3). On one side, this variety is described as a contamination or spoilage yeast and is undesirable in breweries. But in time of craft brewing *S. cerevisiae* var. *diastaticus* attains an image change, since it is used i.e. for the production of Belgian Saison-style beers. This beer style is characterized by a high attenuation (according to http://www.fermentis.com). This is a mixed blessing in case of the variety *diastaticus*. For breweries it is quite important to have no contaminations, because of a possible product damage and loss of image (Meier-Dörnberg et al., 2017b). MALDI-TOF MS enables a rapid differentiation between top-fermenting *S. cerevisiae* and the variety level. However, in the spirit of craft brewing and the development of new beer styles it is possible to match *S. cerevisiae* var. *diastaticus* to a specific application potential. It is useful to classify them according to their common property of high attenuation namely "high attenuator" (HT). This name includes on one side the contamination of *S. cerevisiae* var. *diastaticus*, but on the other hand the application potential to produce beer styles, which are characterized by a dry and winey body with a noticeable phenolic off flavor (Meier-Dornberg et al., 2018).

In case of MALDI-TOF MS, *S. cerevisiae* (var. *diastaticus*) strains were characterized according to their sub-proteomic spectra to various application potentials. This includes different top-fermenting beer styles as well as the potential distinction of brewing and non-brewing yeast strains. With the establishment of a MALDI-TOF MS spectra database based on the genus *Saccharomyces*, unknown samples can be identified on species / variety level and predicted to an application potential in a rapid and easy way.

5.2.2 Classification of wild yeast isolates

The database was applied for the identification and classification of wild yeast isolates. Six yeast strains (Table 3.4) were isolated from various natural sources by the supplier (Table 3.4) and were provided for upcoming analysis by MALDI-TOF MS.

Two out of six samples were identified on species level. The remaining four samples could only be identified on genus level as *Saccharomyces*. Those samples yielded in a mix up of identification results between *S. cerevisiae* and *S. pastorianus*, which is also reflected by the low score values (yellow; probable species identification). This indicates that these strains are

Saccharomyces yeasts, but the species is unclear. After an in-house sequencing, the four strains were matched to *Saccharomyces paradoxus*, which is the closest relative to *S. cerevisiae* (Goddard and Burt, 1999, Johnson et al., 2004, Martini, 1989). The differentiation of *S. paradoxus* to *S. cerevisiae* and *S. pastorianus* is shown within the hierarchical cluster analysis (Figure 4.18), which is visualized by an own clade. Considering those results, MALDI-TOF MS has proven once more its discriminatory power to differentiate on species level between *S. cerevisiae, S. pastorianus* and *S. paradoxus* equally to Blattel et al. (2013). At the end of each identification, the software of MALDI-TOF MS generates a table, which includes the two best identification results as well as a separate top ten list of best matches. Only the observation of the top ten list reveals the mix up between *S. cerevisiae* and *S. pastorianus* for all four *S. paradoxus*. Not only the low score value, but also the identification as two species in the top ten list is a sign of the unreliable identification. It shows that the results of MALDI-TOF MS have to be observed completely. One argument may be a low quality of recorded spectra, which would hinder a comparison to the database entries. However this was not the case. All spectra were recorded automatically and possess a uniform appearance. In fact, the availability of approx. 70 *S. cerevisiae* (combination of Bruker Daltonics and the in-house database) and 21 *S. pastorianus* database entries has to enable a correct identification to this species. The four strains were identified only on genus level, but not on species level. This is similar to the findings of Blattel et al. (2013). All species of *Saccharomyces* were identified by the Bruker Daltonic database, but only a genus level identification was achieved except for *S. cerevisiae* strains. This shows on the one hand side the possibility of MALDI-TOF MS for a fast and reliable identification of unknown samples. On the other side, the disadvantage of this method is highlighted namely the quality of the database. In fact, this part can be avoided with an ongoing expansion of the database to various species of *Saccharomyces* or other microorganisms. Considering the statement before, four database entries were generated for the sequenced wild yeasts of *S. paradoxus,* which will close one small gap within the MALDI-TOF MS database. It shall be noted that for an industrial identification approach it is enough to identify three biological samples of one sample, which are recorded on one day and not on different days. Thereby, a fast identification can be accomplished.

5.2.3 Sub-proteomic biodiversity of *Saccharomyces* on behalf of their application potential

Saccharomyces strains were characterized successfully to various application potentials. Thereby, strains of the brewing environment and non-brewing segment were classified according to their recorded sub-proteomic spectra by MALDI-TOF MS. Legras et al. (2007),

Gallone et al. (2016) and Goncalves et al. (2016) observed on genomic analysis also the separation of *S. cerevisiae* strains to specific industrial application. On the other hand, sub-proteomic spectra of industrial *S. cerevisiae* strains were recorded by MALDI-TOF MS similar to this study and a differentiation to specific fields were achieved by Moothoo-Padayachie et al. (2013). In this scientific work, the wine yeast strains were divided from the brewing yeast strains. The forming of a mixed cluster of different applications was also detected (Moothoo-Padayachie et al., 2013). Considering the dendrogram shown in Figure 4.19, a separation of of *S. cerevisiae* from different applications is achieved similar to Moothoo-Padayachie et al. (2013). Furthermore, a division on species level like Blattel et al. (2013) visualized for all species of *Saccharomyces*, is illustrated also in Figure 4.19. The difference between these three approaches of characterization of *Saccharomyces* strains using MALDI-TOF MS are considered subsequently: (I) Blattel et al. (2013) applied a different sample preparation to analyze the sub-proteomic patterns of *Saccharomyces* strains. Cell material of strains was transferred in test tubes and mixed directly with 70% formic acid, which was followed by acetonitrile addition. For a small set of yeast strains this sample preparation was investigated in the current study, but it wasn't possible to record reliable and automatic spectra by MALDI-TOF MS. Because of that, this sample preparation was not used further. (II) Blattel et al. (2013) and Moothoo-Padayachie et al. (2013) compared the database entries of each *Saccharomyces* species and strain to each other using the MALDI Biotyper 3 software for the creation of a dendrogram. In the current study, 30 single spectra for each strain were recorded to have a quantity of replicates, which covers the variety of peak intensities and mass to charge deviation (600 ppm). The 30 single spectra of each strain were summarized to one mean spectra. These were compared using bioinformatic tools and resulted in the dendrogram shown in Figure 4.19. Figure 4.19 clearly shows the discriminatory power of MALDI-TOF MS on species level. Furthermore, MALDI-TOF MS enables to display the variations within the *S. cerevisiae* strains, which can be labeled to their application sector. The differentiation between brewing and non-brewing *S. cerevisiae* strains is illustrated in Figure 4.17 and discussed in chapter 5.2.1. However, the visual classification in Figure 4.19 is helpful to understand the relation of *S. cerevisiae* strains to their application potential.

5.3 Novel diagnostic marker genes

Considering DMGs for *Saccharomyces* strains, some genes were successfully used to match yeast strains to species level, varieties, stress response to air-drying and groups of hybridization events (Brandl, 2006, Capece et al., 2016, Hutzler, 2009, Monerawela et al., 2015). In this study, two DMGs were successfully applied to perform a classification of *Saccharomyces* strains with respect to their application potential.

5.3.1 PCR-screening

AMD2 putatively encodes for an amidase (EC 3.5.1.4), which belongs to the nitrilase superfamily (Chang and Abelson, 1990, Monerawela and Bond, 2017b, Pace and Brenner, 2001). The enzyme catalyzes the breakdown of aliphatic acylamides to the corresponding carboxylic acids and ammonia (Hirrlinger et al., 1996, Pace and Brenner, 2001, Wu et al., 2017). In this study, 54 % of the tested yeast strains from the species *S. cerevisiae* or *S. pastorianus* possess *AMD2*. Nakao et al. (2009) described the amidase encoded gene as one of the lager-specific genes, which is present in lager brewing yeast but absent in *S. cerevisiae* S288c (TMW 3.0308). Most of the lager brewing yeast strains, except TMW 3.0357, possess this gene, and as Nakao et al. (2009) mentioned, no *AMD2* was found in *S. cerevisiae* S288c (Figure 4.22). Considering *S. cerevisiae*, Monerawela et al. (2015) showed that ale yeast (Foster O) and stout yeasts have different patterns of lager-specific genes. Thereby, *AMD* genes were detected within the *S. cerevisiae* class of stout and other applications (Monerawela et al., 2015). Different industrial *S. cerevisiae* strains were also analyzed and a variation of *AMD2* was found within those strains equal to Monerawela et al. (2015). 25 *S. cerevisiae* strains, mainly used for the production of wheat beer, seven *S. cerevisiae* var. *diastaticus*, some Ale strains and five non-brewing yeast strains have *AMD2*. 38 of the tested strains don't have this gene, which are either classified to be German Alt-Kölsch, Ale or wine strains. This is similar to the results of Monerawela et al. (2015), where they found *S. cerevisiae* strains from the brewery sector without *AMD2*.

The second DMG used to classify most yeasts of chapter 3.1, is *GEX1* (YCL073C) encoding a glutathione antiporter (Dhaoui et al., 2011). Dhaoui et al. (2011) describes the main function of the glutathione exchanger being the import of glutathione from the vacuole and export through the plasma membrane. The proton antiporter is related to resistance to oxidative stress caused by bivalent heavy metals (for example cadmium) or hyper peroxide to achieve a detoxification of the cell (Dhaoui et al., 2011, García et al., 2012, Mendoza-Cozatl et al., 2005, Ortiz et al.,

1992, Stephen and Jamieson, 1996). Compared to *AMD2*, no PCR screening tests for *GEX1* are mentioned till now for yeasts of the genus *Saccharomyces*. Of the tested 83 yeast strains, all bottom-fermenting *S. pastorianus* strains were negative for *GEX1* (Figure 4.22).

In case of *S. cerevisiae* strains, a discrimination to application potentials was discovered. No strain of the German Alt-Kölsch style possesses *GEX1* and approx. 91% of the Ale style are negative as well. In total, 26 of the tested *S. cerevisiae* strains don't have *GEX1*, which are mainly applied in the brewing sector. Considering the remaining 36 positive *S. cerevisiae* strains, three major ecotypes were found namely wheat beer, *S. cerevisiae* var. *diastaticus* and the winery sector, which possess *GEX1*. Dhaoui et al. (2011) used the laboratory strain of *S. cerevisiae* BY4741 to analyze the glutathione exchanger, which is a derivative of S288c. Using the PCR screening *GEX1* was found in S288c (TMW 3.0308), as expected. In all winery yeast strains *GEX1* is detectable, which is suggested to increase the glutathione concentration in wine (Kritzinger et al., 2013a, Kritzinger et al., 2013b). However, the exact mechanism of glutathione export and the relative importance of *GEX1* is still unclear (Cordente et al., 2015, Kritzinger et al., 2013b). In case of S288c, it was possible to verify the PCR screening to the specific DMGs, because of the findings of Nakao et al. (2009), Monerawela et al. (2015) and Dhaoui et al. (2011). The actual origin of the laboratory strain S288c is notable, since it is a derivate of a *S. cerevisiae* (EM93) which was isolated from rotten figs (Landry et al., 2006, Mortimer and Johnston, 1986). *GEX1* shows to be present in *S. cerevisiae* strains which have their origin from fruits for example figs or grapes as well applied in the winery sector.

Both genes were found in all wheat beer strains, which is quite interesting, because of the mosaic genome of those strains. This cross probably took place between a wine and an ale strain of *S. cerevisiae* (Gallone et al., 2016). All wine yeast strains possess *GEX1* as well as wheat beer strains, which seems to be a parental marker for this cross. In case of *AMD2*, it seems that this part comes either from an ale strain of *S. cerevisiae* like Gallone et al. (2016) described or from bread yeasts. Goncalves et al. (2016) showed this using *S. cerevisiae* TMW 3.0866 (TUM 507), a strain with high genomic similarities to wheat beer strains. Actually, two positive results to both DMGs would be expected for this strain, because this strain is used for the wheat beer production, but this was not the case. Only *AMD2* was detectable. The presence of one of these genes might give a clue about this wheat beer hybridization event. All other Ale and special yeast strains with one positive result for *AMD2* have more similarities to the bread clade on a genomic level (Goncalves et al., 2016).

The DMGs enable the classification of domesticated *S. cerevisiae* strains with respect to industrial application types. The wide distribution of the lager-specific gene *AMD2* within industrial yeast strains was shown, similar to Monerawela et al. (2015). Additionally, for the first time the occurrence and absence of *GEX1* was visualized within the testing set. Furthermore, the DMGs can be applied to distinguish a *S. cerevisiae* strain not only to one specific beer style, but also to different application potentials by a PCR-screening. Four different classifications are possible: (1) both DMGs are detectable, which classify one strain very likely to the wheat beer application potential (WBAP); (2) a positive detection for *GEX1* and negative for *AMD2* classify one strain to the non-brewing application potential of wine (NBAP-W); a positive detection for *AMD2* and negative for *GEX1* in *S. cerevisiae* classify one strain to the non-brewing potential of bread with a high probability (NBAP-B); (4) both DMGs are not detectable, which classify a strain with the highest probability to the German Alt-Kölsch / Ale style.

Furthermore, a combination of three DMGs is suggested, which differentiates all wheat beer strains from *S. cerevisiae* var. *diastaticus* strains, because of the presence of *AMD2* and *GEX1* within this groups. The expansion of the PCR screening with *STA1* (Brandl, 2006, Yamauchi et al., 1998) shall distinguish between wheat beer strains and *S. cerevisiae* var. *diastaticus*. Two positive detections for *AMD2* and *GEX1* are expected and a negative one for *STA1* for wheat beer strains. *S. cerevisiae* var. *diastaticus* strains will have three positive results. In case of German Alt-Kölsch / Ale style, the absence of all DMGs is expected.

5.4 Cadmium tolerance of *S. cerevisiae*

Cadmium (Cd^{2+}) is described as one of the most toxic heavy metals and to possess no physiological importance for *S. cerevisiae* (Tchounwou et al., 2012, White and Munns, 1951). It causes oxidative stress (Brennan and Schiestl, 1996), inhibition of protein function / activity (Chrestensen et al., 2000) and impairs DNA repair mechanisms (Bravard et al., 2006, Jin et al., 2003). Another negative effect is the apoptosis of yeast cells (Nargund et al., 2008) including an endoplasmic reticulum stress (Gardarin et al., 2010). The mechanism of cadmium uptake and detoxification of *S. cerevisiae* was studied (Wysocki and Tamas, 2010) and assigned to different transporting systems as well as neutralization through chelation to reduced glutathione (GSH) (De Vero et al., 2017, Gomes et al., 2002, Wysocki and Tamas, 2010). Figure 5.1 shows the general mechanisms of toxic metal uptake and detoxification for *S. cerevisiae* (Wysocki and Tamas, 2010).

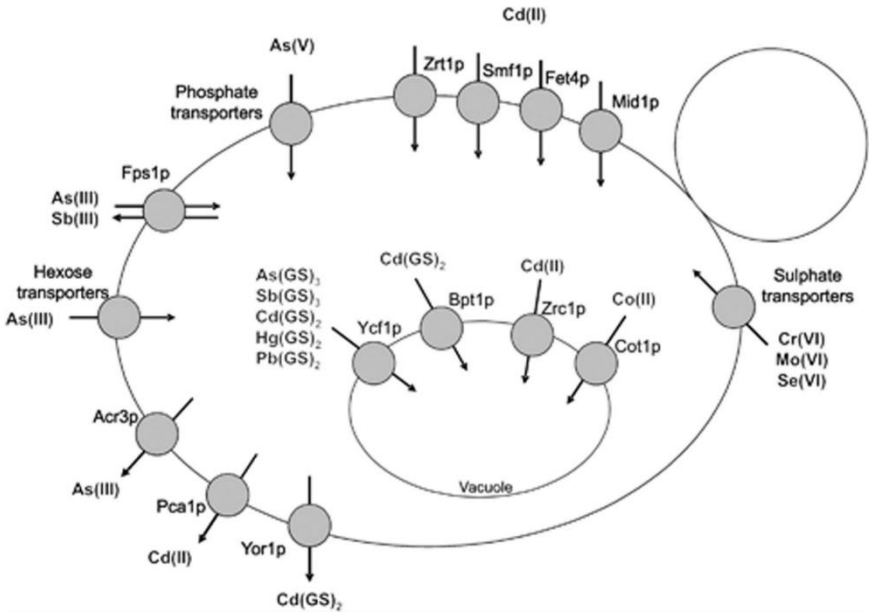

Figure 5.1: Transporters mediating the uptake and detoxification of toxic metals in *S. cerevisiae*, which is illustrated by Wysocki and Tamas (2010). All explanations of chemical and protein abbreviations are given in Wysocki and Tamas (2010).

Besides the cadmium detoxification by different transporting systems like *YOR1* or *YCF1* (Figure 5.1), *GEX1* was described as transporting system for a possible detoxification of cadmium by Dhaoui et al. (2011). In chapter 4.3, *GEX1* was used as a novel DMG to classify *S. cerevisiae* strains with respect to application potentials. Wheat beer strains as well as *S. cerevisiae* var. *diastaticus* possess this gene (Figure 4.22), while it is not present in German Alt-Kölsch and Ale strains. Dhaoui et al. (2011) showed that a laboratory strain possessing this gene grew better on 5 µM Cd_2SO_4 plates than after the deletion of *GEX1*. This leads to the hypothesis that *S. cerevisiae* strains, which possess *GEX1* are less sensitive to a non-lethal concentration (Jin et al., 2003) of 5 µM Cd_2SO_4. In a proof of concept, eight strains (chapter 4.4)

were exposed to different concentrations of Cd_2SO_4. German Alt-Kölsch and Ale strains grew on 5 µM Cd_2SO_4 YPD plates as well at higher concentrations (Figure 4.29). This was unexpected, since these strains don't possess *GEX1*. In contrast, *S. cerevisiae* var. *diastaticus* strains shows to have a high tolerance equal to German Alt-Kölsch and Ale strains, but the variety possess *GEX1*. Wheat beer strains are hypersensitive and differentiate to the other beer styles as well as variety even though *GEX1* is present. This shows that the presence of *GEX1* does not indicate the tolerance to Cd_2SO_4.

Considering these results, an inhibitory effect of Cd_2SO_4 was observed for all strains, which is detectable visually by the formation of smaller colonies (Figure 4.27). Similar to Dhaoui et al. (2011), a negative effect of cadmium was detected. Gallone et al. (2016) showed that many *S. cerevisiae* strains from different industrial clades performed poorly in general stress conditions like Cd_2SO_4 concentrations from 300 µM to 500 µM. However, similar to the proof of concept of chapter 4.4 Gallone et al. (2016) found *S. cerevisiae* strains possessing a higher tolerance to concentrations from 300 µM to 500 µM. In addition, a poor performance was observed for wheat beer strains for the proof of concept approach likewise to Gallone et al. (2016). The main difference between these two studies is that the high sensitivity of wheat beer strains to the usually non-lethal concentration of 5 µM Cd_2SO_4 was visualized by the proof of concept experiment. As described in chapter 5.2.1, wheat beer strains of *S. cerevisiae* are hybrids of two different *S. cerevisiae* strains (Gallone et al., 2016). It seems that a plating test with YPD containing 5 µM Cd_2SO_4 is useful to characterize a *S. cerevisiae* strain to their mosaic genome status, which reflects its potential for the production of wheat beer.

The background of the low tolerance of wheat beer strains to Cd_2SO_4 can only be speculated upon. The genetic background might be one reason. Figure 5.1 illustrates that a lot of transporting systems are either responsible for the uptake or export of cadmium. Moreover, *S. cerevisiae* produces GSH, which plays an important role in the detoxification of toxic heavy metals. One of the substrates for GSH synthesis is cysteine, which requires the sulfate assimilation and cysteine biosynthetic pathways (Mendoza-Cozatl et al., 2005). It is possible that cadmium inhibits the biochemical and genetic regulation of these pathways in wheat beer strains. Another possibility is the fact that cadmium causes oxidative stress and yeasts grown in the absence of oxygen were more resistant to the heavy metal (Brennan and Schiestl, 1996). In relation to the traditional wheat beer production, the fermentation of this top-fermenting beer style is realized by means of an open vessel (Kunze and Manger, 2011), because of that there is an ongoing exposure to oxygen. In contrast, the fermentation of other beer styles is applied in closed tanks, which might cause a better resistance to cadmium. A possibility to confirm the

oxidative stress proposal is to expose wheat beer strains to different concentrations of hydrogen peroxide (H_2O_2) (Dhaoui et al., 2011), which is not toxic and not carcinogenic like cadmium. The expected results shall be that all wheat beer strains are inhibited by a low concentration of H_2O_2 and other brewing yeast strains will possess a high tolerance to the stressful environment. This will characterize *S. cerevisiae* to the wheat beer application style and differentiate them from other application potentials by a selective media.

5.5 Differences and overlapping of all classifications to their true application potentials

The "golden-standard" for the classification of *S. cerevisiae* to application potentials are sensorial and technological tests e.g. pilot fermentations (personal communication to Mathias Hutzler, Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany), which shows the final impression to each product. Though this method is laborious, time-consuming, expensive, and the classification to a specific application depends on the experience of the testing panel, which typically includes trained employees. Furthermore, a result is neither achieved with respect to species level nor the genomic background of an applied yeast.

The various molecular characterizations of yeasts of the genus *Saccharomyces* showed that every method on sub-proteomics, genetic or physiological properties enables a classification of yeasts with respect to their application potential. A classification of the *Saccharomyces* strains was achieved on genomics similar to the sub-proteomic approach by MALDI-TOF MS (Table 4.12). It was possible to match those strains to various application potentials like wheat beer or Ale using both methods. MALDI-TOF MS enables a finer sub-division to German Alt-Kölsch, whereas the applied DMGs show no major differences between Ale and German Alt-Kölsch. Furthermore, DMG *AMD2* and *GEX1* are not useful for the identification on species or variety level like it is realized with *COX2* (*S. cerevisiae*), *STA1* (*S. cerevisiae* var. *diastaticus*), *LRE1* or BF-300 (*S. pastorianus*) (Brandl, 2006, Hutzler, 2009, Rainieri et al., 2006, Yamauchi et al., 1998). On the contrary, MALDI-TOF MS enables a fast, reliable and low cost identification and differentiation between species (*S. pastorianus* and *S. cerevisiae*) and variety level as well as a potential classification to an application type. A separation of *S. cerevisiae* strains to different beer styles as well as a sub-division to wine styles (wine, sparkling wine, special wine) and other non-brewing sectors were achieved. Though the sub-proteomic approach shows no genetic background of the yeasts it is useful to combine MALDI-TOF MS with the genetic approach of DMGs. Yeasts of *Saccharomyces* are identified and characterized with respect to their application potential within the brewing or non-brewing environment by these two molecular methods.

Considering the total count of classifications to the industrial application, 10 out of 11 *S. cerevisiae* strains were matched correctly to the wheat beer application by MALDI-TOF MS and DMGs. TMW 3.0866 (TUM 507) is described as wheat beer strain (Goncalves et al., 2016), but MALDI-TOF MS classified this strain to the Ale application and only one DMG is detected, *AMD2*. In contrast, both DMGs are present in all other wheat beer strains and on sub-proteomics

wheat beer strains distinguish from all other application potentials. This shows that the hybrids of wheat beer (Gallone et al., 2016) are matched to their application potential with the molecular characterization. Furthermore, the possibility is displayed that Ale strains can be used to produce wheat beer, because of similar characteristics. Goncalves et al. (2016) illustrated the POF characteristic of some strains. For example, TMW 3.0866 possesses the ability to produce POF similar to wheat beer strains. Both methods enabled a correct classification of all German Alt-Kölsch strains according to the industrial application. Within the Ale application eight strains possess no DMGs similar to German Alt-Kölsch and were classified more accurately to this application by MALDI-TOF MS. Besides TMW 3.0866, two more Ale strains have at least one DMG either *AMD2* or *GEX1* and are matched to the Ale application by MALDI-TOF MS. In this case, the sub-proteomic level illustrated their application potential, however the finding of the DMGs displayed the link to the non-brewing sector. Based on genomics analysis those two strains are also linked either to the wine (TMW 3.0673; *GEX1*) or bread (TMW 3.0262; *AMD2*) application (Goncalves et al., 2016). It is possible that those two strains reached the brewing process centuries ago and brewers applied these strains for the brewing process. In the middle ages, it was a common practice that breweries and bakeries shared one yeasts (Krauss, 1994). Because of that, yeast strains from the non-brewing sector may have found their way to the brewing process. Within the special strains including Belgian and African beer styles a concurrence between industrial experience and molecular characterization couldn't be achieved. This can be solved with the expansion of the reference database with strains used for Belgian beer styles and African beer strains. MALDI-TOF MS enabled a correct classification of all *S. cerevisiae* var. *diastaticus* strains, which correspond to the brewer experience. However, an expansion of the two DMGs to three is useful to achieve a separation to wheat beer and non-brewing strains. As stated in chapter 5.3.1, an expansion of the DMGs with *STA1* (Brandl, 2006, Yamauchi et al., 1998) will characterize this variety on genetic level.

Both techniques characterized non-brewing *S. cerevisiae* strains quite well on genetic and sub-proteomic level. Strains applied for the production of winery products like wine, sparkling wine and special wine distinguish from the brewing sector on sub-proteomic and genetic level. Furthermore, the results for this application range to achieve a better separation of these groups. Especially distillery strains, because of wide differentiation between these two strains on genetic level possessing either both DMGs (TMW 3.0928) or no DMG (TMW 3.0265) and a very good strain level identification for both strains was achieved by MALDI-TOF MS.

This shows that it is useful to perform a wide molecular characterization of yeast strains to achieve a good identification combined with a prediction to application potentials with focused

pilot fermentations. Those fermentations are needed to have a phenotypic characterization of each strain and this can also be combined with different testing with selective media like POF or Cd_2SO_4. Figure 5.2 displays the workflow for a molecular characterization of *Saccharomyces* strains on genetic level (DMGs), sub-proteomic level (MALDI-TOF MS) and phenotypic characteristics (POF and Cd_2SO_4), which leads to a classification according to their application potential. After several days, pilot fermentations to a focused application potential can be performed, which is illustrated in Figure 5.3, to access the knowledge about the fermentation performance of each yeast.

5.5.1 Workflow for a molecular characterization of yeasts

It was shown that different molecular methods enable a characterization of yeasts of the genus *Saccharomyces*, especially for *S. cerevisiae* strains. The applied procedures can be combined and expanded with other molecular methods to characterize industrial yeast strains or wild isolates.

Figure 5.2 visualizes the workflow to match one yeast strain to an application potential. After it was incubated on YPD plate for two or more days, depending on genus, species, and strain level, colonies can be picked and inoculated in YPD media for 18 h for the MALDI-TOF MS identification as well as classification. Subsequently, the YPD working plate can be used for the genetic approach for the detection of DMGs, which is described in chapter 3.6.4 and chapter 3.6.5. Though the YPD working plate should not only be used for the sub-proteomic and genetic characterization, it is practical to determine the phenotypic characteristic POF, which is stated in Goncalves et al. (2016) and Meier-Dörnberg et al. (2017a).

After the incubation of the liquid media, samples can be prepared for the MALDI-TOF MS identification and classification as stated in chapter 4.1.1. The remaining media should not be discarded, because it is useful to determine the tolerance of yeast strains to 5 µM Cd_2SO_4. For this purpose, samples should be prepared as stated in chapter 3.7.3

Within a week for fast growing yeast strains, it is possible to characterize them by molecular methods and classify them to a focused application potential and assess their influence on the aroma profile. The information helps to select a focused pilot fermentation combined with a tasting panel, which is shown in Figure 5.3. This reflects the fermentation performance of each strain and completes the characterization. Furthermore, it is useful to compare all results to check the accuracy of each molecular approach.

Besides the molecular characterization of yeast strains the economical perspectives have to be taken into consideration as well. Thanks to a previously conducted classification, users are now able to choose an appropriate fermentation process without wasting any further time on trial and/or error experiments. Alongside this essential point of saving time, it is possible to keep the costs low, because of a specific application of raw materials for a pilot fermentation as well as an inexpensive characterization of yeasts. This also includes the usage of a working plate or incubated liquid for several analyzing steps. Furthermore, the analysis is realized with a small expenditure and can easily be done by any laboratory staff. In the end, the results reflect the link of yeast strains to each other, their true application potential as well as the introduction of aromatic compounds to the final product. The latter can be expanded with further analyses. A final test for the fermentation behavior is inevitable, but preliminary molecular methods can serve as a starting point for the classification of yeast strains with respect to their usefulness for the production of food and beverages.

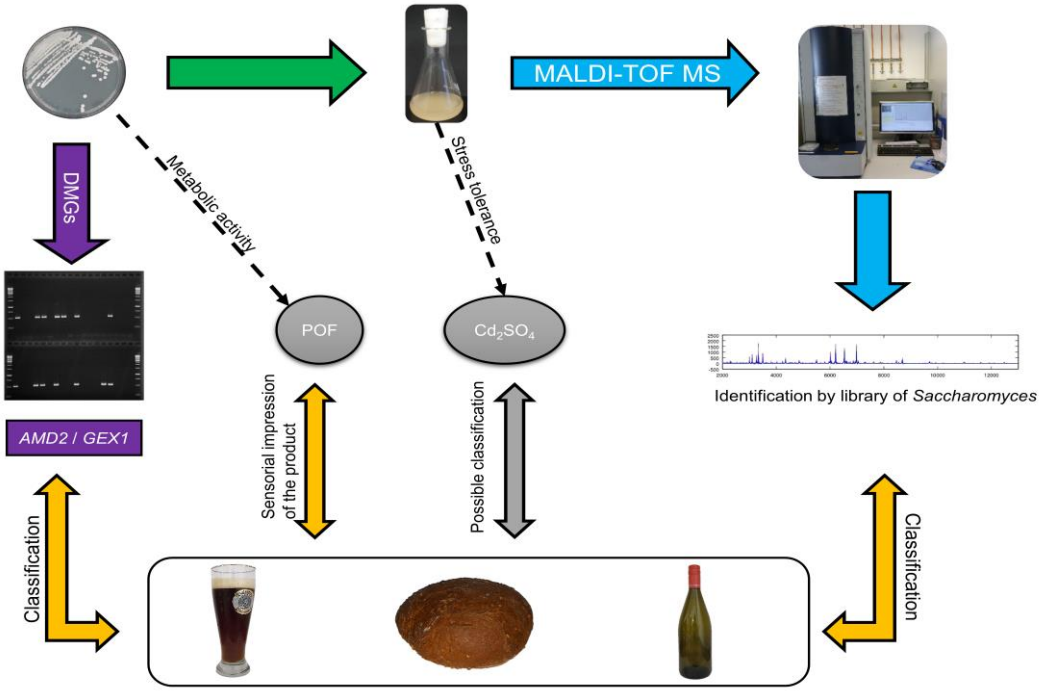

Figure 5.2: Rapid workflow for the molecular characterization of yeasts with respect to their application potential. This workflow includes the characterization with the DMGs *AMD2* and *GEX1* (purple arrow), MALDI-TOF MS classification and identification (light blue) and phenotypic characterization (grey) to phenolic off-flavor (POF) properties and tolerance to cadmium sulfate (Cd_2SO_4). Furthermore, it shows the characterization either from agar plates (DMGs and POF) as well as inoculated YPD media (MALDI-TOF MS and Cd_2SO_4).

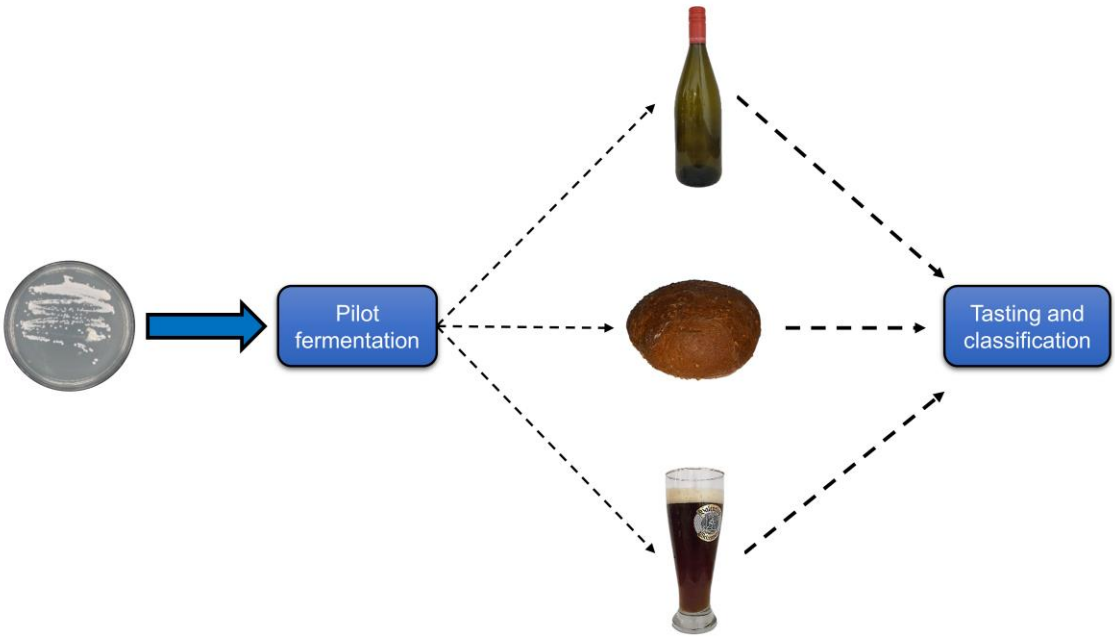

Figure 5.3: Classical classification of a yeast to an application potential by pilot fermentation, which is combined with a final tasting and resulting classification.

6 Summary

An increasing number of publications is observed on various *Saccharomyces* strains for industrial applications as well as laboratory strains, which aim to understand the importance of yeasts for mankind. The characterization of those yeasts for brewing or other food / beverage application is important to select the most appropriate strain. In this case, pilot fermentations are the standard procedure to select the perfect strain.

In this study, the potential is demonstrated of different molecular methods to alternatively characterize *Saccharomyces* strains with respect to their application potential. This characterization reflects the relation of strains to a specific application without any pilot fermentation. The focus is on the proteomic level by the Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and on genetic level by diagnostic marker genes (DMGs). This is achieved by classification of 89 yeasts of the genus *Saccharomyces* along their application potential by MALDI-TOF MS. Afterwards, the BIAst Diagnostic Gene findEr (BADGE) was used to predict DMGs from the genome sequence of top-fermenting *S. cerevisiae* strains to distinguish between beer styles. This was followed by a proof of concept study to correlate one DMG to a phenotypic characteristic by using a selective media. Finally, the results were reflected and compared to the industrial application.

First, the sample preparation was optimized for the MALDI-TOF MS measurement. A major modification of the sample preparation could not be achieved, however, some minor modifications were realized to achieve a simple handling of the samples as well as a better mixing of the samples.

By the means of a standardized sample preparation yeast strains were classified with respect to their application potential by MALDI-TOF MS. The sub-proteomic spectra of 89 strains from different applications were recorded. Besides the recording of 30 spectra per strain, database entries were also recorded for all strains. An almost 100% separation of bottom-, top-fermenting, *S. cerevisiae* var. *diastaticus* and non-brewing yeast strains of *S. cerevisiae* was achieved. Considering brewing yeast strains, Ale strains show a high degree of dissimilarity with regard to their sub-proteome. In contrast, wheat beer strains differed from all others and formed their own cluster. A good strain level identification for all brewing yeast strains could not be achieved, but on the other hand for non-brewing yeast strains of *S. cerevisiae*. Comparing the MALDI-TOF MS results of the yeasts with the experience of the yeast supplier, 92% were correctly classified to their application potential. This shows the discriminatory potential of

MALDI-TOF MS to characterize yeasts to various application potential in a rapid, easy way, and focus fermentation trails accordingly.

In another approach, it was tested by a genome-based method to characterize yeasts of the genus *Saccharomyces* with the respect to application potentials by the use of DMGs. The software tool BADGE and the genome sequences of 25 brewing yeast strains were used to predict DMGs for the discrimination of *Saccharomyces* strains according their beer style. A first PCR-screening of 25 top-fermenting *S. cerevisiae* strains, using the DMG-specific primer pairs for *AMD2* and *GEX1*, distinguished yeast strains of the wheat beer style to 100% from all other beer styles. The PCR-screening of an extended set displayed a more variable distribution of the selected DMGs to different application potentials. 62 out 89 strains were positive for at least one DMG. The DMGs proved to be useful for the differentiation of wheat beer strains to German Alt-Kölsch as well Ale strains. It was also shown that only the detection of *GEX1* seems to be marker gene for strains applied in the non-brewing sector of wine.

Based on the results of the genetic analysis, it was attempted to correlate a DMG with a phenotypic trait under defined conditions. A selective medium containing a different concentration of cadmium sulfate hydrate was used to link the tolerance or sensitivity of eight selected yeast strains to *GEX1*. The presence of *GEX1* shall improve the tolerance of yeast strains to the toxic heavy metal. Taking into account the genetic analysis, both wheat beer and *S. cerevisiae* var. *diastaticus* strains possess *GEX1*, but the selected Alt-Kölsch and Ale-strains do not. It was expected that a concentration of 5 µM cadmium sulfate hydrate has a lethal effect to yeast strains of German Alt-Kölsch and Ale. The results showed actually that only wheat beer strains were highly sensitive to 5 µM cadmium sulfate hydrate and were not able to grow at this concentration. This visualized that *GEX1* cannot be correlated to the resistance to this toxic heavy metal. However, this proof of concept showed the possibility to differentiate wheat beer strains from other *S. cerevisiae* of various beer styles by a selective medium.

The correlation of all results showed that on sub-proteomics as well genetic level different groupings could be achieved. The MALDI-TOF MS approach enabled a finer sub-division of *S. cerevisiae* strains either to different beer styles or non-brewing applications. The use of DMGs proved that marker genes differentiate between *S. cerevisiae* on four ways: both DMGs are present; either *GEX1* or *AMD2* was detected; no DMG could be detected. However, the DMGs enabled a classification to application potential like wheat beer, German Alt-Kölsch / Ale or non-brewing sector. Yeasts of *Saccharomyces* are characterized with respect to their application potential within the brewing or non-brewing environment by these two molecular

methods. 90% were correctly assigned to one application potential compared to the information of the yeast supplier. In addition, those purposes helped to characterize wild isolates to their application potential as well as to differentiate them on species level. A workflow based on molecular methods enables a characterization of yeast strains within several days and classify them to a focused pilot fermentation.

Besides the molecular characterization of yeast strains the economical perspectives have to be taken into consideration as well. Thanks to a previously conducted classification, users are now able to choose an appropriate fermentation process without wasting any further time on trial and/or error experiments. Alongside this essential point of saving time, it is possible to keep the costs low, because of a specific application of raw materials for a pilot fermentation as well as an inexpensive characterization of yeasts. This also includes the usage of a working plate or incubated liquid for several analyzing steps. Furthermore, the analysis is realized with a small expenditure and can easily be done by any laboratory staff. In the end, the results reflect the link of yeast strains to each other, their true application potential as well as the introduction of aromatic compounds to the final product. The latter can be expanded with further analyses. A final test for the fermentation behavior is inevitable, but preliminary molecular methods can serve as a starting point for the classification of yeast strains with respect to their usefulness for the production of food and beverages.

7 Zusammenfassung

Eine zunehmende Anzahl von Publikationen beschäftigt sich mit *Saccharomyces*-Stämmen für Industrie sowie Labor-Anwendungen, mit dem Ziel die Bedeutung von Hefen für die Menschheit zu verstehen. Die Charakterisierung von *Saccharomyces*-Stämmen zum Beispiel für das Brauen ist wichtig, um den am besten geeigneten Stamm auszuwählen. In diesem Fall ist die Pilot-fermentation normalerweise der goldene Standard, um den perfekten Stamm auszuwählen.

In dieser Arbeit wurde das Potenzial verschiedener molekularer Methoden zur Charakterisierung von *Saccharomyces*-Stämmen hinsichtlich ihres Anwendungspotentials aufgezeigt. Diese Charakterisierung muss spezifisch genug sein, um die Beziehung der Stämme zu einer spezifischen Anwendung ohne Pilotfermentation widerzuspiegeln. Der Schwerpunkt wurde dabei auf der Proteomebene durch die Matrix-unterstützte Laser-Desorption / Ionisations-Flugzeit-Massenspektrometrie (MALDI-TOF MS) und auf genetischer Ebene durch diagnostische Markergene (DMGs) abgedeckt. Dies wurde durch eine Einteilung von 89 Stämmen in ihr Anwendungspotential durch MALDI-TOF-MS erreicht. Danach wurde der BlAst-Diagnostic-Gene-FindEr (BADGE) verwendet, um DMGs aus der Genomsequenz von obergärigen *S. cerevisiae* vorherzusagen, um zwischen Bierstilen zu unterscheiden. In einer weiteren Studie wird ein DMG mit einem phänotypischen Merkmal unter definierten Bedingungen korreliert. Abschließend wurden die Ergebnisse mit der industriellen Anwendung verglichen.

Zunächst sollte die Probenvorbereitung für die MALDI-TOF-MS-Messung optimiert werden. Zusammenfassend wurde eine wesentliche Änderung der Probenvorbereitung nicht erreicht, verglichen mit beschriebenen Methoden in wissenschaftlichen Arbeiten. Es wurden jedoch geringfügige Änderungen vorgenommen, um eine einfachere Handhabung sowie eine bessere Durchmischung der Proben zu erreichen.

Mittels der standardisierten Probenvorbereitung sollten Hefestämme hinsichtlich ihres Anwendungspotentials durch MALDI-TOF MS klassifiziert werden. Die Subproteom-Spektren von 89 Hefestämmen wurden aufgenommen. Neben der Aufnahme von 30 Spektren pro Stamm wurden auch Datenbankeinträge für alle Stämme angelegt. Eine fast 100%ige Abtrennung von ober- und untergärigen, *S. cerevisiae* var. *diastaticus*- und nicht-Brauhefen von *S. cerevisiae* wurden erreicht. Die Ale-Stämme weisen in Bezug auf ihr Subproteom eine große Unähnlichkeit auf. Im Gegensatz dazu unterschieden sich die Weizenbierstämme von allen

anderen und bildeten ein eigenes Cluster. Neben der Klassifizierung zu verschiedenen Anwendungsbereichen sowie Speziesebene, wurde ebenfalls die Identifizierung auf Stamm-Ebene betrachtet. Hierbei wurden Unterschiede zwischen Brau- und nicht-Brauhefen ausgemacht. Eine gute Bestimmung des Stammspiegels für alle Brauhefestämme konnte nicht erreicht werden, aber für nicht-Brauhefen. Bei Vergleich der MALDI-TOF MS Ergebnisse der Hefen mit der Erfahrung des Hefehändlers wurden 92% korrekt auf ihr Anwendungspotential klassifiziert. Dies zeigt das Potential von MALDI-TOF MS, um Hefen für verschiedene Anwendungspotenziale auf schnelle und einfache Weise zu charakterisieren und Fermentationsversuche entsprechend zu bestimmen.

In einem anderen Ansatz wurden auf genetischer Ebene Hefen der Gattung *Saccharomyces* basierend auf DMGs nach Anwendungspotentiale differenziert. Mit Hilfe des Softwaretools BADGE und Genomsequenz von 25 Brauhefestämmen wurden DMGs für die Unterscheidung von *Saccharomyces*-Stämmen gemäß ihrem Bier Stil identifiziert. Zwei DMGs wurden für das PCR-Screening ausgewählt, um das Vorhandensein dieser Gene innerhalb von 89 Stämmen zu testen. Ein erstes PCR-Screening von 25 obergärigen *S. cerevisiae*-Stämmen unter Verwendung der DMG-spezifischen Primerpaaren *AMD2* und *GEX1* unterschied Stämme des Weizenbier-Stils zu 100%. Das erweiterte PCR-Screening zeigte eine variablere Verteilung von *AMD2* und *GEX1* auf unterschiedliche Anwendungspotentiale. 62 von 89 Stämmen waren für mindestens ein DMG positiv. Die DMGs erwiesen sich als nützlich für die Differenzierung von Weizenbierstämmen gegenüber Alt-Kölsch- sowie Ale-Stämmen. Es wurde auch gezeigt, dass der Nachweis von nur einem der beiden Gene Stämme identifiziert, die im Nicht-Braubereich angewendet werden.

Ausgehend von den Ergebnissen der genetischen Analyse wurde versucht *GEX1* mit einem phänotypischen Merkmal unter definierten Bedingungen zu korrelieren. Dabei wurde ein selektives Medium verwendet, das eine unterschiedliche Konzentration von Cadmiumsulfat-Hydrat enthielt, um die Toleranz von acht ausgewählten Stämmen mit *GEX1* zu verknüpfen. Das Vorhandensein von *GEX1* soll die Toleranz gegenüber dem toxischen Schwermetall verbessern. In diesem Fall wurden Stämme ausgewählt, die entweder *GEX1* codieren oder für dieses Gen negativ sind. Unter Berücksichtigung der genetischen Analyse besitzen sowohl Weizenbier als auch *S. cerevisiae* var. *diastaticus*-Stämme *GEX1*, jedoch die ausgewählten Alt-Kölsch und Ale-Stämme nicht. Es wurde erwartet, dass eine Konzentration von 5 µM Cadmiumsulfat-Hydrat eine letale Wirkung auf Alt-Kölsch und Ale Stämme hat. Die Ergebnisse zeigten jedoch, dass nur Weizenbierstämme bei einer Konzentration von 5 µM Cadmiumsulfat-Hydrat nicht wachsen konnten. Dies zeigte, dass *GEX1* nicht mit der Resistenz

gegenüber diesem toxischen Schwermetall korreliert werden kann. Jedoch zeigte die Studie die Möglichkeit, Weizenbierstämme von anderen *S. cerevisiae* durch ein selektives Medium zu unterscheiden.

Der Vergleich aller Methoden zeigte, dass sowohl auf Sub-Proteomik als auch auf genetischer Ebene ähnliche Gruppierungen erreicht werden konnten. Der MALDI-TOF-MS-Ansatz ermöglichte eine feinere Unterteilung von *S. cerevisiae*-Stämmen entweder in verschiedene Biersorten oder Nicht-Brauanwendungen. Die Verwendung von DMGs bewies, dass Markergene auf vier Arten zwischen *S. cerevisiae* unterscheiden: beide DMGs sind vorhanden; entweder *GEX1* oder *AMD2* wurde nachgewiesen; kein Gen. Die DMGs ermöglichten jedoch eine Einordnung in Anwendungspotenziale wie Weißbier, Alt-Kölsch / Ale oder Nicht-Brauwesen. *Saccharomyces*-Hefen werden durch diese beiden molekularen Methoden hinsichtlich ihres Anwendungspotentials innerhalb der Brau- oder Nicht-Brauumgebung charakterisiert. 90% der Stämme wurden im Vergleich zu den Angaben des Hefehändlers korrekt einem Anwendungspotential zugeordnet. Darüber hinaus trugen diese Zwecke dazu bei, Wildisolate auf ihr Anwendungspotential zu charakterisieren und sie auf Speziesebene zu differenzieren. Ein auf molekularen Methoden basierender Arbeitsablauf ermöglicht es, Hefestämme innerhalb weniger Tage zu charakterisieren und einer gezielten Pilotfermentation zuzuordnen.

Neben der molekularen Charakterisierung von Hefestämmen müssen auch die wirtschaftlichen Perspektiven berücksichtigt werden. Als Folge einer Klassifizierung können Anwender die richtige Pilotfermentation wählen, ohne Zeit zu verschwenden. Neben diesem wesentlichen Punkt der Zeitersparnis ist es möglich, die Kosten niedrig zu halten, durch einen gezielten Einsatz von Rohstoffen sowie eine kostengünstige Charakterisierung von Hefen. Dies schließt auch die Verwendung einer Arbeitsplatte oder einer inkubierten Flüssigkeit für mehrere Analyseschritte ein. Darüber hinaus wird die Analyse mit geringem Aufwand realisiert und kann von jedem Laborpersonal durchgeführt werden. Am Ende spiegeln die Ergebnisse die Verbindung der Hefestämme zueinander, ihr wahres Anwendungspotential sowie die Einführung aromatischer Verbindungen in das Endprodukt wider, die durch weitere Analysen erweitert werden können. Ein letzter Test für das Fermentationsverhalten ist immer erforderlich, aber vorläufige molekulare Methoden können als Ausgangspunkt für die Klassifizierung von Hefestämmen hinsichtlich ihrer Nützlichkeit für die Herstellung von Nahrungsmitteln oder Getränken dienen.

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9 Appendix

9.1 Figures and Tables

Appendix 9.1: BADGE settings, which are changed from default value to changed value. For further explanations about the settings we refer to Behr et al. (2016)

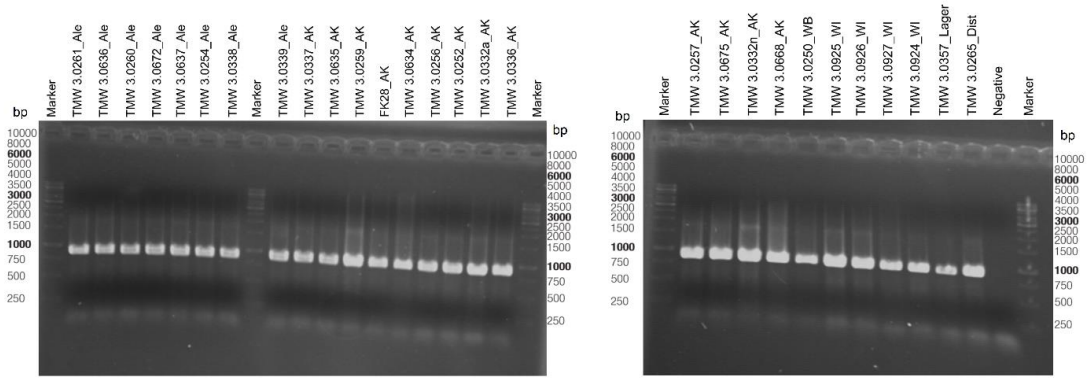
Setting name in BADGE	default value	Changed to
megablast_perc_identity_cut	95	90
megablast_within_group_qscov	0.95	0.90
dc_mode	false	true
dc_filter	true	false
blastn_filter	true	false

Appendix 9.2: DMG specific primer pairs used for the PCR screening and primer sequence *ITS5* / *ITS4* (White et al., 1990) for positive DNA control

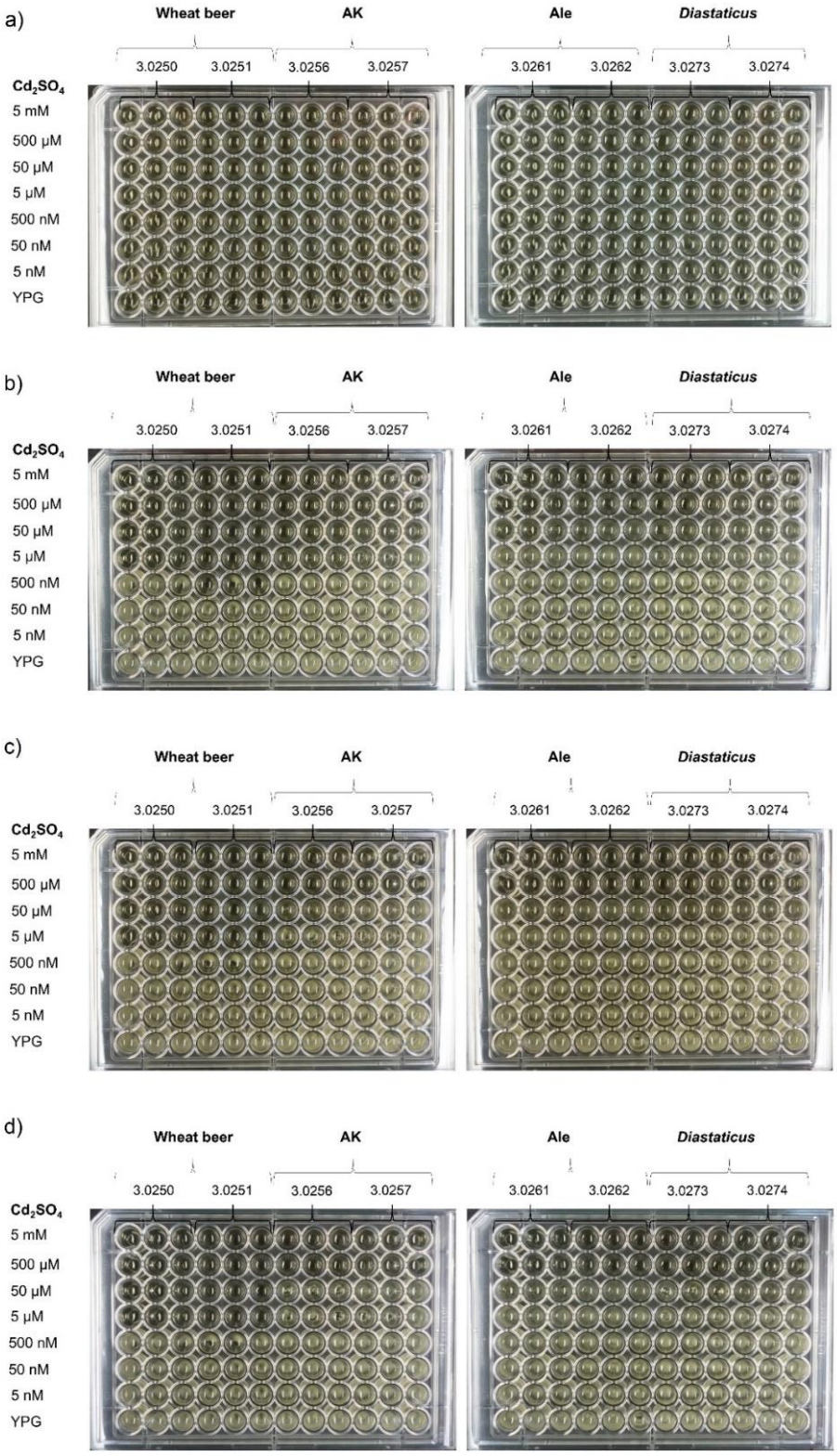
Primer pair	forward primer	reverse primer	product size (bp)
AMD2	TCATCTGGTAGTGCCAGTTC	TCCCAATCCCAGCCATTATC	500
GEX1	GAACGGGCGGATTATTTGAG	GTAGCAGCAACAAAGTCAGG	407
ITS5 / *ITS*4	GGAAGTAAAAGTCGTAACAAGG (*ITS5*)	TCCTCCGCTTATTGATATGC (*ITS4*)	(White et al., 1990)

Appendix 9.3: Brewing yeast strains of the genus *Saccharomyces* used for the expanded classification. Strains added to the expanded classification are highlighted with the bold type; TMW = Technische Mikrobiologie Weihenstephan

Brewing yeasts of major beer styles of the genus *Saccharomyces* (TMW coding)												
S. cerevisiae - wheat beer	3.0250	*S. cerevisiae* - Ale	3.0254	*S. cerevisiae* - Alt beer	3.0252	*S. cerevisiae* - Kölsch	3.0256	*S. cerevisiae* var. *diastaticus*	3.0273	*S. pastorianus* – flocculation yeast	**3.0275**	*S. pastorianus* – *powdery yeast*	**3.0285**
	3.0251		3.0260		3.0257		3.0332a		3.0274		**3.0276**		**3.0286**
	3.0253		3.0261		3.0259		3.0332n		3.0624		**3.0277**		**3.0351**
	3.0255		3.0262		3.0336		**3.0961**		3.0625		**3.0278**		**3.0352**
	3.0258		3.0338		3.0337				**3.0628**		**3.0279**		**3.0354**
	3.0343		3.0339		3.0634				**3.0811**		**3.0280**		**3.0356**
	3.0666		3.0636		3.0635				**3.0812**		**3.0281**		**3.0357**
	3.0667		3.0637		3.0668						**3.0282**		**3.0358**
	3.0669		**3.0672**		3.0675						**3.0283**		**3.0359**
	3.0674										**3.0284**		
											3.0813		
											3.0938		



Appendix 9.4: DNA control with a positive control on ITS5 / ITS4 (White et al., 1990) to check the presence of genomic DNA for negative results. TMW = Technische Mikrobiologie Weihenstephan; WB = wheat beer; AK = German Alt-Kölsch; Dist = Distillery; bp = base pair; Marker = molecular weight showing the range from 250 – 10000 bp



Appendix 9.5: Visual evaluation of the growth behavior within a microwell plate until 72 h. All yeasts are labeled with the strain ID (TMW = Technische Mikrobiologie Weihenstephan) and their ecotype, which is written above (AK = German Alt-Kölsch; *Diastaticus* = *S. cerevisiae* var. *diastaticus*); on the left side the Cd_2SO_4 (cadmium sulfate) concentrations are listed including the positive control YPD without Cd_2SO_4; three biological replicates were analyzed per strain; a) starting plate 0 h; b) after 24 h incubation time; c) after 48 h incubation time; d) after 72 h incubation time

9.1.1 Sequence of DMGs

> (WB_marker I) putative Amidase

TUM175_ERR1352847_6137:4537-6334

ATGGGATTTGAAATGGAGCTCAGATTGGCAGACCATCAAGTAGTTATGAGAATCA
AAGACGTTGTGTTATTAGGGCTATCATTGACATCAGTGGAAGCCACGCCAGTTAA
AAAAATATGGAAACGGTTTTTAGCCAGTATACCAAGTGAAACCCAAACAGTAAA
CACGACTACTTTTGTATATCCTCAAACGCAGTCAGGCGAACTATTCCCGATGGAT
ATGTGTAAAGGTATTACGTTAGAAGATGCCACGATAGACCAATTACAGGGTTACT
TTGATAAAGGTCTCCTTACGTCAGAGGATGTTGTGCGCTGCTACCTGGACCGGTA
TTTCCAACTGAATTCGTATGTCAATGGTATATTGCAGGTCAATCCTGATGCTATTT
CAATTGCTCAGGAGAGGGATCGTGAGCGGGCAGCAGGAGTAGTTAGAAGTTCAT
TGCATGGAATTCCATTTTTAGTTAAGGATAATTATGCTACAAAGGATAAAATGGA
CACTACTTGCGGTTCATGGATGCTTTTAGGTTCAGTAGTGCCACGAGATGCTCATG
TTGTATCCAAATTAAGAGATGCTGGCGCTGTATTGTTTGGTCACTCCACATTAAGT
GAATGGGCAGACATGAGATCATCAGATTACTCTGAGGGCTACTCCGCTAGAGGTG
GCCAAGCACGTTGTCCTTTCAATCTTACTACCAATCCAGGAGGTAGTTCATCTGGT
AGTGCCAGTTCTGTAGCTGCCAATATGATCATGTTTTCGCTAGGAACCGAAACTG
ATGGTAGTATTATTGATCCAGCGATGAGGAATGGTGTTGTTGGTTTTAAACCCAC
TGTTGGTTTAACCTCAAGGTCTGGTGTAATTCCAGAGTCCGAGCATCAGGATTCT
ACTGGACCTATGGCTAGAACTGTCCGCGACGCAATATATGCTTTCCAATATATGT
GGGGGGTCGATGAGAAAGACGTGTATACCTTGAATCAGACCGGTAAAGTTCCAG
ATGATGGTGATTACTTGAAATATTTAACTGACAAAAGTGCTCTGAAAGGTGCAAG
ATTTGGTTTGCCATGGAAAAAGCTTTGGTCATACGCCAAAACCGATGAAATTCCT
AGACTTTTAGAAGTCATTAAAGTTATTGAAGACGCAGGAGCTACAATTTATAATA
ATACCGACTTTGGAAATTTGGATGTTATTTCAGATAATGGCTGGGATTGGGACTTT
GGACCAGCAAATGAGAGTGAATTCACTGTGGTGAAAGTTGACTTCTATAATAACA
TTAAGTCCTACTTGAACGAATTAGAAAACACAAATATACGCTCCTTAGAGGACAT
TGTTGCATATAACTATAATTTCACCGGTAGCGAAGGCGGATACAATAATACACAT
CCTGCTTTTTCATCTGGTCAAGATTCCTTCTTAGACTCTCTTGCGTGGGGAGGTAT
CAAGAATGCGACCTACTGGGAAGCTGTAGAATTTGTTCAAAGAACCTCCAGAGAT
GAAGGCATTGACCATGCACTTAACTACACCGATCCTAACACCGGTGAAAATTTTA
AGCTGGATGGTCTTTTGGTTCCAAGTGGTTTGTCAATCACATATCAGCAAGCCGCT
AAAGCTGGTTATCCGATGATAACATTACCGATAGGTGTAAAAAAGGCTAATGGC

AGACCTTTTGGTCTTGGTATAATGCAATCTGCTTGGCAAGAACCTCAGCTGATCA
AATACGGCTCAGCCATTGAAGATTTATTAAGTTACAAATGCAAGCCACAGTATTA
TGAATATGTGGCAAAAAATATCCCCGTTATATGA

> WB_marker II → YCL073C → Glutathione exchanger 1 (*GEX1*)

TUM205_ERR1352846_23320:8-1712 [revcomp]

ATGTCTGCACAGTTTGATTCCTTGAAATATAAAATTCTACTGATAAGTACCGCGTT
TGTATGTGGGTTTGGAATTAGTTTAGACTACACACTTAGATCGACCTATACGGGC
TATGCGACGAACTCATATTCAGAACACTCCTTACTTTCAACTGTCCAAGTTATCAA
TGCTGTTGTAAGTGTCGGATCCCAAGTTGTCTACTCCAGACTCTCTGACCACTTCG
GAAGACTAAGGCTTTTTTTAGTTGCAACTATTTTTTATATAATGGGAACCATCATT
CAATCACAGGCGACCCGTCTCACAATGTATGCAGCAGGATCGGTTTTCTATAACT
GTGGATACGTCGGAACAAATCTGCTCCTGACATTAATACTTTCTGATTTCTCCTCC
TTGAAGTGGAGAATGTTTTACCAGTACGCCTCATATTGGCCATATATCATAATAC
CATGGATTTCAGGTAATATTATCACAGCAGCAAATCCTCAGAAAAACTGGTCCTG
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TATTCTTTATATGAAGTACAAATCTTCAAAGACTGCTGAGTGGAGATCTCTCAAA
GAACAGGCTAGAAAGGAAAGAACGGGCGGATTATTTGAGAATTTGGTGTTTCTAT
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CTTGTCCCTTTGACGTTGGCTAATGAGACATCACAGAAGTGGCACAATTCAAAAA
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CCAAATTTGCCAAATCTCCTCTTCTACCGTTCAAATTACTAAGTGATCGTGGAATT
TGGGCACCCCTTGGTGTTACTTTTTTCAATTTTTTCACCTTTTTCATTTCGTGTGAC
TATCTGTATCCTGTTTTGCTGGTATCGATGAAAGAATCGTCCACTTCGGCTGCTCG
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TGGTGGCAAAGACGAGGAAACTGAAACTTTCTGTAATCGGAGGTTGTGCTGCATG
GATGGTGTACATGGGCCTTTTTTACAAATACAGAGGAGGATCCGGGTCTCATGAA
GGTGTTATCGCTGCATCTGTTATCATGGGTTTGAGCGGTCTCCTATGCAGCAATTC
AGTGATCGTCATACTGCAAGCCATGACTACGCATAGTAGGATGGCTGTAATAACC
GGCATCCAATATACCTTTTCGAAGCTAGGCGCTGCTATCGGTGCCTCCGTTTCTGG
TGCCATATGGACACAAACCATGCCCAACCAACTCTACAAGAACCTTGGAAACGAT
ACATTGGCAGAAATAGCATATGCATCACCTTATACATTCATTAATGATTATCCTTG
GGGCTCACTCGAAAGAGATGCTGTGGTTGAATCTTACAGATATGTTCAACGAATA
ATAATGACGGTTGGCTTGGCATGTACGGTACCGTTCTTTGCGTTTACAATGTTCAT
GAGAGATCCGGAACTAATAGACAAGGCGACACACGAAGAATTCACTGAAGATGG
TTTGGTCGTCTTGCCAGATGAGGAAAACATTTTCTCTCAAATCAAGGCACTTTTTA
AACATAATCGAAGTGACAAGGAATCAGGATGTTGA

10 List of publications and student theses

Peer-reviewed Journals

Alexander Lauterbach, Julia C. Usbeck, Jürgen Behr, Rudi F. Vogel. MALDI-TOF MS typing enables the classification of brewing yeasts of the genus *Saccharomyces* to major beer styles. PLoS ONE 12 (8) (2017): e0181694. https://doi.org/10.1371/journal.pone.0181694

Alexander Lauterbach‡, Andreas J. Geissler‡, Lara Eisenbach, Jürgen Behr, Rudi F. Vogel. Novel diagnostic marker genes for the differentiation of Saccharomyces with respect to their potential application. Journal of the Institute of Brewing (2018) 124: 416 – 424. https://doi.org/10.1002/jib.525.

‡: shared first authorship

Alexander Lauterbach, Caroline Wilde, Dave Bertrand, Jürgen Behr, Rudi F. Vogel. Rating of the industrial application potential of yeast strains by molecular characterization. European Food Research and Technology (2018) 244: 1759 – 1772. https://doi.org/10.1007/s00217-018-3088-2

Sedjro Emile Tokpohozin, Alexander Lauterbach, Susann Fischer, Jürgen Behr, Bertram Sacher, Thomas Becker. Phenotypical and molecular characterization of yeast content in the starter of "Tchoukoutou," a Beninese African sorghum beer. European Food Research and Technology (2016) 242: 2147 – 2160. https://doi.org/10.1007/s00217-016-2711-3

Oral presentations:

Jürgen Behr, Alexander Lauterbach. MALDI-TOF Analytik in der Brau- und Getränketechnologie. Oral presentation by Dr. Jürgen Behr and Alexander Lauterbach at the Seminar Hefe und Mikrobiologie (Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität), Freising, Germany, 25.03.2015 to 26.03.2015

Alexander Lauterbach, Jürgen Behr, Rudi F. Vogel. MALDI-TOF - Brauereihefen Barcoding. Oral presentation at the Seminar Hefe und Mikrobiologie (Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität), Freising, Germany, 15.03.2016 to 16.03.2016

Alexander Lauterbach, Jürgen Behr, Carola C. Kern, Julia C. Usbeck, Rudi F. Vogel. Biotyping of microorganisms by MALDI-TOF MS – Identification and classification of food born isolates. Oral presentation at the Seminar Advanced Mass Spectrometry (Bavarian Center of Biomolecular Mass Spectrometry), Freising, Germany, 08.11.2017

Poster presentations:

Alexander Lauterbach[‡], Tim Meier-Dörnberg[‡], Jürgen Behr, Rudi F. Vogel, Mathias Hutzler. The potential of MALDI-TOF MS for sensotyping of brewing yeasts. Poster presented at World Brewing Conference, Denver, Colorado, United States of America, 13.08.2016 to 17.08.2016.

[‡] : shared first authorship

Student theses:

The following student theses were supervised.

Master theses:

Jessica Lea Magdalena Schneider. Untersuchung der Korrelation von Stoffwechseleigenschaften und MALDI-TOF MS Biotypen bei Nicht-*Saccharomyces* Hefen. 2016

Carmen Sarah Susanne Henkel. Die mögliche Detektion der Stabilität einer *S. pastorianus* Bruchhefe sowie verschiedener Industriehefen nach mehrmaligen Passagieren mittels MALDI-TOF MS. 2017