TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Regulation of PAU5 – a gushing-reducing protein in sparkling wine – by *Saccharomyces cerevisiae*

Magdalena Agnes Mann

Vollständiger Abdruck der von der TUM School of Life Sciences der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Dieter Langosch
Prüfer der Dissertation:	1. Prof. Dr. Rudi F. Vogel
	2. Prof. Dr. J. Philipp Benz

Die Dissertation wurde am 21.05.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 21.07.2021 angenommen.

„Wine is sunlight, held together by water.”

-

Galileo Galilei

Danksagung

Zuallererst möchte ich mich von ganzem Herzen bei meinem Doktorvater Herrn Prof. Dr. Rudi F. Vogel bedanken, der mich zu jedem Zeitpunkt mit seiner exzellenten wissenschaftlichen Meinung und vielen guten Ideen unterstützt hat und ohne den diese Arbeit niemals möglich gewesen wäre. Außerdem geht mein aufrichtiger Dank an meinen Mentor Herrn apl. Prof. Dr. Ludwig Niessen, dessen Tür für jede noch so kleine Frage stets offenstand. Des Weiteren bedanke ich mich bei meinem Zweitprüfer Herrn Prof. Dr. Philipp Benz für das Bewerten der vorliegenden Arbeit und dafür, dass er mich als Betreuer meiner Masterarbeit auf die Promotion vorbereitet hat.

Herzlich bedanken möchte ich mich auch bei Frau Prof. Dr. Rauhut vom Institut für Mikrobiologie und Biochemie sowie Herrn Dipl.-Ing. Wallbraun vom Institut für Önologie der Hochschule Geisenheim University und deren Teams für die Beratung und Unterstützung bei önologischen Fragestellungen, für die lehrreichen Forschungsaufenthalte in Geisenheim sowie für die Durchführung des Schönungsmittelversuch und der NOPA, Ammonium und FTIR Messungen. Dem gesamten projektbegleitenden Ausschuss unter dem Vorsitz von Herrn Dr. Gloden möchte ich für die interessanten Diskussionen danken. Besonders möchte ich hier auch den Industriepartnern Herrn Burger, Herrn Dr. Fröhlich, Herrn Gruber und Frau Dr. Schneider für die großzügige Bereitstellung von Probenmaterial danken.

Herrn Dr. Oreb vom Institut für Molekulare Biowissenschaften der Goethe Universität Frankfurt möchte ich für die Zusendung des *Saccharomyces* Expressionssystems und der zugehörigen Protokolle danken. Des Weiteren bedanke ich mich herzlich bei Kai Büchner vom BGT für die Zusammenarbeit im Rahmen unserer „*Pichia*-Selbsthilfegruppe“.

Ein ganz besonderer Dank geht an meine liebste Kollegin, Projektpartnerin und Büronachbarin Lisa Frisch – ohne Dich hätte ich das nicht geschafft. Vielen Dank für jede einzelne fachliche Diskussion, aber auch dafür, dass ich in Dir eine wahre Freundin gefunden habe. Außerdem bedanke ich mich bei den Mädels vom Büro 36, ihr habt die Zeit am TMW wirklich besonders gemacht und ich hoffe, dass wir auch in Zukunft noch viele Bonding-Events zusammen erleben werden. Darüber hinaus möchte ich mich auch herzlich bei allen anderen TMW Mitgliedern für die stets kollegiale Zusammenarbeit bedanken.

Natürlich möchte ich mich auch bei meiner gesamten Familie, besonders aber bei meinen Eltern Thomas und Barbara Hackhofer bedanken für die unglaubliche Unterstützung während meines gesamten Studiums und dafür, dass ihr mich so weit gebracht habt. Zu guter Letzt gilt mein außerordentlicher Dank meinem geliebten Ehemann Tobias Mann, der mich immer wieder motiviert, mir zuhört, Verständnis zeigt und Erfolge mit mir feiert. Du bist mein Ruhepol und meine Antriebskraft zugleich.

Contents

1 Introduction

1.1 Gushing

1.1.1 What is Gushing?

Gushing is a multi-factorially caused phenomenon, which is defined as spontaneous excessive over-foaming of carbonated beverages due to the sudden pressure release upon opening of a bottle, despite correct handling. An example of its potential extent is shown in Figure 1. It causes severe economic losses and reputational damages to the beverage industry and can occur in all types of carbonated beverages, but is mainly known from beer (Bach, 2001; Schumacher, 2002). Gjertsen et al. (1963) provided a concept to distinguish between primary and secondary gushing. Primary gushing is defined as a periodically and locally limited phenomenon, which is caused by raw materials. Secondary gushing is initiated by production failures like particles in the product. In contrast to primary gushing, secondary gushing is rather easy to cure once its cause has been found.

Figure 1: Gushing red sparkling wine The bottle has been opened after standing static for 2 h at RT.

1.1.2 Gushing in beer

Gushing in beer has been a problem ever since the middle of the 16th century, when bottling of beer became popular in Great Britain (Beattie, 1951). First research on the issue of gushing has been performed roughly a century ago in the 1920's (Windisch, 1923a, 1923b). Since then, a wide variety of studies has been published and revealed a plethora of factors that have an influence on gushing. The phenomenon is very complex and not only dependent on gushing inducers or reducers, but also on physical factors such as the temperature (Bowers et al., 1999), the pH (Curtis & Martindale, 1961), the surface viscosity (Gardner, 1972), the carbon dioxide content, the shape and size of the containers or bottles, the amount of neck space air, and rate of agitation (Amaha & Kitabatake, 1981; Dachs & Nitschke, 1977; Deckers et al., 2013). Rated among secondary gushing inducers are factors such as rough inner bottle surfaces, which may result from residues from improper washing or cracks in the bottle material (Wershofen, 2004). Some examples are shown in Figure 2. Another major problem are particles in the product, which serve as nuclei for bubble formation. Such particles may for example be glass splinters (Beattie, 1951), dust from the crown caps (Brenner, 1957a), or they may come from the bottle washing process (Dachs & Nitschke, 1977) or from contamination by filter aids (Lusk, 2016; Zarnkow & Back, 2001). Another frequent source for solid particles in beer is crystallization (see Figure 2). Often, calcium from the brewing water and oxalate from the malt crystallize into octahedral or

amorphous calcium oxalate microcrystals. Especially the latter ones serve as condensation nuclei. If the precipitation occurs after the filtration process, the nuclei are prevalent in the final product and can cause gushing (Brenner, 1957a, 1957b; Burger & Becker, 1949; Burger et al., 1956; Schur et al., 1980; Zepf & Geiger, 1999). Furthermore, metal ions have been demonstrated to cause gushing in beer. Early studies showed that iron, nickel, cobalt, tin, titanium, and uranium ions provoked gushing and that this effect could be diminished by the addition of EDTA, except in the case of titanium (Gray & Stone, 1956; Gray & Stone, 1957; Gray & Stone, 1960; Stone & Gray, 1955). Other researchers found that nickel and iron induce gushing mainly by composite effects of complexes with hop ingredients, such as iso-humulone (Hudson, 1962; Rudin & Hudson, 1958). On the other hand, Guggenberger and Kleber (1963) suggest that gushing is associated with free iron (III) ions because these can form $Fe(OH)_3$ molecules, which accumulate on the surface of tiny gas bubbles after their solubility is exceeded. In contrast to the earlier studies, the authors found no direct effect of Ni^{2+}, Co^{2+}, Cu^{2+}, or Al^{3+} ions on gushing, but stated that those metal ions can rather displace Fe^{3+} ions from their complexes or chelates and thus have an indirect gushing inducing effect.

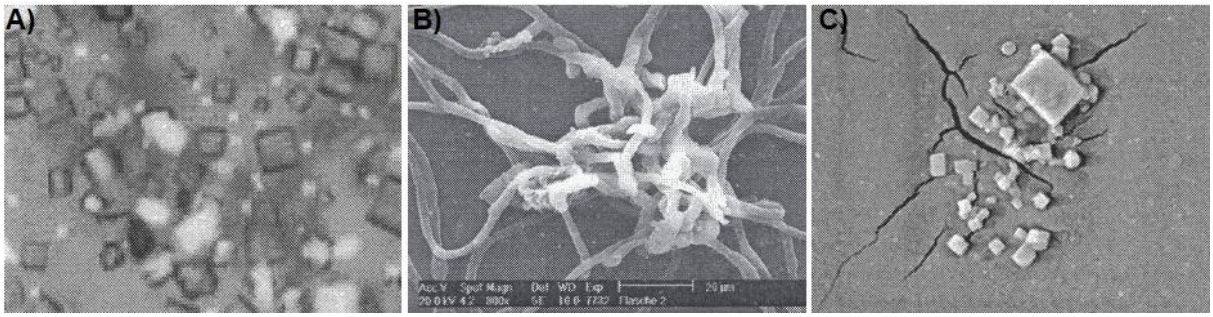

Figure 2: Examples for secondary gushing inducers
Particles in the product or rough inner bottle surfaces can serve as bubble nuclei and induce secondary gushing. A) Dextrin crystals (Zarnkow & Back, 2001), B) REM image of mold residues on an inner buttle surface (Wershofen, 2004), C) damaged inner PET bottle surface with mineral deposits (Wershofen, 2004).

With regard to primary gushing in beer, weathered barley can be a contributory cause (Gjertsen et al., 1963; Schildbach, 1987). Poor weather conditions enhance the growth of a broad range of filamentous fungi and yeasts on the grains, in the field, and during storage. Many fungal species have been associated with beer gushing: Mainly *Fusarium* species such as *Fusarium graminearum* or *Fusarium culmorum* (Habschied et al., 2014; Narziß et al., 1990; Niessen et al., 1992; Sarlin et al., 2012; Sloey & Prentice, 1962), but also other species like *Trichoderma* sp. (Sarlin et al., 2005), *Stemphylium* sp., *Penicillium* sp., *Rhizopus* sp. (Amaha et al., 1973; Yoshida et al., 1975), *Aspergillus* sp. (Gyllang & Martinson, 1976), *Nigrospora* sp. (Kitabatake & Amaha, 1974, 1976; Yoshida et al., 1975), or *Alternaria alternata* (Niessen et al., 1992). In the mechanism of microbial gushing, fungal hydrophobins play a major role. Those are small hydrophobic proteins (≤ 20 kDa) secreted uniquely by fungi, which are characterized by the presence of eight conserved cysteine residues implicated in the formation of four disulfide bonds. Hydrophobins contribute to the gushing

phenomenon because they can spontaneously assemble into amphipathic monolayers at hydrophobic–hydrophilic interfaces such as gas bubbles (Bayry et al., 2012; Deckers et al., 2010; Kleemola et al., 2001; Linder, 2009; Sarlin et al., 2005). Examples for such hydrophobins are FcHyd5 from *Fusarium culmorum* (Lutterschmid et al., 2010; Stübner et al., 2010; Zapf et al., 2006) or HFBI and HFBII from *Trichoderma reesei* (Lutterschmid et al., 2011; Riveros G. et al., 2015; Sarlin et al., 2005). An indirect effect of barley and wheat pathogens on gushing is the degradation of the non-specific lipid transfer protein 1 (nsLTP1) by heat stable fungal proteases (Hippeli & Elstner, 2002; Hippeli & Hecht, 2008; Stanislava, 2010). Lutterschmid et al. (2011) attributed the plant protein ns-LTP1 in its heat denatured form a reducing capacity on hydrophobin-induced gushing, and Zapf et al. (2005) found a gushing-reducing function of glycosylated wheat ns-LTP1. Similarly, the protein Z4 from barley has a gushing reducing effect, albeit only in its native form and to a lesser extent compared to ns-LTP1 (Specker et al., 2014). Furthermore, hop ingredients influence the gushing behavior of beer. Unsaturated fatty acids, such as iso-α-acids, lupuline, and cohulupone have a gushing inhibiting effect but their oxidation products and some saturated fatty acids may be gushing inducers. The ratio of unsaturated to saturated fatty acids in hops and of oxidized materials to unchanged α-acids, however, is such that hop is supposed to have a rather gushing reducing effect (Carrington et al., 1972; Lutterschmid et al., 2010; M. P. Müller et al., 2010; Sandra et al., 1973). Another gushing promoter described in literature is papainase added to beer to stabilize haze proteins (Amaha & Horiuchi, 1979; Kieninger, 1976).

More recent publications suggest a holistic point of view on gushing instead of focusing on single gushing factors. Accordingly, gushing occurs if a threshold of the so called gushing potential is exceeded, to which different factors can contribute (Gastl et al., 2008). According to this assumption, there is also not a single measure that could prevent gushing, but many different measures can reduce the gushing potential and together they result in a flawless product. Gushing-reducing measures that have been suggested in the literature for the treatment of beer are for example the use of sound barley (Gjertsen, 1967), an increased hopping rate (Gardner et al., 1973), treatment with an anti-foam based on hop-oil (Shokribousjein et al., 2014), treatment with specific adsorbents such as kaolin or activated alumina (Curtis & Martindale, 1961), treatment with cobalt salts (Thorne & Helm, 1957), treatment with silica products enriched with calcium (V. Müller et al., 2013) or treatment with calcium to precipitate calcium oxalate crystals prior to filtration (Zepf & Geiger, 1999). Other methods include proper filtration (Narziß et al., 1990), blending with non-gushing beer (Gjertsen, 1967), germination of barley in the presence of microorganisms which inhibit the growth of hydrophobin-producing fungi, or coating glass bottle necks with hydrophobin-

binding materials (Laitila et al., 2007; Postulkova et al., 2016). However, the gushing problem in beer is neither completely understood nor solved.

1.1.3 Research on gushing in sparkling wine

With regard to gushing in sparkling wine, the majority of research has been focused on secondary gushing and revealed similar factors as in beer gushing - namely particles in the product, crystallization (e.g. tartar), irregularities on the inner bottle surface, paraffin dissolved from the cork, or residuals from the bottle washing process as gushing inducers (Bach, 2001; Henning, 1963; Rankine, 1977; Schanderl, 1964). In contrast, only few studies have been published about primary gushing in sparkling wine and factors such as tannins (Henning, 1963) or fungal spores (Schanderl, 1964) were found. Hydrophobic colloids like wax from the grape cuticle, grape skin cell wall particles, or fatty acids from grape seeds or from the yeast can coagulate and eventually induce gushing (Bach, 2001). As most of those factors come from the grape skin, it is not surprising that red sparkling wine is more prone to gushing than white sparkling wine. Vogt et al. (2017) identified two surface-active proteins from *Penicillium oxalicum* and Frisch et al. (2021) identified a surface-active protein from *Penicillium expansum*, respectively, that can induce gushing in sparkling wine made from infected grapes. Moreover, the protein content and the foaming properties of sparkling wines made from grapes infected with *Botrytis (B.) cinerea* have been demonstrated to be altered due to the secretion of fungal proteases (Cilindre et al., 2007; Richard Marchal et al., 2020; Richard Marchal et al., 2001). According to Kupfer et al. (2017b), botrytized wines are more prone to gushing due to the release of proteinases by *B. cinerea*. The proteolytic activity degrades *inter alia* the yeast mannoprotein Seripauperin 5 (Pau5p) from *Saccharomyces* (*S*.) *cerevisiae*, which was identified as a negative biomarker for gushing. More precisely, the authors analyzed 35 sparkling wines 18 of which were gushing. Among the gushing-negative bottles, 94% contained large amounts of Pau5p, whereas among the gushing-positive bottles 50% lacked Pau5p. This means that the absence of the yeast protein is correlated with a high gushing risk. Furthermore, Pau5p has foam-stabilizing properties and thus has a direct gushing-reducing effect (Kupfer et al., 2017a; Kupfer et al., 2017b). This is in accordance with earlier studies suggesting a gushing-reducing effect of yeast mannoproteins, which could serve as protective colloid and inhibit the formation of other colloids (Bach et al., 2001).

1.2 Sparkling wine production

1.2.1 Definitions of sparkling wine

According to the European Union, sparkling wine is a product, which is obtained by first or secondary alcoholic fermentation from grapes, grape must, or wine and which releases carbon dioxide derived exclusively from fermentation upon opening of the container. Furthermore, it has to have an excess pressure in closed containers of at least 3.0 bar at

20 °C and the total alcoholic strength of the cuvées used for the preparation must be at least 8.5 % vol. (European Union, 2008). There are a number of further classifications such as “quality sparkling wine”, which shall have an excess pressure of minimum 3.5 bar when kept at 20 °C in closed containers (European Union, 2008). Furthermore, several terms indicate the geographical origin of the sparkling wine or are local quality awards. The probably most popular and most prestigious sparkling wine is champagne, which must be produced in the Champagne in France exclusively from the varieties *Pinot Noir*, *Pinot Meunier*, and *Pinot Chardonnay* (Arntz, 1997), whereas Prosecco is a product derived from the *Prosecco* variety in the Veneto region in Italy, and Catalonia in Spain is known for its *DO Cava* sparkling wine (Buxaderas & López-Tamames, 2012). Regarding Germany, the term *Sekt* is often used as a synonym for sparkling wine, but is indeed a designation for a German quality product (*Qualitätsschaumwein*) (Arntz, 1997).

1.2.2 Base wine production

1.2.2.1 Primary fermentation

The first step in wine production is the harvest of the grape *Vitis vinifera*, of which many different varieties are used, as for example Riesling, Pinot Blanc, Muscatel, or Chardonnay for white wines or Pinot Noir for red wines. The ripe grapes are mechanically destemmed and crushed. In the case of white wine (see Figure 3A), the juice is directly gained by pressing and optionally clarified by filtration, centrifugation, or cold settling before it is drained or pumped (racked) into a stainless-steel tank. Next, the yeast starter culture (usually *S. cerevisiae*, for further details see chapter 1.3.2) is added (10^6-10^7 cells/ml) and the juice undergoes alcoholic fermentation at 18-20 °C for at least seven days. As shown in Figure 3B, the order of the wine making process is different in red wine production. Here, the alcoholic fermentation of the must is conducted before removal of the grape skins. Maceration takes place in the fermentation tank, which is the rising of the skins to the top. Therefore, the must is regularly pumped over the resulting cap or the cap is punched down. This process leads to the release of anthocyanin pigments and other phenolic compounds like tannins lending the wine its typical color and astringency (Fleet, 2007).

1.2.2.2 Malolactic fermentation

Malolactic fermentation is optional for white wine production and necessary for almost all red wines. Lactic acid bacteria, mainly *Oenococcus oeni,* naturally present in the wine can induce this additional fermentation, but wine makers often add starter cultures. The main reaction in malolactic fermentation is the decarboxylation of L-malate to L-lactate. The process also leads to an increased pH, reduces the amount of residual sugar, and releases aromatic compounds (Bauer & Dicks, 2004; Lonvaud-Funel, 1995).

1.2.2.3 Fining and aging

Fining agents can be added to the grapes, must, young wine, or wine. For further information on fining agents in sparkling wine production, see chapter 1.2.4. More information about the aging process can be found in chapter 1.3.2.3.

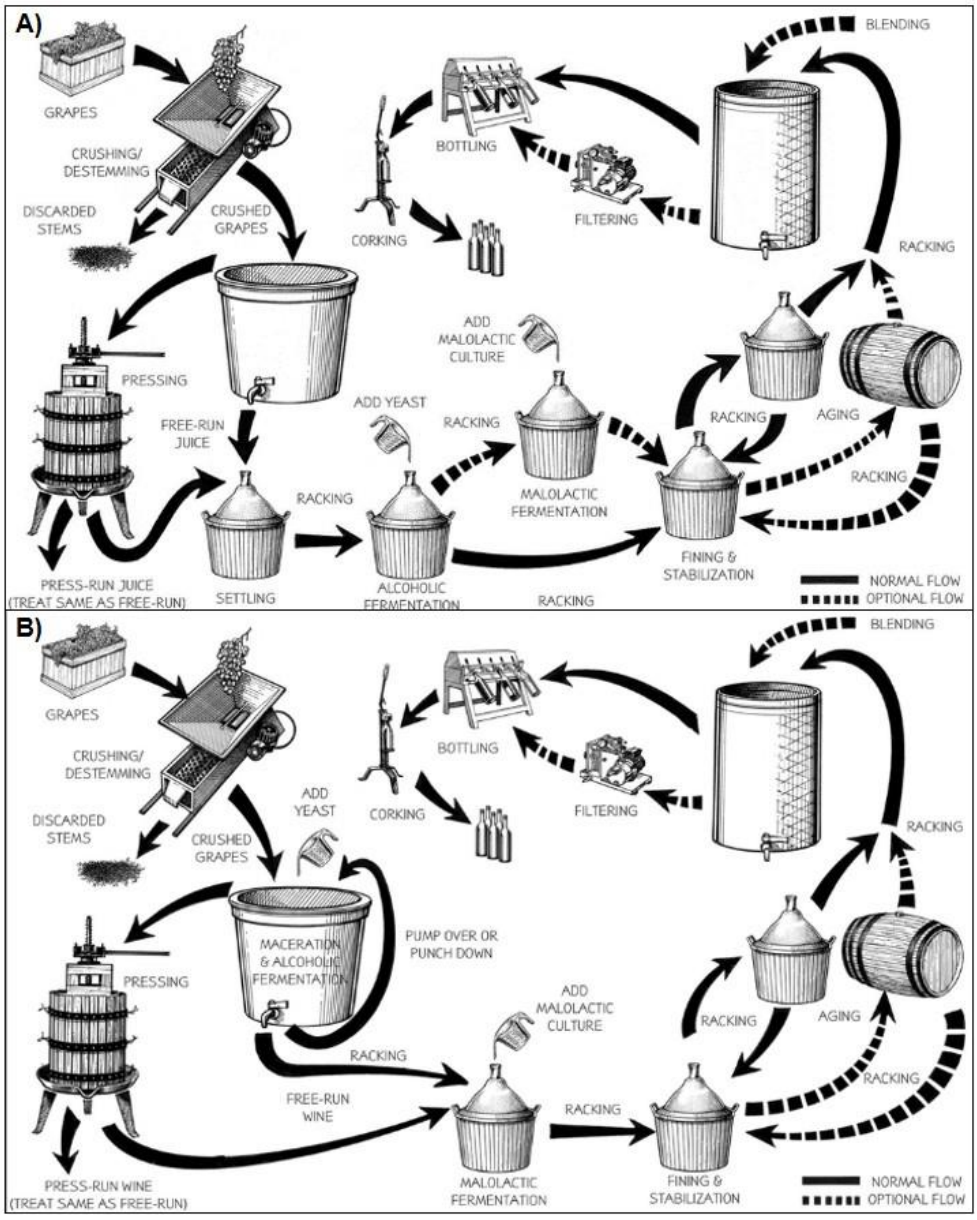

Figure 3: Flow charts of white and red wine production
The production process of white wine (**A**) and red wine (**B**) are displayed schematically (Northeast Winemaking, 2021).

1.2.3 Secondary fermentation

In industrial sparkling wine production, several different base wines are blended. The aim is to create cuvées with similar sensory properties each year to produce sparkling wines with consistent taste and quality (Jährig & Schade, 1993).

1.2.3.1 Champenoise method

This method is also called traditional method and refers to traditional bottle fermentation. The base wine is supplemented with up to 24 g/L sugar, nutrients, optional excipients like riddling aid (see chapter 1.2.4.3), and yeast starter culture consisting of rehydrated active dry yeast. The mixture is then filled into bottles and fermentation takes place at 10-15 °C for at least 90 days in a horizontal bottle position. The sparkling wine can then age on the lees and long aging is associated with higher organoleptic quality. In the case of *Sekt*, the wine must lay on the lees for a statutory minimum of nine months. To remove the yeasts, bottles are historically riddled manually in a rack. In this process, also called remuage, the bottles are stacked in a horizontal position for two weeks, in the first of which they are not moved to allow the yeast to settle, and in the second week they are periodically twisted alternately 1/8 to the right and to the left to narrow the area on which the settled yeasts lay in the bottle. In the following two weeks they are placed a little steeper with each turning until they are almost vertical. In this process, the sediment moves completely to the bottle neck. Before disgorgement, the necks are dipped in a calcium chloride of ethylene glycol freezing bath. Upon opening of the bottles, which are sealed with a bidule and a crown cap, the frozen yeast plug is pushed out of the bottle due to the overpressure inside. In modern sparkling wine production, automated riddling and disgorgement machines decrease the time requirement drastically. Finally, the dosage (*expedition liquor*) is added, which consists mainly of wine or grape must, sugar, and sulfur dioxide to adjust the desired sweetness and ensure microbial stability, before corking, placement of capsule and wire (called agraffe), and labelling (Arntz, 1997; Bach et al., 2010; Buxaderas & López-Tamames, 2012; Fleet, 2007; Jährig & Schade, 1993).

Figure 4: Sparkling wine bottles in a riddling rack
Bottles from sparkling wine production with the traditional method are historically riddled manually to settle the yeast sediment in the bottle neck before disgorgement.

1.2.3.2 Transfer method

In the transfer method, the preparation of the cuvée and the first dosage is the same as for the traditional method and the secondary fermentation also takes place in glass bottles, although those often have the two- or three-fold volume of normal sparkling wine bottles. Instead of riddling and disgorgement after a short maturation on the lees, the sparkling wine is filled into a pressure tank where the dosage is added. Yeasts are removed by filtration before the product is bottled, corked, and labeled (Fleet, 2007; Jährig & Schade, 1993).

1.2.3.3 Charmat method

In this method, also called Granvas or bulk method and often applied by large modern sparkling wine producers, the secondary fermentation takes place in large isobaric tanks. Those are equipped with agitators and temperature control systems that allow a faster fermentation than in the bottles. It can be distinguished between short and long Charmat method, in which the wine is on the lees in the tank for 1-3 moths or up to 6 months, respectively. The first results in a young and fruity sparkling wine, the latter has a more evolved aroma and resembles wine produced by bottle fermentation. Afterwards, the sparkling wine is decanted in a second tank, which contains the dosage. Clarification is conducted by centrifugation and/or filtration and the product is bottled under a counter-pressure. The advantages of this method lie in the possibility of continuous controls of the fermentation process, in a guarantee of complete fermentation, and in an economic benefit (Buxaderas & López-Tamames, 2012).

1.2.4 Fining and fining agents

1.2.4.1 The principle of fining

Fining agents are reactive or adsorptive substances, which shall reduce the concentration of undesirable molecules from the wine. Especially colloidal salts are to be removed to clarify wines and protect them against natural colloidal haze, which cannot be ensured by manual techniques such as centrifugation or filtration alone. The fining agents form a floccular precipitate after a settling period, which can last from hours to weeks, depending on the agent used. Afterwards, the fining agents must be removed completely, as they are no wine ingredient but technical “assistants” or adjuvants. The wine can therefore be decanted and/ or filtrated resulting in a permanently clarified and protein as well as tartare stabilized wine. There are different classes of both organic and inorganic fining agents including earths (bentonite, kaolin), animal or plant proteins (gelatin, isinglass, caseins, pasteurized milk, albumens, or proteins from cereals, grape seeds, potatoes, legumes, etc.), polysaccharides (alginates, gum arabicum), carbons (wood charcoal), synthetic polymers (PVPP, nylon), silica gel (silicon dioxide), tannins, and others (including metal chelators, blue fining, and enzymes) (Marangon et al., 2019; Richard Marchal & Jeandet, 2009; Zoecklein et al., 2013). They can be added at diverse steps of sparkling wine production. A common practice is sulphuration of the grapes or addition of charcoal or bentonite to the grapes to avoid oxidation processes. In white wine production, fining of the must is very frequently applied. In red wine production however, this is impossible but fining agents can be added to the mash during maceration. In both cases, the fining at this production step is advantageous because no additional process time or step is necessary. Furthermore, young wine can be fined to stabilize the product immediately. Anyhow, most fining agents are added to the wine at the end of the production process. At this stage not only protein and tartar stability can be

achieved, but also sensory wine faults can be corrected. Aside from the addition of fining agents, physical measures to obtain tartar stability can also be applied. One common example is cold stabilization. Its principle is based on the fact, that tartaric acid has a lower solubility at lower temperatures. Thus, the wine can be cooled down to the coldest expected storage temperature and then filtered at this temperature. By this, cold-induced tartar formation can be avoided (Steidl, 2004).

1.2.4.2 Bentonite fining

The most common fining agent is bentonite, which is a volcanic material weathered over millions of years into a mineral. More precisely, it is a complex hydrated aluminum silicate with exchangeable cationic components like sodium or calcium. It exists as small plates (1 nm x 500 nm), which upon rehydration form a structure with a huge negatively charged surface and small positively charged edges (see Figure 5). Proteins, which are mostly positively charged in wine due to the low pH, can be adsorbed at this negative surface. Indirectly, phenolic compounds may be adsorbed if they are complexed with proteins. Three-quarter of the proteins in wine react with bentonite within a few minutes, allowing for rapid processing (Zoecklein, 1988).

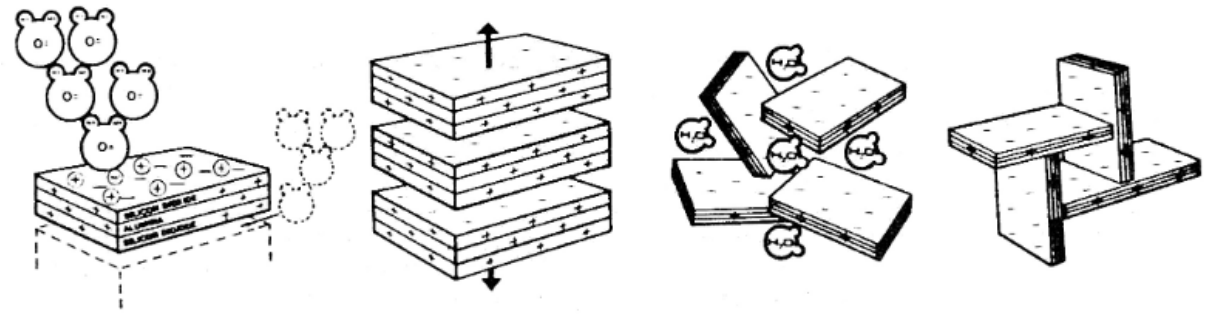

Figure 5: Bentonite rehydration
Upon rehydration, bentonite nanoplates form a “house of cards” – a molecular structure with a large negatively charged surface and high binding affinities for positively charged proteins (Zoecklein, 1988).

Many different forms of bentonite exist depending on their different geographical locations and depths where they are mined from, the level of purity, the particle size, and other processing parameters. Thus, the type and source of bentonite affects the adsorption capacity and swelling ability and therewith also the protein removal capacity. Generally, sodium bentonite has a great swelling power. In contrast, calcium bentonite tends to agglomerate, which reduces the exposed surface area and thereby protein binding. As a result, calcium bentonite produces more compact lees than sodium bentonite, but precipitation takes longer (Zoecklein, 1988). In sparkling wine production, the use of a mixture of bentonite together with other fining agents such as activated charcoal and PVPP or potassium caseinate and cellulose microcrystalline can enhance the fermentation kinetic,

improve the foam stability, and reduced the browning ability (Gava et al., 2020; Puig-Deu et al., 1999).

1.2.4.3 Riddling aid

Technically spoken, riddling aids are fining agents. They are, however, applied to improve the riddling ability and disgorgement in sparkling wines produced with the Champenoise method. The most common riddling aids are different bentonite-based products, especially for young white wines. Calcium bentonites are preferred over sodium bentonites because they form more compact lees. At the applied amounts they have a neglectable influence on the sensory quality of the wine (Zoecklein, 1988, 1998). Other examples are tannins or gelatin used especially for older or aged wines (Zoecklein, 1998) or potassium alginate used in the organic wine production (Bioland, 2019).

1.2.4.4 Mannoproteins

Mannoproteins are highly glycosylated yeast proteins, which consist of approximately 90% sugars, mainly mannose. As major cell wall components, they account for 25-50% of the cell wall dry weight and up to 20% of the cell dry weight (Martínez et al., 2016; Pozo-Bayón et al., 2009). Located in the outer cell wall layer and connected to a matrix of amorphous β-1,3-glucan by covalent bonds, they play a major role in cell wall stability and porosity (Torresi et al., 2011).

During fermentation and aging on the lees, they are released to the medium and thus play an important role in the vinification process. Mannoproteins account for approximately 35% of the polysaccharide content in wine. Their functions include, *inter alia*, wine enrichment and reinforcement of aromatic compounds, adsorption of toxic substances possibly present in wine such as orchatoxin A, an increased growth of malolactic bacteria, reduction of the astringency, and prevention of tartar crystallization and of protein haze (Caridi, 2006; Pérez-Serradilla & de Castro, 2008; Quirós et al., 2010). A proposed theory on the mechanism of haze is a competition between mannoproteins and other wine proteins for unknown non-proteinaceous wine components, which are required for the precipitation of denatured proteins into large insoluble aggregates (Dupin et al., 2000; Waters et al., 2005). Several attempts including UV mutagenesis or hybridization have been made to increase the mannoprotein release by industrial *S. cerevisiae* wine yeast strains resulting in improved mannoprotein-related characters of wines (González-Ramos et al., 2010; Pérez-Través et al., 2016; Quirós et al., 2010). Another possibility is the direct application of yeast mannoprotein-containing preparations during vinification as fining agent (Lochbühler et al., 2015; Richard Marchal & Jeandet, 2009) or as protection during active dry yeast rehydration and as enhancers of alcoholic or malolactic fermentation (Pozo-Bayón et al., 2009). As such, yeast mannoproteins are rated among the most used additives for improving either

technological processes or sensory characteristics of wine (Pozo-Bayón et al., 2009). The European Union allows the addition of yeast mannoproteins as stabilizing agents to partially fermented must, wine, and sparkling wine. Moreover, yeast autolysates in fresh grapes and grape must as well as yeast cell walls and inactivated yeasts in fresh grapes, grape must, wine, and sparkling wine are authorized as processing aids (European Union, 2019).

1.3 Wine yeasts

1.3.1 *S. cerevisiae* - an important industrial tool and powerful model organism

The yeast species *Saccharomyces cerevisiae*, shown in Figure 6, belongs to the division Ascomycota in the subkingdom Eumycota of the kingdom of Fungi. It has been biotechnologically used for thousands of years, albeit unconsciously in the past. The most prominent applications lie in the food and beverages industry, especially in baking and fermentation of beer, wine, and cider, but it is also applied for biofuel production. Whereas the yeast was traditionally insect-born, it is deliberately added to these processes as starter culture in modern industry. During the last decades, uncountable pure-breed high-performance strains of *S. cerevisiae* with distinct characteristics have been carefully cultured, selected, and purified, so that the species is regarded as domesticated (Duina et al., 2014; Gallone et al., 2016; Jährig & Schade, 1993; Parapouli et al., 2020).

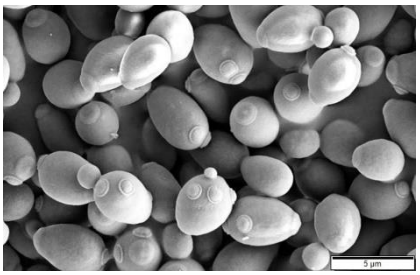

Figure 6: *Saccharomyces cerevisiae*
Electron microscopy of a fresh yeast culture (Murtey & Ramasamy, 2016).

Apart from the biotechnological importance, *S. cerevisiae* is also a valuable model organism. It has been the first eukaryotic organism whose genome, which consists of 12068 kilobases (kb) encoding for approximately 6000 genes organized in 16 chromosomes, has been fully sequenced (Goffeau et al., 1996). Furthermore, it has the most advanced selection of genetic tools available for any eukaryotic organism (Duina et al., 2014). Therefore, it has frequently been applied to study eukaryotic biology, as for example cell aging, human diseases, or specific processes in species with a long generation time or development time, such as plants (Bilinski et al., 2017; Fabrizio & Longo, 2003; Karathia et al., 2011; Menezes et al., 2015; Nielsen, 2019).

1.3.2 *S. cerevisiae* in wine production

1.3.2.1 Alcoholic fermentation

One of the most important features of *S. cerevisiae* for wine production is the fact that the yeast converts glucose and fructose into ethanol and carbon dioxide by alcoholic fermentation. Even under aerobic conditions, the respiratory pathway is repressed at high sugar levels such as present in grape must. This so called “Crabtree effect”, shown in Figure

7A, is named after the biochemist Herbert Grace Crabtree and results in a drastically reduced biomass production compared to Crabtree negative organisms, which convert the sugar completely into biomass by respiration (Barford & Hall, 1979; Crabtree, 1929; Dashko et al., 2014).

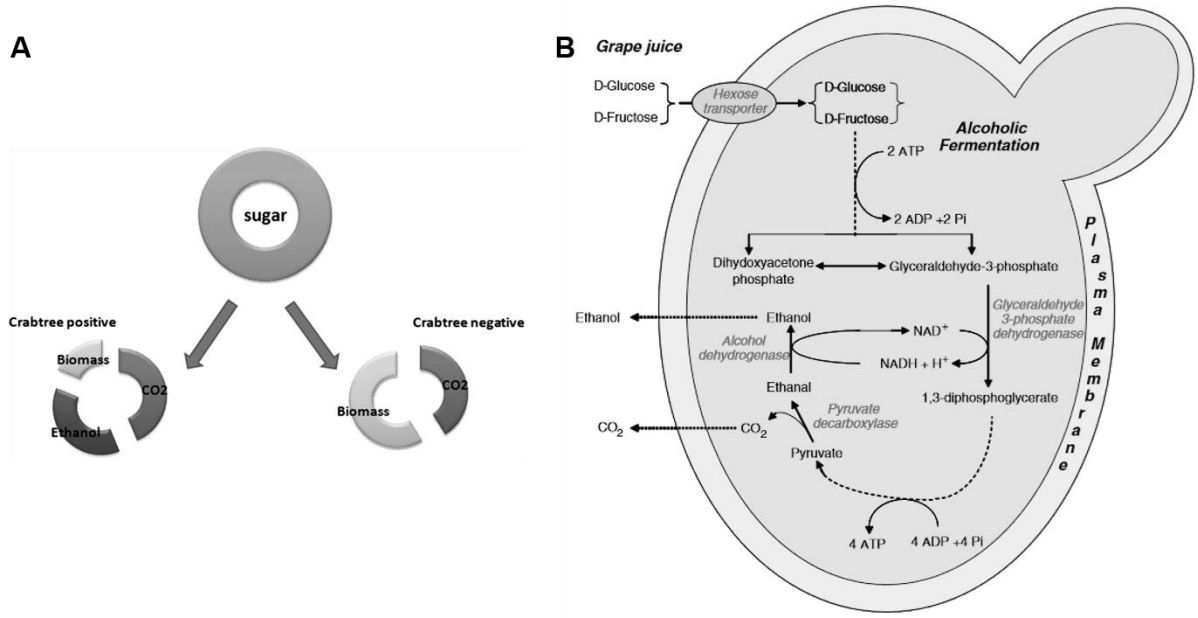

Figure 7: Crabtree effect and alcoholic fermentation
A) *S. cerevisiae* is Crabtree positive, which means that at high sugar levels, respiration is repressed and the sugars are mainly converted into ethanol and carbon dioxide resulting in a reduced biomass production compared to Crabtree negative organisms, which use the respiratory pathway under aerobic conditions (Dashko et al., 2014). **B)** The biochemistry of alcoholic fermentation (Zamora, 2009).

The main pathway for sugar consumption in yeasts is glycolysis. This is also a major part of alcoholic fermentation or respiration and consists of the intracellular transformation of glucose or fructose into pyruvate. It is performed by eleven successive biochemical reactions, the first of which is the phosphorylation of the hexoses under ATP consumption. This keeps the glucose and fructose levels inside the cytoplasm lower than the external sugar concentrations, allowing for hexose uptake by facilitated diffusion without expenditure of energy. The resulting hexose-6-phosphates are not only intermediate products in glycolysis but are also necessary as secondary metabolites in cell wall construction. In the next steps of glycolysis, however, each of these molecules is metabolized into two pyruvate molecules under the consumption of two NAD^+ molecules. Pyruvate can serve as substrate for respiration as well as for alcoholic fermentation. Hence, this is where alcoholic fermentation really starts by decarboxylation of pyruvate to ethanal releasing carbon dioxide. Next, ethanal is reduced to ethanol by an alcohol dehydrogenase which recycles NADH to NAD^+ (Zamora, 2009). Finally, ethanol is carried into the extracellular space where it inhibits growth of other microorganisms. Its own high ethanol tolerance together with its ability to grow under anaerobic conditions is how *S. cerevisiae* can compete with Crabtree negative bacteria or yeasts albeit its reduced biomass production, and thus usually becomes the

predominant species in wine fermentation, even without the addition of starter cultures (Dashko et al., 2014).

1.3.2.2 Necessary nutrients

1.3.2.2.1 Nitrogen

S. cerevisiae needs to assimilate nitrogen for biomass production. There is a variety of nitrogen compounds in grape juice such as ammonia, amino acids, peptides, or proteins of which only ammonia and all proteinaceous amino acids but proline are easy to assimilate for the yeast cells. Thus, those substances are referred to as easily-assimilable nitrogen (EAN), of which rather low concentrations are present in grape juice or must. However, in literature the term yeast assimilable nitrogen (YAN) is more common, as its concentration can be determined by NOPA analysis (see chapter 2.2.5.3) (Dukes, 2010). The YAN concentration influences the sugar consumption rate, the fermentation rate, and whether completion of the alcoholic fermentation can be achieved. That is why winemakers often supplement their grapes or musts with either inorganic nitrogen in the form of ammonium salts or organic nitrogen in the form of inactive dry yeasts. The YAN concentration should be higher than 130 mg/L to ensure a complete alcoholic fermentation, albeit the nitrogen assimilation is also yeast strain dependent. Excess ammonium, on the contrary, may lead to microbial instability and negatively influence the palatability of the wine because less aromatic secondary products are built by deamination of amino acids (D'Amato et al., 2006; Martí-Raga et al., 2016; Mendes-Ferreira et al., 2004; Taillandier et al., 2007; Zamora, 2009). Furthermore, the initial YAN concentration is a major factor influencing the production of aroma compounds by *S. cerevisiae* (Rollero et al., 2015). In sparkling wine production, nitrogen is often added to the cuvées prior to secondary fermentation. It has been examined that the nitrogen source during acclimation of the inoculum has an effect on the kinetic of the secondary fermentation (Martí-Raga et al., 2016) and that the addition of inactive dry yeast to the base wine can lead to increased polysaccharide contents and foaming properties of the sparkling wine (Martí-Raga et al., 2016).

1.3.2.2.2 Other nutrients

Apart from nitrogen, yeasts need a lot of vitamins and minerals which grape juice is often lacking. A nutrient deficiency, however, leads to sluggish or stuck fermentations. To overcome this problem, a lot of different commercial yeast activators exist, which often contain ammonium and thiamine (vitamin B1) and sometimes also other substances like sterols, unsaturated fatty acids, minerals, or panthothenic acid (Zamora, 2009). Some intracellular enzymes also need special cofactors. For example, the pyruvate decarboxylase needs magnesium and thiamine pyrophosphate (S. Hohmann, 1996) and the alcohol dehydrogenases *ADH1* and *ADH3* are dependent on zinc (Bird et al., 2006).

1.3.2.3 Aging and autolysis

Aging *sur lies* is mandatory for sparkling wine production with the traditional method, whereas it is skipped or shortened in the Charmant method. But it has also gained popularity in the elaboration of still wines. The wine lees consist mainly of yeast cells and their remnants, tartaric acid crystals, and possibly clarifying agents like bentonite. During the contact with the lees, mostly unwanted molecules are adsorbed whilst other are released, partly by autolysis. Autolysis is the enzymatic self-degradation of yeast biopolymers which is associated with the stationary growth phase in alcoholic fermentation and cell death. Released compounds can be grouped by their origin: Polysaccharides like glucans and mannoproteins are cell wall components whereas the compounds released from the cytoplasm include aroma compounds, amino acids, peptides, fatty acids, and nucleotides. The major products of autolysis, however, are peptides that result from the degradation of yeast proteins mainly by proteinase A and other acidic proteolytic enzymes. Long aging on the lees is also associated with higher antioxidant capacity and color stability (Buxaderas & López-Tamames, 2012; Martínez-Rodríguez & Pueyo, 2009). One point here are phenolic compounds from grape skins and seeds, which lead to a high astringency of the wine and upon oxidation lead to unwanted browning of white wines. The lees can adsorb and release phenolic compounds and by autolysis set free enzymes altering the phenolic content. Furthermore, released mannoproteins can also adsorb phenolic molecules. With regard to aroma compounds, there are opposite effects: large amounts of lees promote ester synthesis, which positively influences wine quality, but compounds with detrimental effects on wine quality such as long-chain alcohols and volatile fatty acids are also produced (Pérez-Serradilla & de Castro, 2008). It is generally agreed that aging and autolysis significantly alter sparkling wine composition and quality, but despite extensive investigations the process and its organoleptic consequences are not yet completely understood and some controverse findings exist. But overall, long aging *sur lies* is an important quality attribute of sparkling wines. Because of the costs and time needed for this long process, some attempts have been made to reduce the production time of aged wine by increasing the speed of autolysis. Induced autolysis, which is frequently used in industry to manufacture yeast extracts or culture medium, takes place in only 48-72 h. In the natural process in sparkling wine production, the conditions are far from optimal for autolysis and thus responsible for the long duration. Such attempts include the addition of yeast autolysates, an increased temperature during aging, the selection of natural or mutated yeast strains with increased autolytic capacity, or the use of killer and sensitive strains. However, the sensory effects of those strategies have not been fully investigated (Gonzalez et al., 2003; Lombardi et al., 2015; Martínez-Rodríguez & Pueyo, 2009).

1.3.3 Non-*Saccharomyces* yeasts in winemaking

More than 40 different yeast species have been isolated from grape surfaces. As such, a high number of different yeasts is present also in grape must. There is naturally a sequential succession of yeast species during fermentation dependent on their metabolic properties and many non-*Saccharomyces* species (especially species of *Hanseniaspora, Candida, Pichia* and *Metschnikowia*) are known to initiate spontaneous fermentation. In spontaneous fermentation as well as in inoculated fermentation, however, *S. cerevisiae* will very soon be the dominant species due to ethanol accumulation. The contribution by non-*Saccharomyces* yeasts to wine palatability is dependent on their activity. It is well known that they play a role in the release of flavor compounds including terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid (Fleet, 2008; Jolly et al., 2014). Furthermore, they are a source of mannoproteins, nitrogen, enzymes, and antimicrobial compounds (Domizio et al., 2014; Vejarano, 2020). Nowadays, many winemakers consider non-*Saccharomyces* yeasts as important tool to produce “natural” wines with enhanced flavor and aroma complexity. So, an array of yeast products with non-*Saccharomyces* strains including dry active yeasts is available on the market. Fermentation with solely wild yeasts is possible, but mixed fermentation with a *Saccharomyces* yeast in sequential or co-inoculation is preferred. One of the first species commercially available was *Torulaspora* (*T*.) *delbrueckii*, which is suitable for wine and sparkling wine production in mixed or sequential fermentation with *S. cerevisiae*. This results in a beverage with increased glycerol concentration, decreased volatile acidity, and favorable foaming properties. Another example is *Metschnikowia* (*M*.) *pulcherrima*, which has a high ß-glucosidase activity and produces medium-chain fatty acids, alcohol, acetate, acetate esters, polysaccharides, and terpenols. Furthermore, it has an antimicrobial effect on spoilage organisms and can be applied for ethanol reduction in the final product (Ivit & Kemp, 2018; Romano et al., 2019). The use of combined fermentation with *Saccharomyces* and non-*Saccharomyces* yeasts bears a great potential for the production of high quality wines with a complex aroma, but further elucidation of the complex interactions is required for the spread of mixed-culture techniques (Sablayrolles, 2009).

1.4 The yeast protein Seripauperin 5

1.4.1 The seripauperin gene family

PAU5 belongs to the largest multi-gene family in *S. cerevisiae*, comprising 24 genes with high sequence similarity, which are distributed across all 16 *S. cerevisiae* chromosomes (Goffeau et al., 1996; Luo & van Vuuren, 2009; Viswanathan et al., 1994). The *PAU* gene family is closely related to the *TIR* and *DAN* gene families with a common N-terminal sequence homology (Abramova et al., 2001). All these genes are stress-related and mainly

upregulated under anoxic conditions. Their promoter regions contain binding motifs for Upc2p and Mot3p, a heme-inhibited activator and a heme-induced repressor, respectively (Abramova et al., 2001; Luo & van Vuuren, 2009). *PAU* genes are regulated in a Rox1p independent manner, but are aerobically depressed by Tup1p (Rachidi et al., 2000b).

1.4.2 Seripauperin 5

In contrast to most *PAU* genes, which are located in subtelomeric regions, *PAU5* is located close to the centromere of chromosome VI (Luo & van Vuuren, 2009). Pau5p consists of 122 amino acids and has a grand average of hydropathy (GRAVY) value of 0.307, thus being a hydrophobic integral membrane protein in its non-mannosylated state. However, Pau5p can be *O*-mannosylated at 23 serine / threonine residues. The resulting soluble glycoprotein is secreted via its predicted signal peptide at amino acids 1-19 (Luo & van Vuuren, 2008; Viswanathan et al., 1994). The amphiphilic structure of mannoproteins with a hydrophobic protein moiety and a hydrophilic sugar moiety allows them to contribute to foam formation (Blasco et al., 2011). The glycoprotein Pau5p has demonstrated to stabilize foam and is a negative biomarker for gushing in sparkling wine (Kupfer et al., 2017a).

1.5 Motivation of this study

The spontaneous intense over-foaming of sparkling wine upon opening of a bottle – known as gushing – is responsible for excessive losses to the beverage industry. According to the *Verband Deutscher Sektkellereien e.V.* (VDS) up to two percent of the annual production capacity of their member companies can be affected in a typical gushing vintage (personal communication VDS, 12.09.2017). As gushing regularly leads to consumer complaints, the producers must face not only economical but also reputational damages. The industry therefore seeks for practical solutions to reduce the gushing potential of their products. This study was part of and funded by a project of the Research Association of the German Food Industry (FEI) via the German Federation of Industrial Research Associations (AiF) [grant AiF 19952 N] within the program for promoting the Collective Industrial Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi) based on an enactment of the German Parliament. The aim of this project was the development of diagnostic and technologic strategies to reduce gushing in sparkling wine. In a previous project, the yeast protein Pau5p has been identified as negative biomarker for gushing in sparkling wine and its presence was also found to have a gushing reducing effect. The current thesis is focused on this biomarker and has the global aim to find ways of increasing the Pau5p content in sparkling wine and therewith reduce the risk of gushing. The thesis has been based on the following hypotheses:

- The knowledge of the presence or absence of Pau5p in base wines can serve to improve quality control and to forecast the gushing potential of sparkling wines produced from them.
- An antibody can be generated against Pau5p with which a simple and fast immunochemical detection method can be established.
- Typical *Saccharomyces cerevisiae* (sparkling) wine yeast strains have different production potentials for the gushing reducing protein Pau5p.
- The use of high Pau5p producing yeast strains can increase the Pau5p content in sparkling wine and thereby reduce the risk of gushing.
- Certain treatment procedures can be identified which have a positive effect on the Pau5p content in sparkling wine and thereby decrease its gushing potential.

In order to test these hypotheses, the following objectives should be persued in the current study:

- Cloning, recombinant expression, and purification of Pau5p.
- Generation of specific antibodies against Pau5p.
- Development of an immunochemical detection assay for Pau5p for easy on-site application.
- Screening of *S. cerevisiae* (sparkling) wine yeast strains for their potential to form Pau5p.
- Identification of fermentation parameters that increase the Pau5p production in *S. cerevisiae* strains.
- Analysis of the effect of different treatment procedures on the Pau5p content in sparkling wine production under practical conditions.

Some of the above-mentioned objectives could only be addressed in an interdisciplinary approach. We therefore collaborated with the Department of Microbiology and Biochemistry and the Department of Enology from the Hochschule Geisenheim University, which have the necessary facilities for (sparkling) wine production under practical conditions. Prof. Dr. Doris Rauhut and Michael Wallbraun and their teams kindly helped with performing the practical sparkling wine experiments and measured the internal bottle pressures. Prof. Dr. Doris Rauhut furthermore performed the FTIR, ammonium, and NOPA analyses as well as the bentonite fining, which are displayed and discussed in the present thesis.

2 Material and Methods

2.1 Material

2.1.1 Microorganisms

Microorganisms that were employed for or generated during this study are listed in Table 1.

Table 1: Microorganisms
All microorganisms used in the study are displayed.

Species	Description	Source	TMW number
E. coli	TOP10	TMW strain collection	2.0580
E. coli	TOP10 pPICZαA	TMW strain collection, plasmid from Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA	2.0651
E. coli	TOP10 pRS62K	This study, plasmid from Goethe-Universität-Frankfurt, IMBW, Prof. Boles	2.2105
E. coli	TOP10 pRS62K *PAU5* native	This study	2.2113
E. coli	TOP10 pRS62K *PAU5* HisTEV	This study	2.2116
E. coli	TOP10 pPICZαA *PAU5* native	This study	2.2275
E. coli	TOP10 pPICZαA *PAU5* HisTEV	This study	2.2276
Metchnikowia pulcherrima	White wine	Anonymous manufacturer	3.1016
P. pastoris	X33	Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA	3.0177
P. pastoris	pPICZαA (empty vector integrated)	Dissertation Lisa M. Frisch, Chair of Technical Microbiology, TUM	3.1068
P. pastoris	pPICZαA *PAU5* native	This study	3.1190
P. pastoris	pPICZαA *PAU5* native	This study	3.1191
P. pastoris	pPICZαA *PAU5* native	This study	3.1192
P. pastoris	pPICZαA *PAU5* native	This study	3.1193
P. pastoris	pPICZαA *PAU5* HisTEV	This study	3.1194
P. pastoris	pPICZαA *PAU5* HisTEV	This study	3.1195
S. cerevisiae	TUM68 (wheat beer strain, top-fermented)	TMW strain collection	3.0250
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.0704
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.0705
S. cerevisiae	Sparkling wine yeast	TMW strain collection	3.0933
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.0997
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.0998
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.0999
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1000
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1001
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1002
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1003
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1004
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1005

S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1006
S. cerevisiae	Sparkling wine yeast	Anonymous manufacturer	3.1007
S. cerevisiae	CEN.PK2-1C (MATa; ura3-52; trp1-289; leu2-3,112; his3delta 1; MAL2-8C; SUC2)	Goethe-Universität-Frankfurt, IMBW, Prof. Boles	3.1008
S. cerevisiae	Rosé and red wine	Anonymous manufacturer	3.1014
S. cerevisiae	Sparkling wine	Anonymous manufacturer	3.1015
S. cerevisiae	White wine	Anonymous manufacturer	3.1018
S. cerevisiae	White and red wine	Anonymous manufacturer	3.1020
S. cerevisiae	Sparkling wine	Anonymous manufacturer	3.1021
S. cerevisiae	White and sparkling wine	Anonymous manufacturer	3.1022
S. cerevisiae	Rosé and sparkling wine	Anonymous manufacturer	3.1023
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.1024
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.1025
S. cerevisiae	Fruit and sparkling wine yeast	Anonymous manufacturer	3.1026
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.1028
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.1029
S. cerevisiae	Isolated from gushing Auxerrois (2018)	This study	3.1039
S. cerevisiae	Isolated from gushing Riesling (2018)	This study	3.1040
S. cerevisiae	Fruit wine yeast	Anonymous manufacturer	3.1051
S. cerevisiae	pRS62K *PAU5* native	This study	3.1196
S. cerevisiae	pRS62K *PAU5* native	This study	3.1197
S. cerevisiae	pRS62K *PAU5* native	This study	3.1198
S. cerevisiae	pRS62K *PAU5* native	This study	3.1199
S. cerevisiae	pRS62K *PAU5* native	This study	3.1200
S. cerevisiae	pRS62K *PAU5* native	This study	3.1201
S. cerevisiae	pRS62K *PAU5* native	This study	3.1202
S. cerevisiae	pRS62K *PAU5* native	This study	3.1203
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1204
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1205
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1206
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1207
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1208
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1209
S. cerevisiae	pRS62K *PAU5* HisTEV	This study	3.1210
S. cerevisiae (var. bayanus)	Sparkling wine	Anonymous manufacturer	3.1012
S. cerevisiae (var. bayanus)	Sparkling wine	Anonymous manufacturer	3.1013
S. pastorianus	TUM34/70 (lager strain, bottom-fermented)	TMW strain collection	3.0257
Torulaspora delbrueckii	White and rosé wine	Anonymous manufacturer	3.1017

2.1.2 Primers

All primers utilized for cloning of *PAU5* and for sequencing are listed in Table 2.

Table 2: Primers
The primers and their sequences are listed in this table. The uppercase letters of the sequence bind directly to the target DNA whereas the lowercase letters are overhangs added by the primers during PCR, resulting in two different melting temperatures (T_m). The first temperature refers to the first cycles of PCR, when only a part of the primer can bind. The second temperature applies after the first cycles of amplification, when the whole primers binds. Fwd = forward primer; rev = reverse primer.

Name	Purpose	Sequence 5'-3'	T_m [°C]
P1	pPICZαA *PAU5* insert fwd	cctgaattcATGGTCAAATTAACTTCAATCGC	52.3/59.3
P2	pPICZαA *PAU5* native insert rev	ccgtctagagCTAATTTGCAATAGTGTAGATACCG	51.9/61.4
P3	pPICZαA *PAU5* His insert rev	cgtctagagcATTTGCAATAGTGTAGATACCGTCT	54.0/62.4
P4	pPICZαA *PAU5* HisTEV insert fwd	atcatcatcatcatgaaaacctgtattttcagagcATGGTCAAATTAAC-TTCAATCGC	52.3/65.4
P5	pPICZαA *PAU5* HisTEV insert rev	gctgggccacgtgaattcCTAATTTGCAATAGTGTAGATACC-G	51.9/67.4
P6	pPICZαA *PAU5* HisTEV vector fwd	cggtatctacactattgcaaattagGAATTCACGTGGCCCAGC	55.9/67.4
P7	pPICZαA *PAU5* HisTEV vector rev	ctgaaaatacaggttttcatgatgatgatgatgatgAGCTTCAGCCTC-TCTTTTCTCGAG	58.3/68.0
P14	pRS62K *PAU5* HisTEV insert fwd	taatcaaaaagttaacatgcatcaccatcaccatcacgaaaacctgtattttcagagcGTCAAATTAACTTCAATCG	44.3/67.8
P15	pRS62K *PAU5* native insert fwd	aaacacaaaaacaaaaagttttttaattttaatcaaaaaATGGTCAAATTAACTTCAAT	44.7/58.4
P16	pRS62K *PAU5* insert rev	ggagggcgtgaatgtaagcgtgacataactaattacatgaCTAATTTGCAATAGTGTAGA	44.6/67.3
P17	pPICZαA sequencing fwd	GACTGGTTCCAATTGACAAGC	65.0
P19	pPICZαA sequencing rev	GCAAATGGCATTCTGACATCC	65.0

2.1.3 Enzymes

Enzymes that were applied during this study and their producers are mentioned in Table 3.

Table 3: Enzymes
All enzymes used are listed.

Enzyme	Manufacturer
DraI FastDigest	Thermo Fisher Scientific Inc., Waltham, MA, USA
EcoRI FastDigest	Thermo Fisher Scientific Inc., Waltham, MA, USA
Phusion® High-Fidelity DNA Polymerase	New England BioLabs GmbH, Ipswich, MA, USA
Ribonuclease A (90 U/mol, salt free)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
SmaI FastDigest	Thermo Fisher Scientific Inc., Waltham, MA, USA
T4 DNA ligase	Thermo Fisher Scientific Inc., Waltham, MA, USA
Taq polymerase (*Taq* DNA CORE Kit 10)	MP Biomedicals, Santa Ana, CA, USA
XbaI FastDigest	Thermo Fisher Scientific Inc., Waltham, MA, USA

2.1.4 Antibodies

All used antibodies are listed in Table 4.

Table 4: Antibodies
All antibodies that were generated or used throughout the study are listed.

Antibody	Application	Manufacturer
Anti 6x-His tag, monoclonal, produced in mice	Primary antibody for recombinant protein detection	Thermo Fisher Scientific Inc., Waltham, MA, USA
Pierce® anti-mouse IgG-AP, produced in goat	Secondary antibody for recombinant protein detection	Thermo Fisher Scientific Inc., Waltham, MA, USA
Anti-HAC-AAG-IgG, polyclonal, produced in hen	Primary antibody for the detection of Pau5p	Davids Biotechnology GmbH, Regensburg, Germany
Anti-HAC-DIR-IgG, polyclonal, produced in hen	Primary antibody for the detection of Pau5p	Davids Biotechnology GmbH, Regensburg, Germany
Anti-hen IgY-AP, produced in rabbit	Secondary antibody for the detection of Pau5p	Sigma-Aldrich GmbH, Schnelldorf, Germany

2.1.5 Kits

All kits that were applied in the study are listed in Table 5.

Table 5: Kits
Names, catalogue numbers, and manufacturers of the used kits.

Kit name	Catalogue number	Manufacturer
Ammonia Assay Kit		Megazyme Ltd., Wicklow, Ireland
Enzymatic Deglycosylation Kit for N-Linked and Simple O-Linked Glycans	GK80110	Agilent Technologies, Santa Clara, CA, USA
GeneJet Gel Extraction Kit	K0691	Thermo Fisher Scientific Inc., Waltham, MA, USA
Protein Deglycosylation Mix II	P6044	New England BioLabs GmbH, Ipswich, MA, USA
QIAprep Spin MiniPrep Kit	27104	Qiagen N. V., Venlo, Netherlands
QIAquick PCR Purification Kit	28104	Qiagen N. V., Venlo, Netherlands

2.1.6 Chemicals

The chemicals used within the study and their manufacturers are listed in Table 6.

Table 6: Chemicals
All chemicals used in this study and their manufacturers are displayed.

Chemical	Purity	Manufacturer
2-Mercaptoethanol	BioReagent, 99%	Sigma-Aldrich, St. Louis, MO, USA
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)		Gerbu Biotechnik GmbH, Heidelberg, Germany
5x Phusion® HF Buffer		New England BioLabs GmbH, Ipswich, MA, USA

6x DNA loading dye		Thermo Fisher Scientific Inc., Waltham, MA, USA
Acetic acid	Rotipuran®, 100%, p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetone	≥ 99.5%, for synthesis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acetonitrile (ACN)	ROTISOLV® ≥ 99.9%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamide/Bis solution	30%, 37.5:1	SERVA Electrophoresis GmbH, Heidelberg, Germany
Agar agar	BioScience grade, granulated	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agarose Biozym LE	For gel electrophoresis	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Ammonium persulfate (APS)	≥ 98%, p. a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium sulfate	For enzymology	Gerbu Biotechnik GmbH, Heidelberg, Germany
Ampicillin sodium salt		Gerbu Biotechnik GmbH, Heidelberg, Germany
BD Difco™ Yeast Nitrogen Base (YNB)	Laboratory use, without amino acids and ammonium sulfate	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Boric acid	≥ 99.8%, p. a., ACS reagent, Ph. Eur.	Honeywell International Inc., Morristown, NJ, USA
Bovine serum albumin fraction V (BSA V)	≥ 98%, pulverized, for molecular biology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromphenol blue sodium salt	Electrophoresis grade	AppliChem GmbH, Darmstadt, Germany
Calcium chloride dihydrate	For analysis	Merck KGaA, Darmstadt, Germany
Calcium sulfate dihydrate	≥ 98%, Ph. Eur., extra pure	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Cobalt(II) chloride hexahydrate	ACS reagent, 98%	Sigma-Aldrich, St. Louis, MO, USA
Copper(II) sulfate pentahydrate	ACS reagent	VWR International, Radnor, PA, USA
D-(-)-Fructose	≥ 99.5%, for biochemistry	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-(+)-Biotin	≥ 98.5%, for biochemistry	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D-(+)-Glucose monohydrate	For microbiology	Merck KGaA, Darmstadt, Germany
di-Ammonium hydrogen citrate	≥ 98%, high purity	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)		New England BioLabs GmbH, Ipswich, MA, USA
Dimidium bromide	≥ 95%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
di-Potassium hydrogen phosphate trihydrate	For analysis	Merck KGaA, Darmstadt, Germany
Disodium hydrogen phosphate monohydrate	≥ 98%, p. a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	For microbiology	Gerbu Biotechnik GmbH, Heidelberg, Germany

D-Maltose monohydrate	Biochemical grade, for microbiology	Gerbu Biotechnik GmbH, Heidelberg, Germany
DNA from herring sperm	Lyophylized sodium salt	Hoffmann-La Roche, Basel, Switzerland
dNTP Mix	10 mM each	MP Biomedicals GmbH, Eschwege, Germany
D-Sorbitol	≥ 98%	Sigma-Aldrich, St. Louis, MO, USA
Ethanol	Absolute	VWR International, Radnor, PA, USA
Ethylenediaminetetraacetic acid (EDTA)		Gerbu Biotechnik GmbH, Heidelberg, Germany
FastDigest buffer	10x	Thermo Fisher Scientific Inc., Waltham, MA, USA
Formaldehyde	≥ 37%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
G 418 disulfate salt	powder	Sigma-Aldrich, St. Louis, MO, USA
Gene Ruler DNA Ladder	100 bp Plus; 1 kb	Thermo Fisher Scientific Inc., Waltham, MA, USA
Gluconic acid sodium salt	≥ 99%, for synthesis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glycerol	99.5%, anhydrous, high purity	Gerbu Biotechnik GmbH, Heidelberg, Germany
Glycine	99.56%	Gerbu Biotechnik GmbH, Heidelberg, Germany
HPLC grade water		J.T. Baker, Center Valley, USA
Hydrochloric acid	37%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Iron(II) sulfate heptahydrate	≥ 99.5%, Ph. Eur., USP	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	≥ 99.5%, for synthesis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-(+)-Ascorbic acid	≥ 99%, p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-cysteine hydrochloride monohydrate	≥ 98.5%, for biochemistry	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L-Glutamic acid monosodium salt monohydrate	≥ 98% (NA)	Sigma-Aldrich, St. Louis, MO, USA
Lithium acetate	Dihydrate	Sigma-Aldrich, St. Louis, MO, USA
Magnesium chloride	≥ 98.5%, anhydrous	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium sulfate heptahydrate	For analysis	Merck KGaA, Darmstadt, Germany
Malt extract	Pulverized	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Manganese(II) sulfate monohydrate	≥ 99%, p. a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Meat extract	Dry, granulated, for microbiology	Merck KGaA, Darmstadt, Germany
Methanol	≥ 98.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

MOPS	For laboratory use only	Gerbu Biotechnik GmbH, Heidelberg, Germany
N,N-Dimethylformamide (DMF)	99.8%, anhydrous	Sigma-Aldrich, St. Louis, MO, USA
Nitro blue tetrazolium chloride (NBT)	Analytic grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
PageRuler™ Plus Prestained Protein Ladder	10-250 kDa	Thermo Fisher Scientific Inc., Waltham, MA, USA
PageRuler™ Plus Prestained Protein Marker	10-250 kDa	Thermo Fisher Scientific Inc., Waltham, MA, USA
PEG 4000	ROTIPURAN® Ph. Eur.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Peptone ex soya	papainic digested, for microbiology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Phenol	Roti®-Aqua-Phenol, for RNA extraction	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Potassium acetate		VWR International, Radnor, PA, USA
Potassium dihydrogen phosphate	For analysis	Merck KGaA, Darmstadt, Germany
Potassium sulfate	Powder, extra pure	Merck KGaA, Darmstadt, Germany
Roti®-Phenol:Chloroform: Isoamyl alcohol (25:24:1)	For nucleic acid extraction	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Serva Triple Color Protein Standard 3	5-245 kDa	SERVA Electrophoresis GmbH, Heidelberg, Germany
Silver nitrate	≥ 99.9%, p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium acetate trihydrate	≥ 99.5%, p. a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland
Sodium carbonate	≥ 99.5%, p. a., ACS, anhydrous	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride	≥ 99%, p. a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dihydrogen phosphate	≥ 98%, p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Pellets, research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Sodium hydroxide	≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium iodide	≥ 99%, puriss., Ph. Eur.	Sigma-Aldrich, St. Louis, MO, USA
Sodium molybdate	≥ 99%, puriss. p. a.	Sigma-Aldrich, St. Louis, MO, USA
Sodium thiosulfate pentahydrate	For analysis	Merck KGaA, Darmstadt, Germany
T4 DNA ligase buffer	10x	Thermo Fisher Scientific Inc., Waltham, MA, USA

*Taq p*olymerase buffer with $MgCl_2$	10x	MP Biomedicals GmbH, Eschwege, Germany
Tetramethylethylenediamine (TEMED)	~99%	Sigma-Aldrich, St. Louis, MO, USA
Tricine	Pufferan®, ≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trifluoro acetic acid (TFA)	≥ 99%	Sigma-Aldrich, St. Louis, MO, USA
Tris(hydroxymethyl)-aminomethane (Tris)	Ultrapur, analytical grade	Gerbu Biotechnik GmbH, Heidelberg, Germany
Tris-HCl	For molecular biology	Gerbu Biotechnik GmbH, Heidelberg, Germany
Tryptone/ peptone	From casein, granulated	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween 20	For bacteriology	Gerbu Biotechnik GmbH, Heidelberg, Germany
Tween 80		Gerbu Biotechnik GmbH, Heidelberg, Germany
Urea	Ultrapure reagent	Gerbu Biotechnik GmbH, Heidelberg, Germany
Yeast extract	Micro granulated, for bacteriology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Zeocin	Solution at 100 mg/mL in HEPES buffer	InvivoGen, San Diego, CA, USA
Zinc chloride	p. a., ACS, ISO	Merck KGaA, Darmstadt, Germany
α-Lactose monohydrate	≥ 99%, total lactose basis	Sigma-Aldrich, St. Louis, MO, USA

2.1.7 Disposables

The utilized consumables and their type and manufacturer can be found in Table 7.

Table 7: Disposables
Disposables used in the study.

Material	Type	Manufacturer
Blotting paper	Grade BF3, 330 g/m^2, 200 x 200 mm	Ahlstrom-Munksjö, Stockholm, Sweden
Canister	20 L, with tap	Hornbach Baumarkt AG, Bronheim, Germany
Cannula	Sterican®, 0.6 x 30 mm	B. Braun Biotech International, Melsungen, Germany
Champagne bottles	750 mL, 770 g	O-I Manufacturing France, Villeurbanne, France
Cotton	100% biological cotton, ebelin	dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany
Crown caps	FER E4, 29 mm, seal 802	SOLOCAP-MAB S.A., Contrexéville, France
Cryo tubes	Nunc® CryoTubes®	Sigma-Aldrich, St. Louis, MO, USA
Cuvette	10 x 4 x 45 polystyrole	Sarstedt AG & Co., Nürnbrecht, Germany
Dialysis tube	Membra-Cel®, MWCO 3500,	SERVA Electrophoresis GmbH,

	Ø 16 mm	Heidelberg, Germany
Electroporation cuvette	Gene Pulser®, 2 mm electrode spacing	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Filter for HPLC	Phenex™-NY, 15 mm, 0.2 µm Phenex™-RC, 4 mm, 0.2 µm	Phenomenex Inc., Aschaffenburg, Germany Phenomenex Inc., Aschaffenburg, Germany
Folded filters	Grade 3 hw, Ø 185 mm, 65 g/m^2	MUNKTELL & FILTRAK GmbH, Bärenstein, Germany
Gauze	100 % polyester, elastic, sterile	Altapharma Naturprodukte, Hamburg, Germany
Glass beads	Ø 0.5 mm	Scientific Industries, Bohemia, NY, USA
Immun-Blot™ PVDF membrane	For protein blotting, 10 x 15 cm, Ø 0,2 µm	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Inoculation loops	10 µL, 20 µL	VWR International, Radnor, PA, USA
Parafilm®	4'' x 125'	Bemis Company, Inc., Oshkosh, WI, USA
PCR tube	0.2 mL, 8-Strip	STARLAB GmbH, Hamburg, Germany
Petri dish	92 x 16 mm	Sarstedt AG & Co., Nürnbrecht, Germany
Pipette tips	TipOne (1000, 200, 100, 20, 10 µL), 5 mL	STARLAB GmbH, Hamburg, Germany
Reaction tubes	1,5 mL, 2 mL, 15 mL, 50 mL	Sarstedt AG & Co., Nürnbrecht, Germany
Sea sand		Merck KGaA, Darmstadt, Germany
Sparkling wine	Nymphenburg Crystal Cabinet dry	Sektkellerei Nymphenburg GmbH, München-Haar, Germany
Sterile filter	Filtropur S 0.2 µm; CytoOne® Bottle Top Filtration Unit, 0.2 µm, 500 ml	Sarstedt AG & Co., Nürnbrecht, Germany; STARLAB GmbH, Hamburg, Germany
Sugar	Feinster Zucker	Südzucker AG, Mannheim, Germany
Syringe	Injekt 20 mL/ 2 mL; HSW NORM-JECT 50 mL	B. Braun Melsungen AG, Melsungen, Germany; Henke Sass Wolf, Tuttlingen, Germany
Toothpicks	NatureStar	Franz Mensch GmbH, Buchloe, Germany
Verex HPLC cap	Ø 11 mm	Phenomenex Inc., Aschaffenburg, Germany
Verex HPLC vial	2 mL	Phenomenex Inc., Aschaffenburg, Germany
Whatman™ 3MM Chr chromatography paper	46 x 57 cm	Thermo Fisher Scientific Inc., Waltham, MA, USA
White grape juice	100% NFC	Eckes-Granini Deutschland GmbH, Nieder-Olm, Germany

2.1.8 Enological products

Enological products were kindly supplied by our industrial project partners. The products used in this work and their principal compositions are listed in Table 8. The product specification may slightly vary from batch to batch.

Table 8: Enological products used in this work
Enological materials and excipients used in the study and their principal compositions are listed.

Enological product type	Specification
Base wine	Silvaner 2018 Silvaner 2019
Bentonite	Ca-bentonite (powder – raw material for granulate production) Na/Ca-bentonite (granulate) Na-bentonite (powder)
Riddling aid	Silicate-clarifying suspension
Yeast nutrients	DAP (granulated) DAP + 0.13% thiamine hydrochloride (granulated) Nutrient A (inactivated yeasts) Nutrient B (inactivated yeasts, yeast cell walls, DAP, thiamin hydrochloride) Nutrient C (inactivated yeasts, yeast cell walls, DAP, thiamin hydrochloride)

2.1.9 Devices

Table 9 shows all devices used during the study.

Table 9: Devices
All devices utilized in the study are listed.

Device	Type	Manufacturer
Agarose gel chamber		Peqlab Biotechnologie GmbH, Erlangen, Germany
Autoclave	Systec VX-150	Systec GmbH, Linden, Germany
Bead beating grinder	FastPrep-24™	MP Biomedicals GmbH, Eschwege, Germany
Camera (in UV chamber)		Intas Science Imaging Instruments GmbH, Göttingen, Germany
Centrifuge	Rotina 380 R; Mikro 200 R; Sigma 6-16K; Sigma 1-14	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany; Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
CO2 measuring device	LAB.CO	ACM GmbH, Vienna, Austria
Computer	Vivobook S	ASUSTeK COMPUTER INC., Taipei, Taiwan
Dotblot apparatus		Stratagene, La Jolla, CA, USA
Drying oven		Memmert GmbH + Co. KG, Schwabach, Germany

Electroporator	Gene Pulser® II Apparatus	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Fermenter	BIOSTAT® A system	Sartorius Stedim Systems GmbH,Guxhagen, Germany
Freeze dryer	FreeZone 2.5	Labconco Corporation, Kansas City, MO, USA
Freezer	Comfort NoFrost GNP 3013-2	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
FTIR spectrometer	System WineScan™ SO2	FOSS GmbH, Hamburg, Germany
Gel electrophoresis system	Mini PROTEAN® Tetra Cell	Bio-Rad Laboratories, Inc., Hercules, CA, USA
HPLC autosampler	Ultimate 3000	Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA
HPLC column	Aeris Peptide XB-C18 3.6 µm 250 x 2.1 mm Aeris Widepore XB-C18 3.6 µm 250 x 2.1 mm	Phenomenex Inc., Aschaffenburg, Germany Phenomenex Inc., Aschaffenburg, Germany
HPLC column compartment	TCC-100	Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA
HPLC detector	Ultimate 3000	Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA
HPLC pre-column	UHPLC C18 peptide, 2.1 mm	Phenomenex Inc., Aschaffenburg, Germany
HPLC pump	Ultimate 3000	Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA
Incubator	TC 135 S; Heraeus B5042E; Unitherm DHP-9162	Tintometer GmbH, Lovibond Water Testing, Dortmund, Deutschland; Heraeus Instruments GmbH, Hanau, Deutschland; UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg, Deutschland
Magnetic stirrer	ARE	VELP Scientifica, Usmate, Italien
Multipette®	Multipette® stream E3/E3x, 1000 µL	Eppendorf GmbH, Wesseling-Berzdorf, Germany
OwlTM semi-dry Elektroblotting System	Hep-1	Thermo Fisher Scientific Inc., Waltham, MA, USA
pH meter	761 Calimatic	Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany
Photometer	NovaSpec Plus	Biochrom Ltd., Cambridge, United Kingdom
Pipettes	Pipetman (5000, 1000, 200, 100, 20, 10 µL)	Gilson, Inc., Middleton, WI, USA
Power supply	Power Pack P25	Biometra GmbH, Göttingen, Germany
Precision scale	SI-234	Denver Instruments, Bohemia, NY, USA

Pulse control	Pulse Controller Plus Model No. 165-2110	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Refrigerator	Profiline	Robert Bosch Hausgeräte GmbH, Munich, Germany
Scale	Scaltec; Kern 572	Denver Instrument, Bohemia, NY, USA; Kern & Sohn GmbH, Balingen-Frommern, Germany
Scanner	Bio 5000, MRS-9600TFU2B	Microtek International, Hsinchu, Taiwan
Shaker	Unimax 2010; Unitwist 300	Heidolph Instruments GmbH & Co.KG. Schwabach, Deutschland; UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg, Deutschland
Spectrophotometer	NanoDrop ND-1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
Sterile bench	HeraSafe	Heraeus Instruments GmbH, Hanau, Germany
Thermal cycler	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
UV transilluminator	UVT-28 M	Herolab GmbH Laborgeräte, Wiesloch, Germany
UV-VIS spectrometer	Biochrom Libra S22	Biochrom Ltd., Cambridge, United Kingdom
Vacuum pump	PC 3003 VARIO	VACUUBRAND GMBH + CO KG, Wertheim, Germany
Vortexer	Vortex Genie 2; Press-to-Mix 34524	Scientific Industries Inc., Bohemia, NY, USA; Snijders Labs, Tilburg, Netherlands

2.1.10 Software and databases

In this section, all software and databases that were necessary for data generation and analysis are outlined in Table 10.

Table 10: Software and databases
The software and databases that were used for data analysis in this study are mentioned.

Software/ Database	Application	Reference/ Source
BIOSTAT® A software	Operation of the fermenter	Sartorius Stedim Systems GmbH,Guxhagen, Germany
Chromeleon™ 6.80	Control and analysis of HPLC experiments	Thermo Fisher Scientific Inc., Waltham, MA, USA
Clustal Omega	Multiple sequence alignments	Madeira et al. (2019)
Intas GDS	Agarose gel documentation	Intas Science Imaging Instruments GmbH, Göttingen, Germany
Matlab	Statistical analyses	The MathWorks, Inc., Natick, MA, USA
Microsoft 365	Analysis of data and thesis writing	Microsoft Corporation, Redmond, WA, USA

Mikrotek ScanWizard	Scanning of polyacrylamide gels	Mikrotek Laborsysteme GmbH, Overath, Germany
Nano Drop 1000	Measurement of DNA concentrations	Thermo Fisher Scientific Inc., Waltham, MA, USA
NCBI database	Obtaining the *PAU5* gene sequence	National Center for Biotechnology Information, U.S. National Library of Medicine, Rockville Pike, MD, USA
Protein GRAVY	Calculation of the Pau5p GRAVY value	Stothard (2000)
SignaP 5.0	Signal peptide prediction	Almagro Armenteros et al. (2019)
SnapGene software	Design of cloning constructs and primers	Insightful Science, San Diego, CA, USA

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Media

All media were prepared with deionized water and autoclaved at 120 °C for 20 min. Sugars and protein components were autoclaved separately and mixed afterwards to avoid the Maillard reaction. For solid medium, additionally 1.5% agar agar were added before autoclaving.

Table 11: Media
Media used for cultivation of microorganisms are listed.

Buffered Complex Glycerol Medium (BMGY)	
Difco™ YNB	1.34%
Biotin	$4 \cdot 10^{-5}$%
Potassium phosphate buffer pH 6.0	100 mM
Yeast extract	1.00%
Tryptone/ peptone	2.00%
Glycerol	1.00%

Buffered Complex Methanol Medium (BMMY)	
Difco™ YNB	1.34%
Biotin	$4 \cdot 10^{-5}$%
Potassium phosphate buffer pH 6.0	100 mM
Yeast extract	1.00%
Tryptone/ peptone	2.00%
Methanol	0.50%

FM22	
Ammonium sulfate	0.50 %
Potassium dihydrogen phosphate	4.29 %
Calcium sulfate dihydrate	0.10 %
Potassium sulfate	1.43 %
Magnesium sulfate heptahydrate	1.17 %
Glycerol	4.00 %
PTM4	0.20 %

***Pichia* trace minerals (PTM4)**	
Copper sulfate pentahydrate	0.50 %
Sodium iodide	0.008 %
Manganese(II) sulfate monohydrate	0.30 %
Sodium molybdate	0.02 %
Boric acid	0.002 %
Calcium sulfate dihydrate	0.05 %
Cobalt(II) chloride hexahydrate	0.092 %
Zink chloride	0.70 %
Iron(II) sulfate heptahydrate	2.20 %
D-(+)-Biotin	0.02 %

Glycerol stock medium	
L-Glutamic acid monosodium salt monohydrate	1.00%
Lactose monohydrate	1.60%
Agar agar	0.10%
Ascorbic acid	0.01%
Glycerol	12.0%

LB Medium	
Tryptone/ peptone	1.00%
Yeast extract	0.50%
Sodium chloride	0.50%
Adjust the pH to 7.5	

Malt extract (ME) medium	
Malt extract	2.00%
Peptone ex soya	0.20%
Adjust the pH to 5.6	

Spicher medium	
Tryptone/ peptone	1.00%
Meat extract	0.20%
Yeast extract	0.20%
Gluconic acid sodium salt	0.20%
Sodium acetate trihydrate	0.50%
Diammonium hydrogen citrate	0.50%
Potassium dihydrogen phosphate	0.25%
L-cysteine hydrochloride monohydrate	0.05%
Tween 80	0.10%
Magnesium sulfate heptahydrate	0.02%
Manganese sulfate monohydrate	0.01%
Ferrous sulfate heptahydrate	0.005%
D-(+)-Glucose monohydrate	0.70%
Maltose	0.70%
Fructose	0.70%
Adjust the pH to 5.4	

YPD medium	
Yeast extract	1.00%
Tryptone/ Peptone	2.00%
D-(+)-Glucose monohydrate	2.00%

YPDS medium	
Yeast extract	1.00%

Tryptone/ Peptone	2.00%
D-(+)-Glucose monohydrate	2.00%
D-Sorbitol	1 M

If selective media were required, the sterile antibiotics listed in Table 12 were added as 1000x stock solutions to the autoclaved and sufficiently cooled media.

Table 12: Antibiotics
Antibiotics used for selective media and their concentrations are outlined.

Antibiotic	Application	Final concentration
Ampicillin (Amp)	pRS62K plasmids in *E. coli*	100 µg/mL
Geneticin (G418)	pRS62K plasmids in *S. cerevisiae*	200 µg/mL
Zeocin (Zeo)	pPICZαA plasmids in *E. coli* pPICZαA integral vectors in *P. pastoris*	30 µg/mL 300 µg/mL

2.2.1.2 Cultivation of Yeasts

If not stated otherwise, yeasts were cultivated in YPD medium at 30 °C. Liquid cultures were shaken overnight at 200 rpm and plates were incubated for 2-3 days.

For the yeast screening and determination of the influence of fermentation conditions on Pau5p release by *S. cerevisiae*, 50 mL white grape juice inoculated with a single colony from an agar plate. These precultures were incubated in 250 mL Erlenmeyer flasks overnight at 180 rpm and 30 °C. On the next day, main cultures of a total volume of 100 mL were prepared in triplicates by inoculating white grape juice to a starting OD_{600} of 0.45-0.55 with preculture. The yeasts were then incubated in 100 mL laboratory bottles with a not tightly closed cap at 20 °C in a darkened incubator for 4 days. Where applicable, some of these standard conditions were adjusted as mentioned in the results part.

For recombinant protein expression, 25 mL BMGY or YPD medium in 250 mL baffled flasks were inoculated with a single colony of a *P. pastoris* or *S. cerevisiae* clone, respectively, and incubated overnight. The cell density of these precultures was determined before cell harvest at 2500 x g. Then the *P. pastoris* or *S. cerevisiae* pellets were resuspended in 100-200 mL of BMMY or YPD medium, respectively, with a cell density of OD_{600} ~ 1. The cultures were incubated in 1 L baffled flasks covered with a sterile gauze. A 50 mL sample was taken after 24 h and 48 h, centrifuged at 10 000 x g and the pellet was stored at -80 °C until further use (cell lysis, see 2.2.3.1) whereas the supernatant was dialyzed (see 2.2.3.2), freeze-dried (see 2.2.3.3) and used for phenolic extraction (see 2.2.3.4). The BMMY cultures were additionally fed with 0.5% methanol after 24 h.

2.2.1.3 Cultivation of E. coli

Bacteria were incubated in LB medium at 37 °C overnight and liquid cultures were shaken at 180 rpm, if not stated otherwise. Storage of plates was conducted at 4 °C.

2.2.1.4 Determination of the cell dry weight

After fermentation, cultures were homogenized by agitation with a magnetic stirrer at 800 rpm for 30 s. Immediately, 50 mL culture were harvested in pre-weighed 50 mL reaction tubes and centrifuged at 10 000 x g for 5 min at 4 °C. The supernatant was used for dialysis and the cell pellet was dried in a drying oven at 95 °C overnight. After 1 h cooling to RT in a desiccator, the tubes were weighed again, and the cell dry weight was calculated by subtracting the empty weight.

2.2.1.5 Cryo-conservation

For bacterial cryo-conservation, 10 mL of an overnight culture were centrifuged at 4000 x g for 5 min. The cell pellet was resuspended in 800 µL fresh medium and the suspension was mixed with 800 µL sterile glycerol (80%), vortexed, and frozen at -80 °C. Yeast cryo-conservation was performed by spreading the cells on ME agar plates and incubation at RT for 2-4 d before they were suspended in 5 mL glycerol stock medium, vortexed, and stored overnight in 15 mL tubes at 4 °C. Finally, the yeasts were vortexed again and aliquots of 1.8 mL were filled into sterile cryo-tubes and stored at -80 °C.

2.2.2 Molecular biological methods

2.2.2.1 DNA Extraction

The DNA concentrations were determined using a NanoDrop spectrophotometer.

2.2.2.1.1 gDNA isolation from yeast

Yeast gDNA was obtained from 3 mL overnight culture, which was centrifuged at 13 000 x g for 5 min at RT in a 2 ml reaction tube. After washing the cell pellet with dH_2O, it was resuspended in 300 µL extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.5) and each 0.1 mL sea sand and glass beads (0.5 mm) were supplemented. The bead beating was performed at 5 m/s for 45 s. Subsequently, 150 µL of 3 M sodium acetate (pH 5.2) were added and the mixture was vortexed. After a cooling step at -20 °C for 10 min, the suspension was centrifuged again as before, and the supernatant was transferred into a new 1.5 mL reaction tube. For precipitation, the same volume of ice-cold isopropanol was added, inverted and frozen overnight at -20 °C. The DNA pellet was gained by centrifugation at 13 000 x g for 5 min at RT and washed with 500 µL ice-cold 70% ethanol. Finally, the pellet was dried and solved completely in 30 µL dH_2O by incubation at 50 °C for 10 min.

2.2.2.1.2 Plasmid preparation from yeast

Either 5 mL of an overnight culture or several yeast colonies, that were scraped directly from an agar plate and suspended in 1 mL sterile dH_2O, were centrifuged at 3000 x g. The supernatant was discarded, and the pellet was washed with 1 mL sterile dH_2O before resuspension in 1 mL resuspension buffer (500 mM EDTA, 1 M Tris-HCl pH 8.0, 100 µg/mL RNase). The cells were mixed with 400 µL lysis buffer (1 M NaOH, 10% (w/v) SDS) and supplemented with approximately 0.5 mL glass beads (Ø 0.5 mm). After cell disruption by vortexing for 8 min at maximum speed at 4 °C, the suspension was centrifuged for 30 s at 15 000 x g and 650 µL of the supernatant were transferred to a new 1.5 mL reaction tube. Next, 325 µL of cold neutralization buffer (3 M KAc, pH 5.5 with acetic acid) were added and mixed gently without vortexing. Precipitation on ice for 10 min was followed by centrifugation at 20 000 x g for 15 min at 4 °C. Then, 700 µL of the supernatant were transferred into a new reaction tube, mixed with the same amount of isopropanol, and incubated at RT for 10 min. The DNA pellet was generated by centrifugation at 20 000 x g for 15 min at RT and washed with 500 µL ice-cold 70% ethanol twice. The pellet was then dried and finally resuspended in 30 µL dH_2O by incubation at RT for 30 min.

2.2.2.1.3 Plasmid Miniprep from bacteria

Bacterial plasmid DNA was observed with the QIAprep Spin MiniPrep Kit according to the manufacturer's instructions. All additional wash steps were performed, and plasmids were eluted with dH_2O.

2.2.2.2 Polymerase chain reaction (PCR)

Generally, PCR products were applied to an agarose gel (see 2.2.2.3) to verify the success of the PCR. If the PCR product was used for downstream experiments, either the band was cut out of the gel and purified via the GeneJet Gel Extraction Kit or the PCR mix was directly applied to the QIAquick PCR Purification Kit.

2.2.2.2.1 Phusion® PCR

The Phusion® High-Fidelity DNA Polymerase was used for cloning and sequencing purposes. Therefore, 50-250 ng genomic DNA or 1 pg-10 ng plasmid DNA were used to prepare 20 µL PCR mixes (see Table 13).

Table 13: Phusion® PCR master mix
The recipe for 20 µL PCR reactions with the Phusion® High-Fidelity DNA Polymerase is shown.

Ingredient	Volume [µL]
5x Phusion® HF Buffer	4
dNTP mix (10 mM each)	0.4
Forward primer (10 pmol/µL)	1
Reverse primer (10 pmol/µL)	1
DMSO	0.6 (optional)
Template DNA	variable
Phusion polymerase	0.2
dH_2O	Fill up to 20 µL
Total volume	**20**

PCR results were optimized by the addition of DMSO in some cases of extremely long primers. For primers with a long overhang, a two-step PCR was performed, whereas for primers without overhang the standard cycler program shown in Table 14 was applied.

Table 14: Phusion® PCR thermal cycler programs
The two-step PCR program was used for primers with a long overhang.

Standard program				Two-step program			
Step	**T [°C]**	**Time**		**Step**	**T [°C]**	**Time**	
Initial denaturation	98	30 s		Initial denaturation	98	30 s	
Denaturation	98	10 s	30x	Denaturation	98	10 s	10x
Annealing	T_m	30 s		Annealing	T_m	30 s	
Elongation	72	15 s		Elongation	72	15 s	
Final Elongation	72	10 min		Denaturation	98	10 s	20x
				Elongation	72	15 s	
				Final Elongation	72	10	

2.2.2.2.2 Colony PCR of *E. coli*

To check whether *E. coli* clones contain a possibly correct insert in the vector, a colony PCR was performed. Therefore, the master mix shown in Table 15 was prepared and filled in PCR tubes. Then, single colonies from the transformation plates were scratched with a pipet tip and first ditched on a new selective medium master plate before putting the tip in the PCR master mix and pipetting up and down several times to shear some cells. The thermal cycler program applied is shown in Table 15.

Table 15: Colony PCR master mix and thermal cycler program
This table shows on the left side the composition of the colony PCR master mix is shown. Instead of isolated DNA, single *E. coli* colonies were scratched directly from the agar plate and sheared by pipetting up and down several times. On the right side, the thermal cycler program for this type of PCR is shown.

PCR master mix (1x)		Thermal cycler program			
PCR master mix (1x)	**Volume [µL]**	**Step**	**T [°C]**	**Time**	
Buffer with $MgCl_2$ (10x)	1.5	Initial denaturation	95	5 min	
dNTP mix (10 mM each)	0.3	Denaturation	95	30 s	
Forward primer (10 pmol/µL)	1	Annealing	T_m	30 s	30x
Reverse primer (10 pmol/µL)	1	Elongation	72	30 s	
Taq polymerase	0.2	Final elongation	72	7 min	
dH_2O	11				
Total volume	**15**				

If the subsequent agarose gel electrophoresis (see 2.2.2.3) revealed a band of correct size, plasmid DNA of this clone was isolated (see 2.2.2.1.3) and sequenced (see 2.2.2.9).

2.2.2.3 Agarose gel electrophoresis

For the separation of DNA molecules by size, 1% (or for small fragments 2%) agarose gels were poured with 1x TAE buffer (2 M Tris, 57.1% (v/v) acetic acid, 50 mM EDTA, pH 8.2). Samples were loaded using a 6x loading dye and 2 µL of either GeneRuler 100 bp Plus DNA Ladder or GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) served as size indicator. With a power supply, 120 V and maximum 500 mA were applied for 40-60 min before gels were stained for 15 min in a dimidium bromide bath. After destaining for 15 min in dH_2O, gels were photographed under UV illumination. If the DNA was to be purified from the gel, bands were cut out with as few UV light exposure as possible and then applied to the GeneJET Gel Extraction Kit.

2.2.2.4 Restriction digest

For classical cloning approaches, the vector and insert DNA were digested with restriction enzymes and afterwards ligated (see 2.2.2.5) for plasmid generation. In case of the insert, the restriction sites have been implemented by PCR primers with corresponding overhangs, whereas the vector contains restriction sites in its multiple cloning site. In reactions of a total volume of 20 µL, 1 µg of DNA, 2 µL FastDigest buffer, and 1 µL of each specific restriction enzyme were added. Incubation at 37 °C for 25 min and subsequent heat inactivation at 80 °C for 5 min were followed by an agarose gel electrophoresis (see 2.2.2.3) from which the DNA band was purified.

2.2.2.5 Ligation

The vector and insert were ligated after restriction digest by mixing them in a 1:3 molar ratio and adding 0.5 µL T4 DNA ligase and 1 µL T4 DNA ligase buffer, filled up to a total volume of 10 µL with dH_2O. After incubation at 25 °C for 10 min and heat inactivation of the enzyme at

65 °C for 10 min, the generated plasmids were briefly cooled on ice and then directly used for chemical transformation in *E. coli*.

2.2.2.6 Vector linearization

Linearization of the pPICZαA vector for transformation into *P. pastoris* was done using the DraI restriction enzyme. Therefore, 5-10 µg plasmid DNA were mixed with 5 µL enzyme and 5 µL FastDigest buffer and filled up with dH_2O to 50 µL. After an incubation at RT for 15 min, the enzyme was heat inactivated at 65 °C for 5 min. Whether the digestion was completely successful was controlled with an agarose gel electrophoresis. If so, a phenol/chloroform extraction followed by adding 1 volume phenol:chloroform:isoamyl alcohol and vortexing for 20 s. After centrifugation at 16 000 x g for 5 min at RT, the upper aqueous phase was carefully transferred to a new reaction tube and precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes ethanol. Incubation for at least 1 h at -80 °C or overnight at -20 °C was followed by centrifugation at 16 000 x g for 5 min at 4 °C and removal of the supernatant. The pellet was washed with 150 µL of 70% ethanol and subsequently dried and solved in 10 µL dH_2O.

2.2.2.7 AQUA cloning

Instead of the standard restriction digest-based method, AQUA cloning was performed to insert additional tags or cleavage sites. In this approach, the insert as well as the vector had to be amplified via PCR, where the primers generated overhangs, so that homologous regions appeared between vector and insert. The plasmid was assembled and ligated directly *in vivo* inside of the bacterial cell without the need for further enzymes. For that, a 10 µL DNA mix containing 12 ng of vector DNA per 1 kb of vector length was prepared. The necessary amount of insert to obtain a 3:1 molar ratio of insert:vector was calculated with Equation 1:

Equation 1: Calculation of the necessary amount of insert DNA to obtain a 3:1 molar ratio of insert:vector for AQUA cloning

$$m_{insert} = \frac{MW_{insert}}{MW_{vector}} \times m_{vector} \times 3$$

This DNA mix was incubated at RT for 1 h and then directly applied on chemical competent *E. coli* cells for transformation.

2.2.2.8 Transformation methods

2.2.2.8.1 Chemical transformation of *E. coli*

For the generation of chemically competent cells, 500 µL of an *E. coli* TOP10 (TMW 2.580) overnight culture were inoculated into 50 mL LB medium and incubated until an OD_{600} of 0.375. Twice 20 mL of the culture were cooled down on ice in reaction tubes for 10 min and subsequently centrifuged at 1600 x g for 7 min at 4 °C. The supernatants were discarded,

and the pellets resuspended in 4 mL sterile $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM MOPS, pH 7.0) each. This step was repeated after centrifugation at 1100 x g for 5 min at 4 °C. The suspensions were cooled on ice for 30 min before a final centrifugation step at 1100 x g for 5 min at 4 °C. The supernatants were discarded, and the cells were resuspended in 800 µL $CaCl_2$ solution each. Aliquots of 50 µL were shock frozen in liquid nitrogen and stored at -80 °C.

For transformation, cells were thawed on ice for 10 min before addition of 1-25 ng of plasmid DNA or 10 µL of an AQUA cloning mix. This mixture was gently stirred with the pipet tip and then incubated on ice for 30 min. The heat-shock was carried out at 42 °C for 30 s and followed by a cooling step on ice for 5 min. Then, 450 µL LB Medium were added, and cells were regenerated at 37 °C on a shaker for 1 h before they were finally plated on appropriate selective LB medium.

2.2.2.8.2 Electroporation of *E. coli*

Electrocompetent bacteria were generated by inoculating 400 mL pre-warmed LB medium with 4 mL of an *E. coli* TOP10 (TMW 2.580) overnight culture and growing the cells until an OD_{600} of 0.6-0.7 was reached. The culture was then transferred to 8 sterile 50 mL reaction tubes and cooled by complete coverage with ice for 30 min. After centrifugation at 4000 x g for 15 min at 4 °C, the supernatant was discarded, each pellet was suspended in 25 mL ice-cold sterile dH_2O and each two cell suspensions were pooled in one tube. This washing step was repeated twice until only one tube remained. A final wash step with 4 mL sterile ice-cold 10% (w/v) glycerol was performed before the cells were resuspended in 4 mL sterile ice-cold 10% (w/v) glycerol and aliquots of 50 µL were frozen at -80 °C.

For transformation, 1 µL DNA from the yeast plasmid preparation were pipetted directly on top of a frozen electrocompetent *E. coli* aliquot. Cells were then thawed on ice, transferred into a pre-cooled 2 mm electroporation cuvette, and electroporated at 2.5 kV voltage, 200 Ω resistance, and 25 µF capacitance. Immediately after the pulse, cells were taken up in 1 mL LB medium, transferred into 1.5 mL reaction tubes, and regenerated for 30-60 min at 37 °C with shaking. The suspension was centrifuged at 3000 x g for 1 min at RT and a major part of the supernatant was discarded. Cells were resuspended in the remaining 100-200 µL of medium and plated on selective LB medium.

2.2.2.8.3 Electroporation of *P. pastoris*

Transformation of *P. pastoris* was conducted according to the Invitrogen pPICZα A, B, and C user manual (Thermo Fisher Scientific Inc., Waltham, MA, USA). Therefore, 100-500 µL of a *P. pastoris* X33 wildtype (TMW 3.1077) overnight culture was added to 500 mL YPD medium and incubated at 30 °C and 180 rpm overnight until an OD_{600} of 1.3-1.5 was reached. Cells were harvested by centrifugation at 1500 x g for 5 min at 4 °C and the supernatant was

discarded. The cell pellet was resuspended in 500 mL ice-cold sterile dH_2O. This washing step was repeated with 250 mL water, 20 mL ice-cold sterile 1 M sorbitol, and finally with 1 mL ice-cold sterile 1 M sorbitol. The electrocompetent yeasts were stored on ice and used freshly on the same day for transformation.

After plasmid linearization (see 2.2.2.6), 10 µL of DNA were mixed with 80 µl of the cells and transferred to an ice-cold sterile 2 mm electroporation cuvette. The filled cuvette was cooled on ice for 5 min before pulsing at 1.5 kV voltage, 400 Ω resistance, and 25 µF capacitance. Then, 1 mL of 1 M sorbitol solution was added quickly, and the suspension was transferred into a 15 mL reaction tube for regeneration at 30 °C for 1-2 h without shaking. Cells were then plated on selective YPDS medium and incubated at 30 °C for 2-5 days.

2.2.2.8.4 Transformation of *S. cerevisiae*

Frozen competent yeast cells (FCC) were prepared in accordance with Gietz and Schiestl (2007). An overnight culture of *S. cerevisiae* (TMW 3.1008) still in the exponential growth phase (OD_{600} ≤ 3) was harvested by centrifugation at 3000 x g for 2 min at RT and the supernatant was discarded. The pellet was washed with 0.5 culture volumes of sterile ddH_2O before resuspension in 0.01 culture volumes of sterile ddH_2O. After another centrifugation step, the supernatant was removed completely by pipetting and cells were resuspended in FCC solution (10% (v/v) DMSO, 5% (w/v) glycerol). The amount of FCC solution was calculated as follows: 0.01 culture volumes x OD_{600} at the time of harvesting. Aliquots of 50 µL were slowly frozen at -80 °C in a Styrofoam box.

Prior to transformation, the DNA mix was prepared in a volume of 54 µL, containing typically 500 ng of vector DNA and insert DNA in a molar ratio of 1:3. Therewith, the transformation mix was made according to Table 16.

Table 16: *S. cerevisiae* transformation mix
The recipe for the transformation mix for *S. cerevisiae* transformation using the prepared DNA mix is shown.

Transformation mix	Volume [µL]
PEG 4000 (50% (w/v))	260
Lithium acetate (1.0 M)	36
Single stranded carrier DNA (herring sperms DNA, 2 mg/mL)	10
DNA mix in sterile dH_2O	54
Total volume	360

The FCC were thawed for 15-20 s in the hand and then centrifuged at 3000 x g for 2 min at RT. The supernatant was removed, and the pellet suspended thoroughly in 360 µL transformation mix by pipetting. A heat-shock was performed at 42 °C for 40 min in the water bath. The transformed cells were centrifuged at 3000 x g for 30 s at RT. The pellet was resuspended in 5 mL YPD medium and regenerated at 30 °C for 2 h while shaking. Finally,

the yeasts were centrifuged at 3000 x g for 2 min at RT, most of the supernatant was discarded, the pellet was resuspended in the remaining 100-200 µL medium, and the cells were plated on selective YPD medium.

2.2.2.9 Sequencing

To determine the correctness of the sequence of DNA molecules after PCR or transformation, either plasmids were isolated, or the sequence of interest was amplified via Phusion® PCR and purified via the QIAquick PCR Purification Kit. Then, 20 µL DNA solved in water at concentrations of 30-100 ng/µL (plasmids) or 10-50 ng/µL (PCR fragments) were sent to GATC Biotech/Eurofins (Luxemburg) together with 20 µL custom primers (10 pmol/µL) for sequencing according to Sanger (SupremeRun Tube).

2.2.3 Protein chemical methods

2.2.3.1 Cell lysis

For the analysis of intracellular recombinant protein, cells were lysed according to the Invitrogen pPICZα A, B, and C user manual (Thermo Fisher Scientific Inc., Waltham, MA, USA). Therefore, the pellets were thawed on ice and resuspended in 100 µL breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM PMSF, 1 mM EDTA, 5 % EDTA) per 1 mL culture from which the pellet resulted. Then, 500 µL of this suspension were supplemented with ~ 500 µL glass beads (Ø 0.5 mm) and treated four times with the bead beating grinder at 5 m/s. Between the repetitions, cells were cooled for 5 min on ice. Finally, the samples were centrifuged at 17 000 x g at 4 °C for 30 min.

2.2.3.2 Dialysis

To remove low-molecular-weight substances, a 3.5 kDa MWCO RC membrane dialysis tube (Serva Electrophoresis GmbH, Heidelberg, Germany) was used. Samples of 50 mL culture supernatant or sparkling wine were dialyzed against the 20-fold volume of deionized water for three days at 4 °C. The water was changed twice a day.

2.2.3.3 Freeze-drying

Dialyzed samples were filled into 250 mL round bottom flasks and frozen under rotation in an ethanol bath at -60 °C. Then, they were either stored at -20 °C or directly attached to the freeze-dryer for 24 h. Lyophilizates were filled in 2 mL tubes and stored at -20 °C until further usage.

2.2.3.4 Phenol extraction

For determination of the Pau5p content via HPLC, the lyophilizate (2.2.3.3) of 50 mL dialyzed (2.2.3.1) culture supernatant was used for phenol extraction according to Vogt et al. (2016). In this protocol, samples were always handled on ice and all centrifugation steps were performed at 4 °C. The lyophilizate was solved in 1 mL buffer P1 (see Table 17) and

vortexed for 1 min before centrifugation at 5000 x g. The supernatant was split in two equal parts of 480 µL each in 2 mL tubes. The same amount of phenol was added, and the tubes were shaken for 30 min at 4 °C. After centrifugation at 6000 x g for 15 min, the upper aqueous phase was discarded, and phenolic phase was washed once. Therefore, 1 mL buffer P1 was added, briefly vortexed, and centrifuged as before. Again, the aqueous phase was discarded. The phenolic phase was mixed with 1.5 mL buffer P2 (100 mM ammonium acetate in methanol) for methanol precipitation overnight at -20 °C. On the next day, a centrifugation step at 20 000 x g for 40 min was followed by complete removal of the supernatant. Then, 1.5 mL buffer P3 (100 mM ammonium acetate, 10 mM DTT in methanol) were added to the pellet and incubated for 60-90 min. The supernatant was completely pipetted off after centrifugation at 13 000 x g for 30 min. This wash step was repeated once with buffer P4 (10 mM DTT in 80% acetone). Finally, the pellet was air-dried at 4 °C for 30-60 min. If used for HPLC subsequently, pellets were stepwise solved in 100 µL of 100 mM sodium hydroxide, 100 µL of 8 M urea, 24 µL acetone, and 576 µL of 0.1% TFA. After the first two additions, the tubes were vortexed for 10 min, respectively.

Table 17: Phenol extraction buffer
The recipe for buffer P1 for phenol extraction according to Vogt et al. (2016) is shown.

Buffer P1	
Tris	100 mM
EDTA	10 mM
ß-Mercaptoethanol	0.4% (v/v)
Potassium chloride	100 mM
Adjust pH to 8.9	
Solve in 90 % volume of HPLC-grade water	
DTT (add 10 % buffer volume of 100 % (w/v) solution freshly before usage)	10% (w/v)

2.2.3.5 Tricine-SDS-PAGE

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in reference to Schägger (2006). First, a 12% or 16% resolving gel (Table 18) was poured between the two glass plates and coated with isopropanol. After polymerization, the isopropanol was removed, and a 5% stacking gel (Table 18) was poured on top with a comb placed inside.

Table 18: SDS-PAGE gels
Composition of gels for SDS-PAGE are indicated.

	Resolving gel (12% Acrylamide, 1.0 M Tris, pH 8.45)	**Resolving gel** (16% Acrylamide, 1.0 M Tris, pH 8.45)	**Stacking gel** (4% Acrylamide, 0.74 M Tris, pH 8.45)
Acrylamide/Bis (30%, 37.5:1)	4 mL	5.3 mL	0.68 mL
Gel buffer (3 M Tris, pH 8.45)	3.33 mL	3.33 mL	1.29 mL
SDS (25% (w/v))	40 µL	40 µL	16 µL
Deionized water	2.56 mL	1.26 mL	3.21 mL
TEMED	7 µL	7 µL	7 µL
APS (10% (w/v))	50 µL	50 µL	33 µL

After assembly of the apparatus (Mini PROTEAN® Tetra Cell, Bio-Rad Laboratories, Inc., Hercules, CA, USA), cathode buffer was filled between the gels, and anode buffer was filled in the outer chamber. Samples were mixed 1:5 with application buffer and loaded on gel. The buffers are shown in the following Table 19.

Table 19: SDS-PAGE buffers
Buffers and solutions used for SDS-PAGE are listed.

Anode buffer	
Tris	200 mM
pH adjusted to 8.9	
Cathode buffer	
Tris	100 mM
Tricine	100 mM
SDS	0.1% (w/v)
pH adjusted to 8.25	
Application buffer	
Tris-HCl (pH 8.45)	250 mM
SDS	7.5% (w/v)
Glycerol	25% (v/v)
Bromophenol blue	0.25 mg/mL
ß-Mercaptoethanol	12.5% (v/v)

If used for silver staining only, 4 µL of PageRuler^{TM} Plus Prestained Protein Ladder was applied. If used for Western blotting, 4 µL of Serva Triple Color Protein Standard 3 were loaded. The gel was run at 80 V for 10 min, followed by 60-90 min at 100 V. Afterwards, the stacking gel was removed, and the resolving gel was put in fixing solution for silver staining (2.2.3.6) or used for Western blotting (2.2.4.1).

2.2.3.6 Silver staining

For visualization of proteins in polyacrylamide gels, silver staining was applied according to Blum et al. (1987). The solvents used and their correct application are presented in Table 20.

Table 20: Silver staining
Solutions used for silver staining of polyacrylamide gels application according to Blum et al. (1987) and their duration of application are listed in the correct order.

Solution	Composition		Duration
Fixative	Ethanol Acetic acid	40% (v/v) 10% (v/v)	> 3 h or over night
Wash solution	Ethanol	30% (v/v)	2 x 20 min
Deionized water			20 min
Thiosulfate solution	Sodium thiosulfate	0.02% (w/v)	40-60 s
Deionized water			3 x 20 s
Silver nitrate solution	Silver nitrate	0.2% (w/v)	20 min
Deionized water			3 x 20 s
Developer	Sodium carbonate Sodium thiosulfate Formaldehyde (37%)	3% (w/v) 0.0005% (w/v) 0.1% (v/v)	< 5 min
Deionized water			3 x 20 s
Stop solution	Glycine	0.5% (w/v)	5 min
Deionized water			5 min

Afterwards, gels were scanned, and the images were processed with Microsoft PowerPoint.

2.2.4 Immunochemical methods

2.2.4.1 Western blot

Immunoblotting of proteins was performed after SDS-PAGE analysis (see 2.2.3.5). Two gels were prepared and loaded in the same manner, one for silver staining (see 2.2.3.6) as a control and the other one for semi-dry Western blotting. Therefore, the Immun-Blot™ PVDF membrane was saturated for 30 s in methanol and washed for 2 min in deionized water. The gel as well as six blotting paper sheets and the membrane were then incubated in transfer buffer (50 mM Tris, 190 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) for 20 min. The soaked sheets were stacked in the following order on the cathode: three blotting paper sheets, the gel, the membrane, three blotting paper sheets. They were topped tightly with the anode. Current was applied for one hour with 50 mA and maximum 24 V. After application of the positive control directly on the membrane, the membrane was immunostained (see 2.2.4.3) with appropriate antibodies.

2.2.4.2 Dotblot

The dotblot was used to test many samples at a time without the need to run an SDS-PAGE beforehand. Therefore, the Immun-Blot™ PVDF membrane was saturated for 30 s in methanol and washed for 2 min in deionized water. Then, a water-saturated blotting paper sheet and the membrane were spanned into the dotblot apparatus. Samples were applied into the holes directly on the membrane and vacuum was applied, so that the samples were pulled through the membrane. Solid particles and proteins were thus attached to the membrane in dots and stained as described in the following chapter 2.2.4.3.

2.2.4.3 Immunostaining

The blotted membrane was incubated in blocking solution overnight on a rocking shaker in the cold room (4 °C). On the following day, the membrane was washed three times in PBS-T buffer for 10 min each. Next, the primary antibody was applied diluted 1:2000 in PBS-T buffer and incubated for 90 min on a rocking shaker. After three more 10 min washing steps with PBS-T buffer, the second antibody was diluted 1:5000 and applied in the same manner as the primary antibody. The membrane was subsequently washed twice with PBS-T buffer, twice with PBS buffer, and once with AP buffer for 5 min each. Finally, the membrane was treated with 15 mL AP buffer mixed with 7.5 µL NBT solution and 30 µL BCIP solution for a few minutes to stain the bound antibodies. The reaction was stopped with deionized water, the membrane was dried, and a photo was taken with a smart phone camera.

Table 21: Immunostaining buffers
All buffers used for Western blotting and dotblotting are described.

Blocking solution	
Tris	20 mM
Sodium chloride	200 mM
BSA	3% (w/v)
pH adjusted to 7.4	

PBS buffer	
Potassium dihydrogen phosphate	4 mM
Disodium hydrogen phosphate monohydrate	16 mM
Sodium chloride	115 mM
pH adjusted to 7.4	

PBS-T buffer	
Potassium dihydrogen phosphate	4 mM
Disodium hydrogen phosphate monohydrate	16 mM
Sodium chloride	115 mM
Tween 20	0.1% (v/v)
pH adjusted to 7.4	

AP buffer	
Tris-HCl	100 mM

Sodium chloride	100 mM
Magnesium chloride	5 mM
pH adjusted to 8.8	
NBT solution	
NBT	75 mg/mL
DMF	70% (v/v)
BCIP solution	
BCIP	60 mg/mL
DMF	100% (v/v)

2.2.5 Analytical methods

2.2.5.1 RP-HPLC analysis

For RP-HPLC analysis, a Dionex Ultimate 3000 System (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a C18 column (Aeris Widepore XB-C18 3.6 µm 250 x 2.1 mm, Phenomenex Inc., Aschaffenburg, Germany) were used. Eluents A (0.1% TFA in HPLC grade water) and Eluent B (0.1% TFA in ACN) were used to establish an ACN gradient during analysis, while Eluent D (50% ACN, 50% HPLC grade water) was used in standby modus and for column storage. All eluents were degassed with N_2 for 10 min and samples were filtered before usage. The gradient used is displayed in Table 22. Pau5p peaks appeared at a retention time of 13.80 min and were integrated with the Chromeleon 6.80 software (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Table 22: RP-HPLC gradient
The ACN gradient for RP-HPLC analysis is described.

Retention time	**Eluent A** (0.1% TFA in HPLC grade water)	**Eluent B** (0.1% TFA in ACN)
0 min	97%	3%
1 min	97%	3%
15 min	50%	50%
19 min	0%	100%
28 min	0%	100%
33 min	97%	3%
40 min	97%	3%

2.2.5.2 FTIR measurement

Fourier-transform infrared spectroscopy (FTIR) was performed to determine enological parameters such as the sugar content (glucose, fructose, total extract), acids, or glycerol in the sparkling wine samples. The analysis was performed by our project partner Prof. Dr. Rauhut at the Hochschule Geisenheim University (HGU) according to Baumgartner et al. (2001) and (Patz et al., 1999).

2.2.5.3 Ammonium and NOPA analysis

The ammonium content in sparkling wine samples was determined with the Ammonia Assay Kit according to the manufacturer's advice with an UV-VIS spectrometer.

As indicator for the yeast available nitrogen content, the amount of α-amino acids in base wine or sparkling wine was determined via a reaction with ortho-phthaldialdehyde (OPA) according to Dukes and Butzke (1998). The resulting NOPA ("nitrogen by OPA") value was calculated in mg/L (Dukes, 2010).

Both analyses were conducted by Prof. Dr. Rauhut and her team (HGU).

2.2.6 Enological methods

2.2.6.1 Rehydration and alcohol adaption of yeasts

Not all yeasts were available as active dry yeast and thus all yeasts were cultivated in the lab prior to sparkling wine production in the first practical experiment (3.5.1). Therefore, 150 mL white grape juice were inoculated with a single colony from an agar plate and incubated in 500 mL laboratory bottles with not tightly closed caps over night at RT with agitation by a magnetic stirrer at 450 rpm. On the next day, the cultures were supplemented first with 50 mL and in the evening with further 100 mL of base wine to adapt the yeasts to higher alcohol levels. After incubation for another night, cells were centrifuged at 4000 x g for 5 min at RT and the medium was discarded. The pellet was resuspended in base wine and the OD_{600} set to approximately 19, of which 10 mL were used per 750 mL bottle of sparkling wine equaling approximately 20 g/hL active dry yeast.

If dry yeast was used (3.5.3 and 3.5.2), 10 g were rehydrated in a mixture of 50 mL base wine, 50 mL tap water, and 3 g sugar and mixed thoroughly in a 500 mL Erlenmeyer flask. After 15 min incubation, the suspension was mixed again and then incubated over night at 20 °C. If not stated otherwise, 2 mL of this mix per liter of sparkling wine were used which is equal to 20 g/hL active dry yeast.

2.2.6.2 Base wine fermentation

Grape musts for base wine fermentation were generated by full grape pressing and sedimented for 16 h (Chardonnay 2020) or 36 h (Riesling 2019), respectively. Then, they were sulfurized with 45 mg/L SO_2 and supplemented with yeast nutrients resulting in 50 g/hL DAP and 65 mg/hL thiamin hydrochloride. Fermentation was performed in batches of 120 L with 20 g/hL active dry yeast for two weeks. Afterwards, the wines were decanted in containers that were filled up to the bung and stored on the yeasts until further use.

2.2.6.3 Bentonite fining of base wines

The bentonite solutions were prepared by mixing 2.5 g of the respective bentonite product with 25 mL of tap water. After 30-40 min, they were mixed again and then left to swell at RT

for 15 h. Of the previously produced base wines, 975 mL were filled into 1 L bottles. The negative controls were supplemented with 25 mL tap water. From each of the residual bottles, 150 mL were put aside. Then, they were supplemented with 25 mL of the respective 10% (w/v) bentonite suspension. The 150 mL base wine were used to rinse the beaker and funnel and to collect the bentonite residues therefrom. The bottles were sealed with a screw cap and shaken. After two days, 450 mL of the wine were centrifuged at 4000 rpm for 10 min at 4 °C. Finally, aliquots of 50 mL were sent from the HGU to our department for Pau5p RP-HPLC analysis (see chapter 2.2.5.1).

2.2.6.4 Sparkling wine filling and fermentation

Base wine was supplemented with 24 g/L sugar and, if not stated otherwise, 50 g/hL of a yeast nutrient resulting in approximately 50 g/hL DAP and 65 mg/hL thiamin hydrochloride and mixed thoroughly in a 20 L canister. Only in the case of the experiment with different ammonium concentrations, 25 g/hL of this nutrient were used, resulting in 32.5 mg/hL thiamin hydrochloride and 25 g/hL DAP. The high ammonium samples in this case were additionally supplemented with 25 g/hL DAP resulting in a total of 50 g/hL DAP. After the crystals were solved completely, yeast was added either directly or after filling. The 750 mL sparkling wine glass bottles were filled manually under non-sterile conditions and closed with a crown cap. Fermentation took place for 6-8 weeks in a horizontal position at 20 °C.

2.2.6.5 Riddling and Disgorgement

After secondary fermentation, sparkling wine bottles were riddled for 2 weeks in a gyro box with increasing angle. For disgorgement, the bottle neck was frozen for 5-10 min in a calcium chloride cooling bath at approximately -18 °C. The salt solution was washed away by dipping the bottle neck into water and the crown cap was removed with a bottle opener. Once the sedimented yeast plug was pushed out of the bottle by over pressure, greater losses of liquid were avoided by holding a thumb on top of the bottle opening. Neither sulfur nor liquor were added.

2.2.6.6 Fermentation graphs

If fermentation curves were generated, a laboratory CO_2 measuring device was used, which allowed for pressure determination without opening of the bottle. The bottles were shaken before each measurement and the CO2 concentration in the neck space was measured spectroscopically. This value was then used to calculate the internal bottle pressure (PB) at the measuring temperature with Equation 2.

Equation 2: Calculation of the internal bottle pressure after spectroscopical measurement of the CO2 concentration in the neck space. PB = internal bottle pressure, CO2 = measured CO2 concentration, T = Temperature while measurement.

$$PB = \left(\frac{CO_2}{10\ x\ 2.71828183^{\frac{-10.608+2557}{T+273.15}}} \right) - 1$$

The internal bottle pressure was transformed into the pressure equivalent at 20 °C with Equation 3 to facilitate comparison of different measurements.

Equation 3: Transformation of the internal bottle pressure into P20. P20 = pressure equivalent at 20 °C, PB = internal bottle pressure, T = Temperature while measurement.

$$\boldsymbol{P20 = PB\ x\ (1.84928571428571 - 0.054492857142857\ x\ T + 0.0006428574286\ x\ T^2)}$$

These values were then processed with Microsoft Excel and fermentation graphs were plotted.

3 Results

3.1 Cloning of *PAU5*

3.1.1 Cloning of *PAU5* in *Saccharomyces cerevisiae*

In order to gain large amounts of Pau5p for the development of an immunochemical detection assay, it was cloned for recombinant protein expression. As Pau5p is a glycoprotein and the glycosylation patterns of different organisms can vary extensively (Goto, 2007), *S. cerevisiae* TMW 3.1008 was chosen as homologous host to which the expression system used by Linck et al. (2014) was applied. It consists of the episomal shuttle vector pRS62K with an ampicillin resistance cassette and a bacterial pBR322 for replication in the intermediate host *E. coli.* Furthermore, the plasmid encodes a geneticin resistance cassette and a 2 micrometer ori and thus is operable in yeast. The gene of interest can be inserted downstream of a truncated version of the constitutive pHXT7 promotor. In this case, the two different *PAU5* constructs shown in Figure 8 were generated: a native one without any tags and one with a N-terminal polyhistidine-tag and a TEV cleavage site, so that the tag can be removed after protein purification.

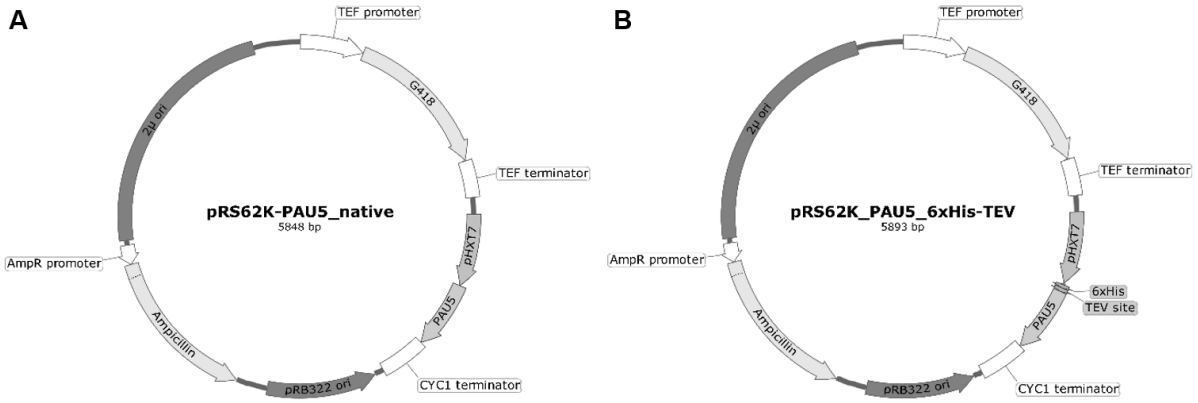

Figure 8: pRS62K *PAU5* constructs with and without 6xHis-tag
Two constructs of the pRS62K vector were cloned for Pau5p expression under the control of the constitutive pHXT7 promoter in *S. cerevisiae*. One of the constructs (**A**) does not encode any tag and thus expressed Pau5p would be in its native state, the other construct (**B**) includes a 6xHis-tag for affinity purification and a TEV protease cleavage site to remove the tag afterwards. Furthermore, both plasmids encode ampicillin and G418 resistance cassettes as well as a bacterial and a yeast origin of replication.

The cloning of these two constructs was performed via recombination-mediated PCR-directed plasmid construction *in vivo* in yeast (Oldenburg et al., 1997). The principle of this strategy is to add overhangs to the insert via the PCR primers that are homologous to the vector sequence. The vector is digested with restriction enzymes and together with the insert directly transformed into the *S. cerevisiae* host strain. The plasmid ligates *in vivo* via homologous recombination events. A DNA extraction from selected yeast transformants and electroporation in *E. coli* allows for plasmid isolation and sequencing. Correctly sequenced plasmids are then transformed into fresh *S. cerevisiae* cells to avoid a genetic background as

for example by incorrect plasmids or genomic integrations. An overview of the workflow is presented in Figure 9.

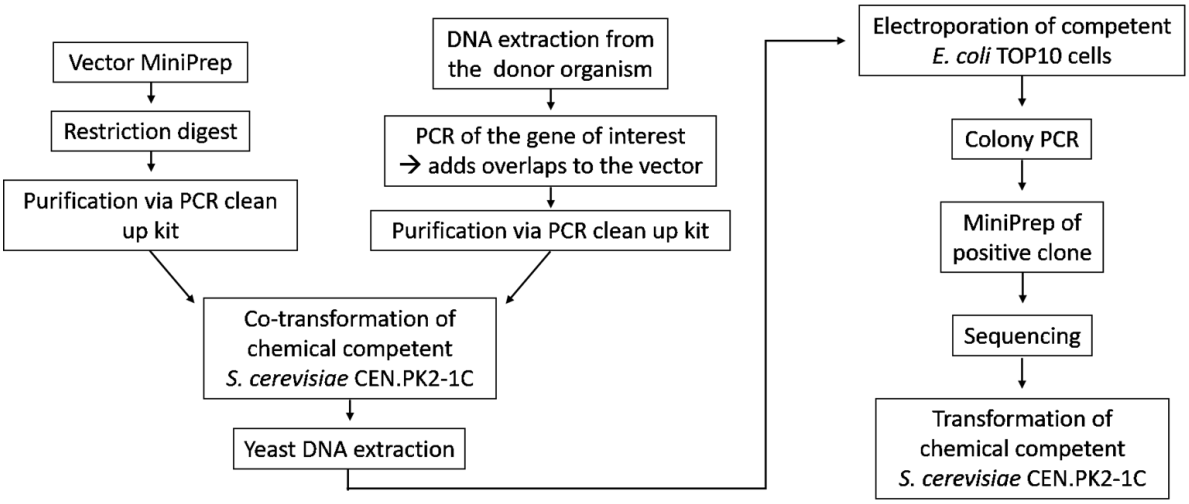

Figure 9: Workflow for *Saccharomyces cerevisiae* pRS62K cloning
The workflow for the pRS62K *PAU5* cloning project via recombination-mediated PCR-directed plasmid construction *in vivo* in yeast according to (Oldenburg et al., 1997) is illustrated.

Firstly, gDNA was received from the donor organism *S. cerevisiae* TMW 3.0933. This was subsequently used as a template for the amplification of the *PAU5* gene with either primer pair P14 and P16 for the *PAU5* HisTEV construct or with primer pair P15 and P16 in case of the native *PAU5* construct (for primer sequences see Table 2). In both cases, the signal peptide was not cut off by the PCR, but the whole gene was amplified. This decision was based on the fact, that the predicted signal peptide was present in the protein sequencing data from Kupfer (2018) for the glycosylated protein isolated from sparkling wine. After an optimization of the polymerase chain reactions shown in Figure 10, 1% DMSO and 10 cycles with an annealing step at T_m = 44 °C followed by 20 cycles of a 2-step PCR without annealing step were chosen for both primer pairs.

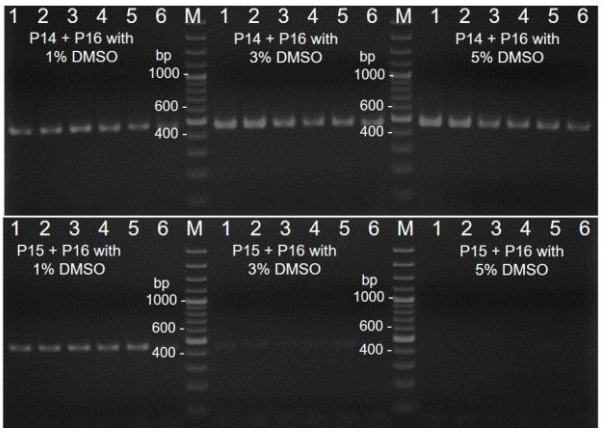

Figure 10: Optimization of the PCR amplification of the insert for the pRS62K cloning
Agarose gel of different PCR reactions for the optimization of *PAU5* amplification from template gDNA with primer pairs P14 and P16 or P15 and P16. 1-4: PCR program with 10 cycles gradient (T_m: 1: 40.0 °C, 2: 42.3 °C, 3: 44.1 °C, 4: 46.1 °C) followed by 20 cycles of a 2-step PCR (without annealing step); 5: 15 cycles with annealing step (T_m: 44.1 °C) followed by 15 cycles 2-step PCR; 6: 5 cycles with annealing step (T_m: 44.1 °C) followed by 25 cycles 2-step PCR; M: Marker (100 bp Plus Gene Ruler DNA Ladder).

The so optimized PCR was repeated, and products were purified with the PCR clean up kit for downstream processing. The vector was gained from *E. coli* TMW 2.2105 via MiniPrep and subsequently linearized with the restriction enzymes EcoRI and SmaI. The digest was controlled with an agarose gel electrophoresis (see

Figure 11) and the correct band was cut out and purified via the GeneJet Gel Extraction Kit.

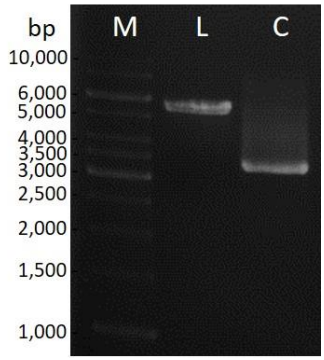

Figure 11: pRS62K plasmid linearization
The linearization of the vector pRS62K with the restriction enzymes EcoRI and SmaI was controlled with an agarose gel electrophoresis. The non-treated plasmid runs much faster through the gel in its coiled-coil structure than the linearized plasmid. M = Marker (1 kb Gene Ruler DNA Ladder), L = linearized plasmid after digest (expected size: 5559 bp), C = circular plasmid.

Afterwards, the inserts were each co-transformed together with the linearized vector into *S. cerevisiae* TMW 3.1008. Positive clones were selected on G418 agar plates and their plasmids were extracted. This DNA was then electroporated into *E. coli*. Positive *E. coli* colonies were selected on ampicillin agar and for both constructs all 9 picked colonies were also positive in the colony PCR (see Figure 12).

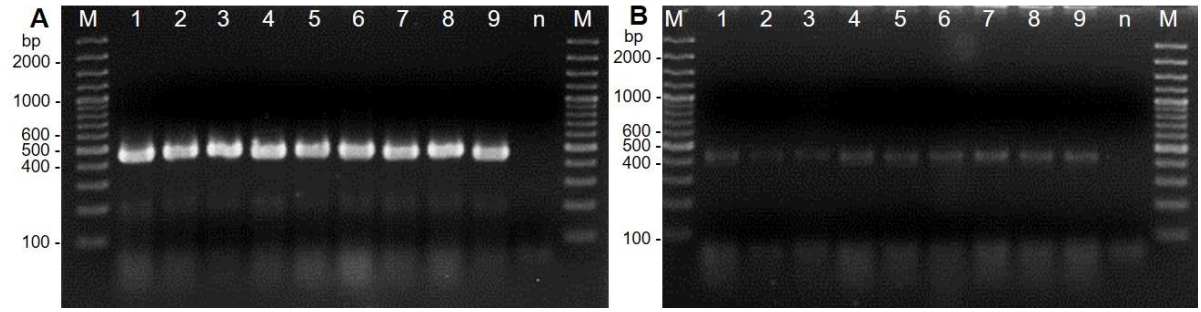

Figure 12: *E. coli* colony PCR of the pRS62K cloning
The agarose gel of an *E. coli* colony PCR after transformation with the pRS62K *PAU5* plasmids constructed via homologous recombination in yeast is shown. (**A**) *PAU5* HisTEV construct, primer pair P14 and P16, expected fragment size: 464 bp. (**B**) *PAU5* native construct, primer pair P15 and P16, expected fragment size: 449 bp. n = negative control (pipet tip dipped on the agar plate), M = Marker (100 bp Plus Gene Ruler DNA Ladder).

Some of the clones were sequenced and the correct strains *E. coli* pRS62K *PAU5* HisTEV clone 2 (TMW 2.2116) and *E. coli* pRS62K *PAU5* native clone 1 (TMW 2.2113) were cryo-conserved. Their plasmids were finally transformed into fresh *S. cerevisiae* TMW 3.1008 cells, and positive clones were picked from selective G418 agar plates and cryo-conserved as TMW 3.1196-3.1203 in the case of *S. cerevisiae* pRS62K *PAU5* native or as TMW 3.1204-3.1210 for *S. cerevisiae* pRS62K *PAU5* HisTEV.

3.1.2 Cloning of *PAU5* in *Pichia pastoris*

Additionally to the *S. cerevisiae* cloning, *PAU5* was cloned into *Pichia pastoris* with the well-established pPICZαA expression system (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). Here, three different constructs were designed: A native one and one with the C-terminal c-Myc epitope and a 6xHis-tag encoded in the vector (both shown in Figure 13A). The third construct was constructed via AQUA cloning (Beyer et al., 2015), so that an N-terminal 6xHis-tag and a TEV cleavage site could be inserted (see Figure 13B). In all cases, the shuttle vector contains the BleoR gene, which confers resistance to Zeocin™ in *E. coli* as well as in *P. pastoris,* and a bacterial origin of replication. The gene of interest is under the control of the *AOX1* promoter and can be induced with methanol. Expressed proteins are supposed to be secreted due to the α-factor signal sequence.

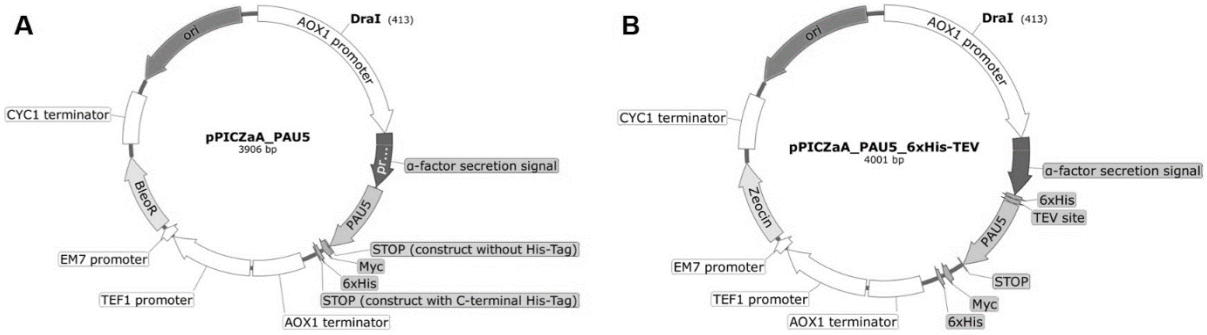

Figure 13: pPICZαA *PAU5* plasmid constructs for *P. pastoris* cloning
The designed pPICZαA constructs are displayed. (**A**) Constructs for the classical ligation-based cloning approach resulting in either a native Pau5p protein or in a C-terminally 6xHis-tagged protein. (**B**) AQUA cloning construct with a N-terminal 6xHis-tag and a TEV protease cleavage site.

For all three constructs, genomic DNA was extracted from the donor organism *S. cerevisiae* TMW 3.0933. The shuttle vector pPICZαA was gained from *E. coli* TMW 2.651 via MiniPrep and *E. coli* TOP10 TMW 2.0580 served as intermediate host whereas *P. pastoris* X33 TMW 3.0177 was the final host. However, two different cloning approaches were used. The overview of the cloning procedure for the native and the C-terminally 6xHis-tagged *PAU5* is shown in Figure 14, an overview for the AQUA cloning approach is shown further below in Figure 18.

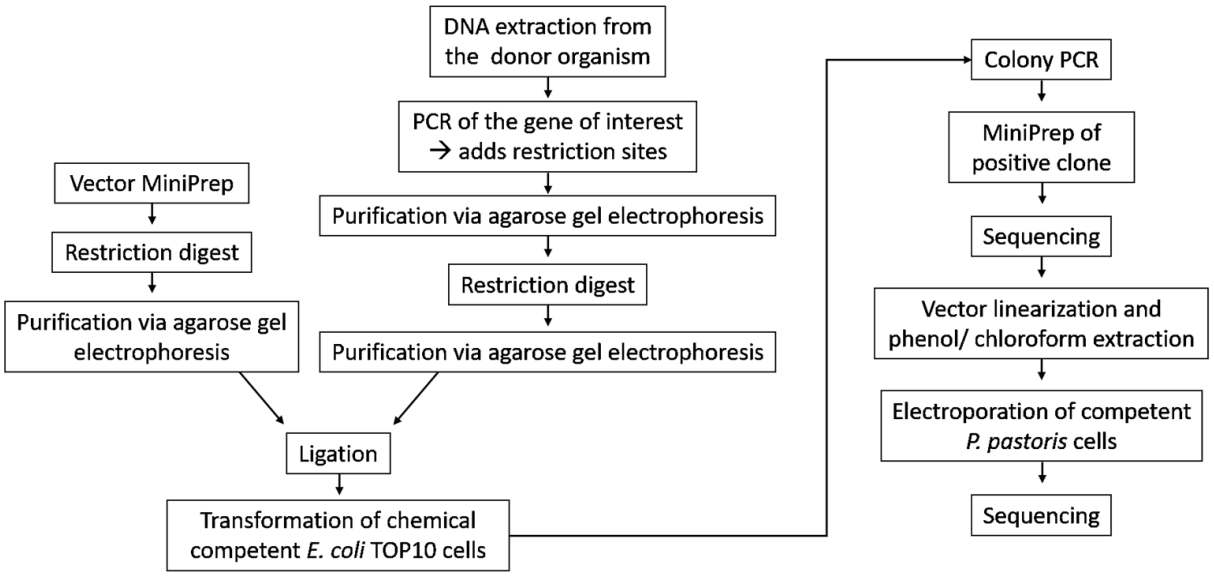

Figure 14: Overview of the ligation-based pPICZαA *PAU5* cloning procedure
The single working steps for the cloning of the native and C-terminally 6xHis-tagged pPICZαA *PAU5* constructs in *P. pastoris* are displayed. This protocol follows the Invitrogen pPICZαA manual (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The insert was amplified with primer pair P1 and P2 to gain a sequence including the native stop codon of the *PAU5* gene (native construct) or with primer pair P1 and P3 resulting in an insert without stop codon but in frame with the vector. Thus, the C-terminal 6xHis-tag and the stop codon from the vector will be translated together with the *PAU5* gene. The PCR primers added EcoRI and XbaI restriction sites via overhangs. Those restriction sites are also present in the multiple cloning site of the vector. Both the vector and the inserts were thus digested, cut out and purified after agarose gel electrophoresis (see Figure 15) and subsequently ligated and transformed into *E. coli*.

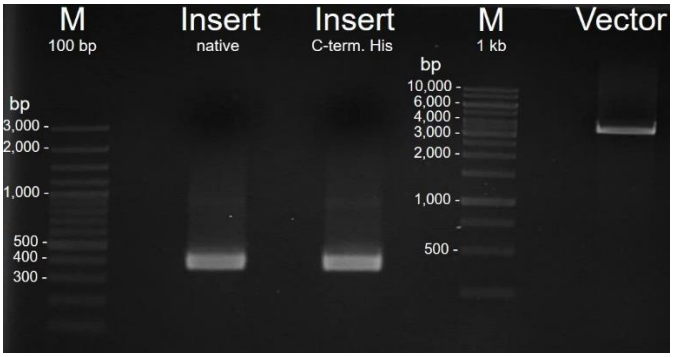

Figure 15: Restriction digest of insert and vector for ligation-based pPICZαA cloning
Agarose gel after EcoRI and XbaI restriction digest of the insert for the native construct (PCR product of *S. cerevisiae* gDNA with primer pair P1 + P2, expected size: 376 bp), the insert for the C-terminally 6xHis-tagged construct (PCR product of *S. cerevisiae* gDNA with primer pair P1 + P3, expected size: 374 bp), and the vector pPICZαA (expected size: 3530 bp). M = Marker (100 bp Plus or 1 kb Gene Ruler DNA Ladder).

Positive *E. coli* clones were selected on Zeocin™ agar and colony PCR revealed each one presumably positive clone (see Figure 16).

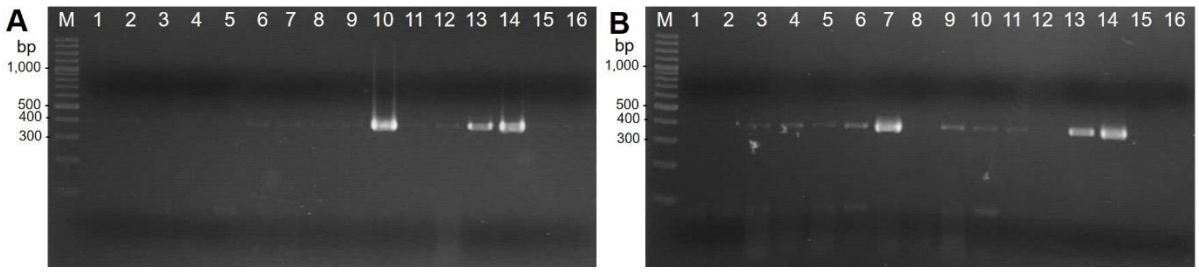

Figure 16: Colony PCR of pPICZαA *PAU5* cloning
Agarose gels of the colony PCR of the *E. coli* pPICZαA *PAU5* transformation. (**A**) 16 colonies of the native construct were tested with primer pair P1 and P2 of which 3 showed a band. (**B**) 3 of the 16 colonies with the C-terminally 6xHis-tagged construct showed a band in the PCR with primer pair P1 and P3. (**A+B**) 1-12: picked clones, 13: positive control (*S. cerevisiae* gDNA), 14: size comparison (*PAU5* HisTEV plasmid, see below), 15: negative control (without DNA), 16: negative control (pipet tip dipped on the agar plate), M: Marker (100 bp Plus Gene Ruler DNA Ladder).

However, subsequent sequencing with primers P17 and P19, which bind in the AOX1 promoter and terminator, respectively, showed massive errors in the *PAU5* sequence in case of the C-terminally 6xHis-tagged construct. Even after several repetitions of the cloning procedure, no correct clone could be gained. In case of the native construct, clone 10 could be confirmed to be correct by sequencing and was cryo-conserved as *E. coli* pPICZαA *PAU5* native TMW 2.2275.

The isolated plasmid of this strain was subsequently transformed into *P. pastoris* and 15 clones were picked for colony PCR (see Figure 17).

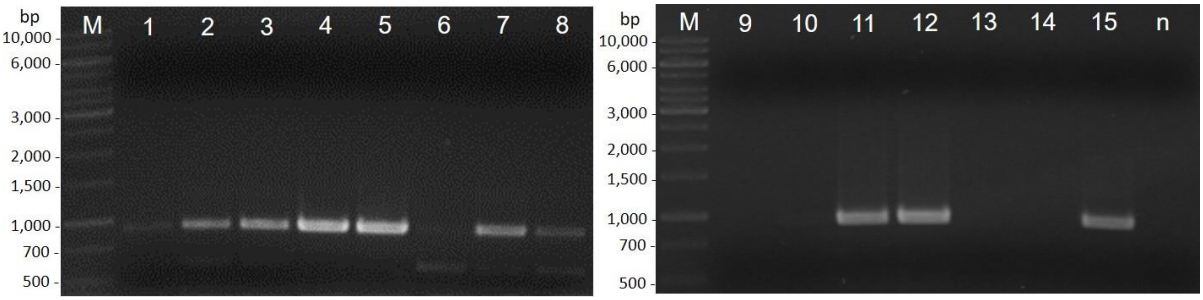

Figure 17: *P. pastoris* colony PCR after pPICZαA *PAU5* native transformation
Agarose gel of the colony PCR of 15 picked *P. pastoris* transformants with primer pair P17 and P19. Expected PCR product size with the correct insert: 902 bp, expected PCR product size of the vector without insert: 589 bp, expected PCR product size of the native AOX1 locus: 2105 bp, n: negative control (*P. pastoris* wildtype strain TMW 3.0177), M: Marker (1 kb Gene Ruler DNA Ladder).

Clones with only the correct band were sequenced with primers P17 and P19 and clone 4, 5, 11, and 12 turned out to correct and were cryo-preserved as *P. pastoris* pPICZαA *PAU5* native TMW 3.1190, 3.1191, 3.1192, and 3.1193, respectively. Because none of these clones showed a second band in the colony PCR with the size of 2105 bp, which would occur if the native *AOX1* locus was still present in the genome, all four of them are Mut^S clones.

The third variant with a N-terminal 6xHis-tag and a TEV cleavage site was constructed following the AQUA cloning workflow in Figure 18.

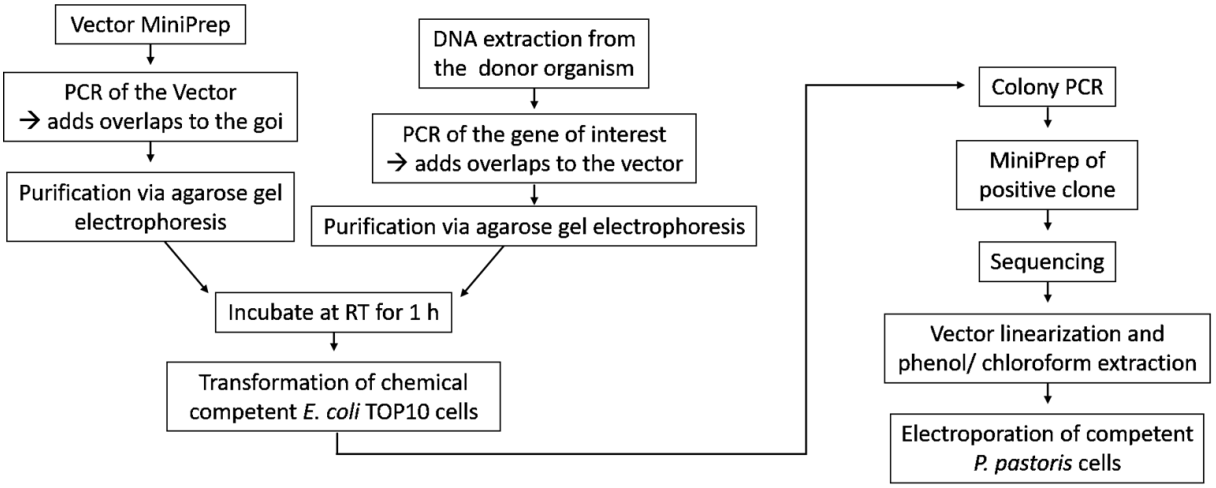

Figure 18: AQUA cloning workflow for pPICZαA *PAU5* HisTEV cloning in *P. pastoris*
A schematic workflow of the "Advanced Quick Assembly" cloning (Beyer et al., 2015) in *E. coli* followed by the standard *P. pastoris* transformation according to the Invitrogen pPICZαA manual (Thermo Fisher Scientific Inc., Waltham, MA, USA) is shown.

For this purpose, the *S. cerevisiae* TMW 3.0933 gDNA was amplified via PCR with primers P4 and P5 whereas the vector was amplified with primers P6 and P7. Each of the primers added overhangs to the resulting sequence, so that the polyhistidine-tag and the TEV cleavage site as well as overhangs to the counterpart of the insert or vector were added, respectively. The so gained and purified PCR products were incubated together and then co-transformed into *E. coli*. The colony PCR (see Figure 19) with both primer pairs P4 and P5 as well as P1 and P2 revealed mostly positive clones.

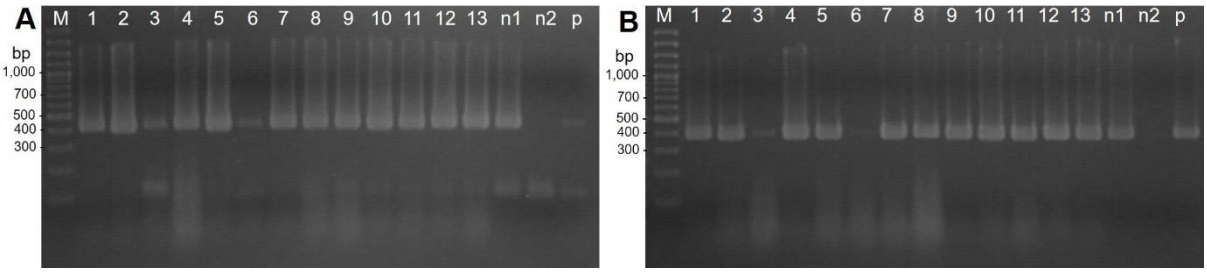

Figure 19: *E. coli* pPICZαA *PAU5* HisTEV colony PCR
Agarose gel of the colony PCR of 13 picked *E. coli* clones after pPICZαA *PAU5* HisTEV AQUA cloning. (**A**) PCR with primer pair P4 and P5, expected size: 422 bp. (**B**) PCR with primer pair P1 and P2, expected size: 370 bp. n1: negative control 1 (pipet tip dipped on agar plate), n2: negative control 2 (water instead of DNA), p: positive control (*S. cerevisiae* TMW 3.0933 gDNA), M: Marker (100 bp Plus Gene Ruler DNA Ladder)

Sequencing with primers P17 and P19 confirmed the correct sequence of clone 12 which was subsequently cryo-conserved as *E. coli* pPICZαA *PAU5* HisTEV TMW 2.2276. The isolated plasmid of this strain was then used for electroporation into *P. pastoris,* which led to three clones, all showing a band in the colony PCR (see Figure 20).

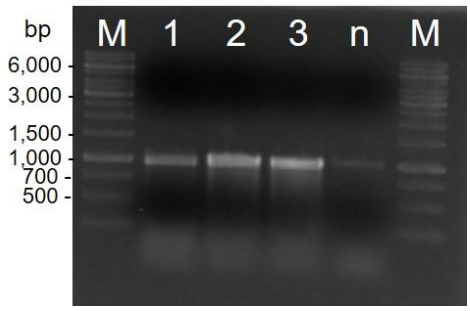

Figure 20: *P. pastoris* colony PCR after pPICZαA *PAU5* HisTEV transformation
Agarose gel of the *P. pastoris* pPICZαA *PAU5* HisTEV colony PCR with primer pair P17 and P19. 1-3: Three colonies, expected PCR product size with the correct insert: 997 bp, expected PCR product size of the native AOX1 locus: 2105 bp, n: negative control (without DNA), M: Marker (1 kb Gene Ruler DNA Ladder).

Clones 2 and 3 proofed to have the correct insertion of the *PAU5* gene into the yeast genome and were cryo-conserved as *P. pastoris PAU5* HisTEV TMW 3.1194 and 3.1195, respectively. Again, those clones did only show one band in the colony PCR and thus the native *AOX1* locus is not present in the genome anymore. This means the clones have a Mut^S genotype.

3.2 Expression of recombinant protein

3.2.1 Homologous expression of *PAU5* in *Saccharomyces cerevisiae*

Positive *S. cerevisiae PAU5* clones were tested for homologous protein overexpression in YPD G418 medium. Detection of His-tagged proteins is possible via Western blot with an anti-His-tag antibody. As a simple screening method, samples of expression cultures of all *S. cerevisiae PAU5* HisTEV clones were applied to a dotblot and stained with the anti-His-tag antibody. A recombinant 6x-His-tagged GFP protein served as positive control. The *S. cerevisiae* wildtype strain TMW 3.1008 served as negative control. The cell culture supernatant samples were all negative, thus containing no or only very few of the desired protein. In contrast, the cell lysates were all positive, including the negative control (see Figure 21).

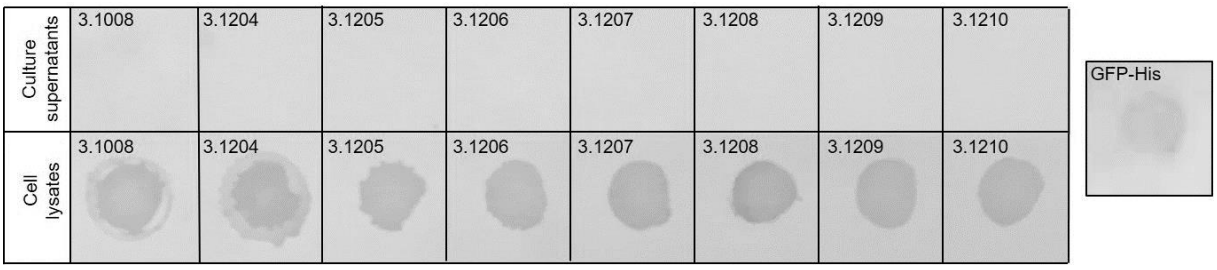

Figure 21: Dotblot of *S. cerevisiae* pRS62K *PAU5* HisTEV samples stained with anti-His-tag antibody
Shown is the dotblot of culture supernatants and cell lysates of *S. cerevisiae* pRS62K *PAU5* HisTEV clones (TMW 3.1204-3.1210) after 48 h incubation in YPD G418 medium stained with the anti-His-tag antibody. Positive control: 6x-His-tagged GFP protein. Negative control: *S. cerevisiae* wildtype (TMW 3.1008).

So, the results of the dotblot were not reliable, and the samples were applied to an SDS-PAGE, but no bands were visible in the following Western blot (data not shown). Exemplarily, samples of one clone (TMW 3.1208) were applied to protein purification via IMAC

chromatography. Subsequent SDS-PAGE showed no additional band of the recombinant strain compared to the wildtype (see Figure 22).

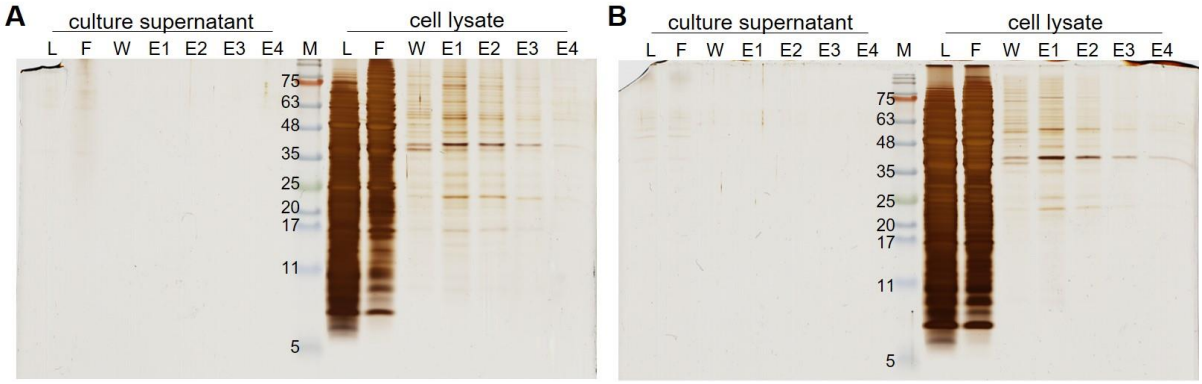

Figure 22: IMAC of *S. cerevisiae* pRS62K *PAU5* HisTEV expression
Silver stained SDS-PAGE of the IMAC protein purification of expression culture samples of (**A**) the *S. cerevisiae* wildtype TMW 3.1008 and (**B**) *S. cerevisiae* pRS62K *PAU5* HisTEV clone TMW 3.1208. L: Load, F: Flow, W: last wash, E1-E4: Elution 1-4, M: Marker (Serva Triple Color Protein Standard 3, SERVA Electrophoresis GmbH, Heidelberg, Germany).

Even after variations of the culture conditions and several attempts, no significant amount of recombinant protein could be detected (data not shown).

Regarding the *S. cerevisiae* pRS62K *PAU5* native strains, the detection of the protein is more complex. As no specific Pau5p antibody was available, the native protein can only be identified, if a distinct SDS-PAGE band is visible, which is absent or at least much less intense in the wildtype. However, this was not the case in several expression experiments (exemplarily shown in Figure 23).

To check if there was a minimal expression of the protein at a level under the detection limit of SDS-PAGE and Western blot or if there was no expression at all, expression cultures of each three recombinant strains per construct were used to take 50 mL samples after 24 h and 48 h. The supernatants of these samples were dialyzed, freeze-dried and phenol extracted. The cell pellets were used for cell lysis. All samples were then applied to SDS-PAGE (see Figure 23A) and in the case of the 6x-His-tagged strains also to Western blot (see Figure 23B).

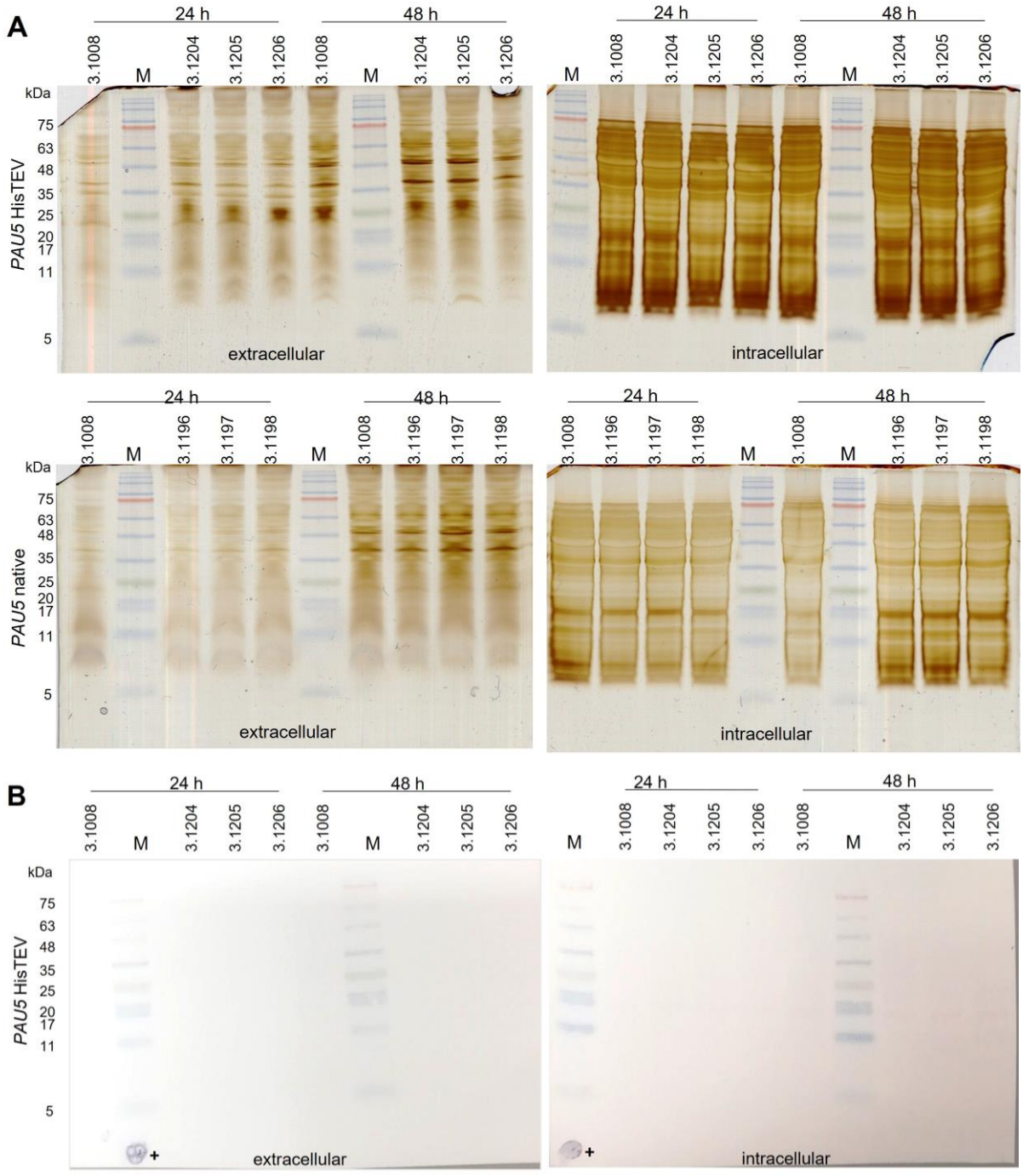

Figure 23: SDS-PAGE and Western blot of recombinant *PAU5* expression in *S. cerevisiae*
S. cerevisiae pRS62K *PAU5* native (TMW 3.1196, 3.1197, 3.1198), *S. cerevisiae* pRS62K *PAU5* HisTEV (TMW 3.1204, 3.1205, 3.1206) and *S. cerevisiae* wildtype (TMW 3.1008) expression studies. 50 mL samples were taken after 24 h and 48 h incubation in YPD G418 medium. The cell pellets were lysed (intracellular), and the supernatants dialyzed, lyophilized, and proteins were extracted with phenol (extracellular). All samples were applied to (**A**) SDS-PAGE and silver staining and samples with 6x-His-tag were additionally used for (**B**) Western blot and immunostaining with an anti-His-tag antibody. M: Marker (Serva Triple Color Protein Standard 3). +: Positive control (6x-His-tagged GFP).

Still, the SDS-PAGE and Western blot did not show any protein expression. Therefore, the technical duplicates of these purified supernatant samples were applied to RP-HPLC analysis, which was more sensitive than SDS-PAGE and Western blot.

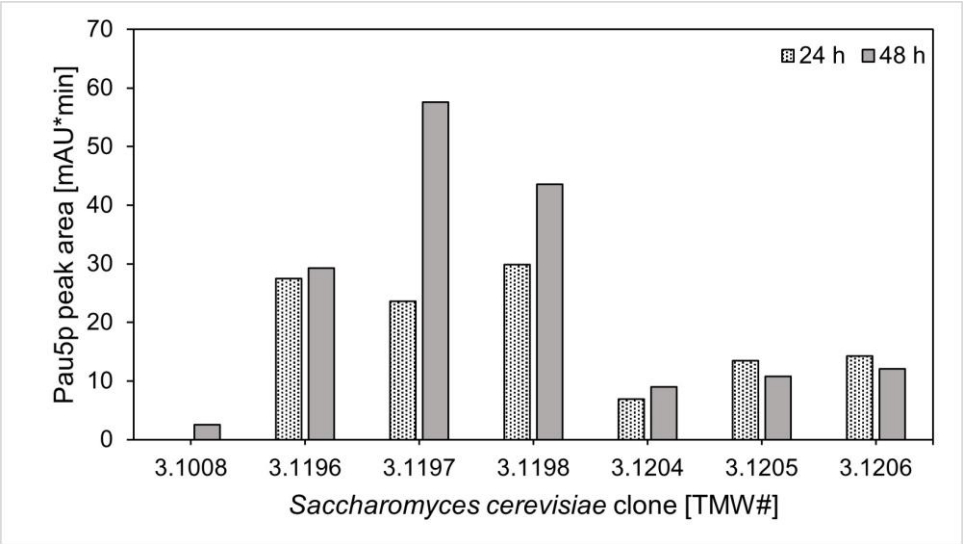

Figure 24: RP-HPLC results of recombinant *S. cerevisiae PAU5* expression
RP-HPLC analysis of expression culture supernatants after dialysis, lyophilization and phenol extraction showed *PAU5* expression in *S. cerevisiae* pRS62K *PAU5* native strain TMW 3.1196, 3.1197, and 3.1198 as well as in *S. cerevisiae* pRS62K *PAU5* HisTEV strains TMW 3.1204, 3.1205, and 3.1206 compared to the *S. cerevisiae* wildtype TMW 3.1008.

As can be seen in Figure 24, all recombinant strains did express and secret low amounts of Pau5p, whereas the wildtype strain expressed (almost) no Pau5p. This means, that the cloning and recombinant protein expression were successful, but no large amounts of protein could be generated in this study.

3.2.2 Heterologous expression of *PAU5* in *Pichia pastoris*

In the case of heterologous expression in *P. pastoris*, the same detection methods as for *S. cerevisiae* expression were available and the same problems occurred. In dotblot analysis the cell lysates were always positive, also from the wildtype (data not shown). Different expression media were tested, including BM, BMM, BMMY, and FM22. A preliminary test with subsequent RP-HPLC analysis showed that the best chance to express protein was in BMMY (data not shown). However, following the Invitrogen pPICZαA expression manual for Mut^+ or Mut^S expression or expression in bioreactors with additional aeration to ensure sufficient oxygen supply and with respiration rate-dependent methanol feeding, no sufficient Pau5p amounts could be expressed to be visible on SDS-PAGE or Western blot (data not shown).

Figure 25 shows an SDS-PAGE and Western blot of cell lysates and dialyzed, lyophilized, and phenol extracted supernatants of expression cultures in BMMY medium (following the Invitrogen pPICZαA Mut^S protocol) after 24 h and 48 h.

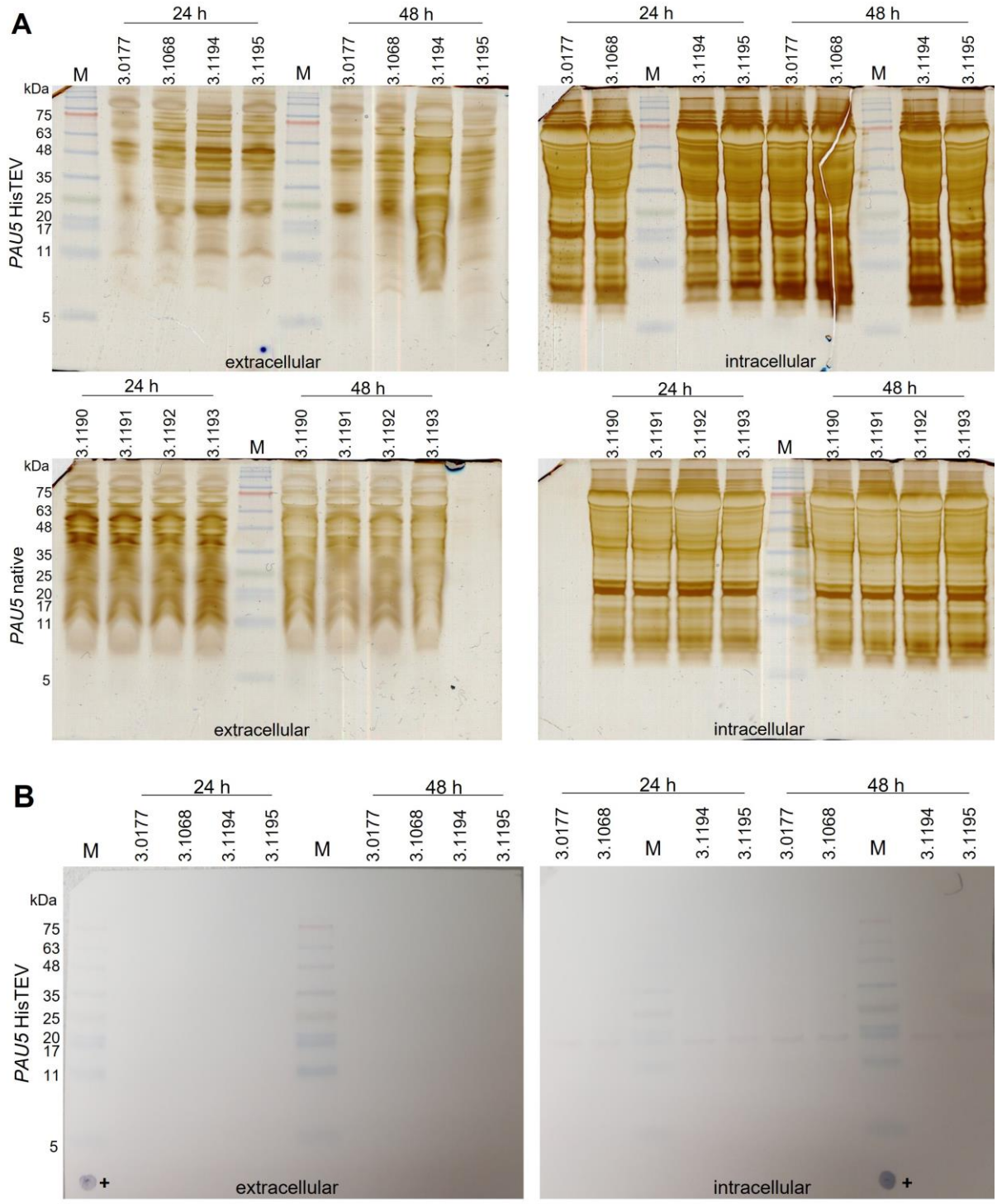

Figure 25: SDS-PAGE and Western blot of recombinant *PAU5* expression in *P. pastoris*
(**A**) SDS-PAGE after silver staining and (**B**) Western blot after immunostaining with an anti-His-tag antibody. Expression culture samples of *P. pastoris* pPICZαA *PAU5* native (TMW 3.1190-3.1193), *P. pastoris* pPICZαA *PAU5* HisTEV (TMW 3.1194, 3.1195), the *P. pastoris* wildtype (TMW 3.0177), and *P. pastoris* pPICZαA (TMW 3.1068) were applied. Therefore, 50 mL samples were taken after 24 h and 48 h incubation in BMMY medium. The cell pellets were lysed (intracellular), and the supernatants dialyzed, lyophilized, and proteins were extracted with phenol (extracellular). M: Marker (Serva Triple Color Protein Standard 3). +: Positive control (6x-His-tagged GFP).

Respectively, one of the technical duplicates of the supernatant phenol extractions was applied to RP-HPLC analysis. The two negative controls *P. pastoris* wildtype (TMW 3.0177) and the *P. pastoris* transformed with the empty pPICZαA vector (TMW 3.1068) did not show

any peaks. All tested recombinant strains did, however, show protein expression, which was significantly increased after 48Figure 26).

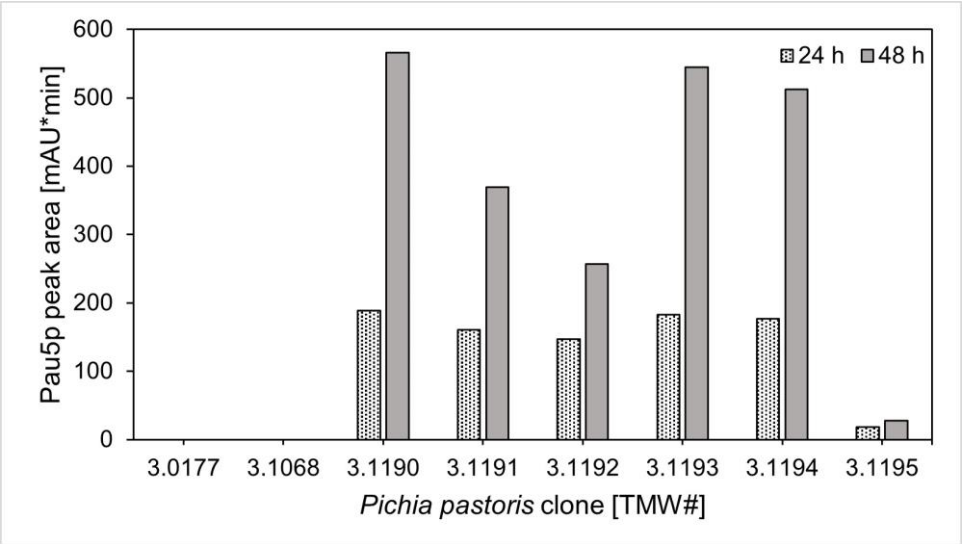

Figure 26: RP-HPLC analysis confirms heterologous expression of *PAU5* in *P. pastoris*
Analysis of dialyzed, freeze-dried, and phenol extracted supernatants of *P. pastoris* pPICZαA *PAU5* native (TMW 3.1190-3.1193) and *P. pastoris* pPICZαA *PAU5* HisTEV (TMW 3.1194, 3.1195) after 24 h and 48 verifies heterologous *PAU5* expression in those strains. The negative controls *P. pastoris* wildtype (TMW 3.0177) and *P. pastoris* pPICZαA (TMW 3.1068) did not show any Pau5p peaks in the RP-HPLC chromatograms.

Taken together, minimal amounts of Pau5p were heterologously expressed in all *P. pastoris* transformants, but despite many attempts to optimize the expression and induction conditions, no large amounts of recombinant protein could be gained.

3.3 Pau5p detection and quantification

3.3.1 Anti-Pau5p antibody development

For the rapid detection and quantification of Pau5p, an immunochemical detection assay should be developed. Therefore, a specific anti-Pau5p antibody was required, and Davids Biotechnologie GmbH (Regensburg, Germany) was commissioned to generate a polyclonal peptide antibody in hens. So, the Pau5p amino acid sequence was analyzed, and the two most promising peptides proposed by Davids Biotechnologie are shown in Table 23.

Table 23: Predicted peptides for anti-Pau5p antibody development
Both peptides predicted to be suitable for hen immunization to gain anti-Pau5p antibodies by Davids Biotechnologie GmbH are mentioned. Antigenicity: algorithms to find sequences, which are predicted to be antigenic and induce a good antibody response. Solubility: algorithms to find sequences, which are predicted to be on the surface of a peptide. Epitope prediction: algorithms to find sequences, which are predicted to be an epitope for inducing B-cells to produce specific antibodies.

Peptide	Sequence	Antigenicity	Solubility	Epitope pred.
HAC-DIR	DIRAHLAEYYSFQAAHPTE	Medium	Medium	Good
HAC-AAG	AAGASAAATTTLSQSDER	Medium	Low	Good

Due to its better solubility, the peptide HAC-DIR was chosen for immunization. Davids Biotechnologie synthetically produced this peptide and conjugated it to the carrier protein

keyhole limpet hemocyanin (KLH). Four immunization boosts with this carrier were injected into a hen at days 1, 14, 28, and 35. At days 45 to 56, 8-10 eggs were collected for IgG/IgY antibody preparation from egg yolk. Those were affinity-purified before shipping of the product with an antibody titer of 32 000 (0.54 mg/mL) and the peptide HAC-DIR (10 mg/mL, 2219.42 g/mol (MS), 95% (HPLC)) to our institute. The antibody was tested for specific Pau5p binding via Western blot. Therefore, small amounts of Pau5p were gained by manual collection of Pau5p RP-HPLC peaks of sparkling wine samples, which were pooled, dialyzed against dH_2O, lyophilized, and solved in dH_2O. This resulted in a clear band in the SDS-PAGE which could not be immunostained on the Western blot (see Figure 27). The peptide HAC-DIR, which served as positive control, was stained intensively.

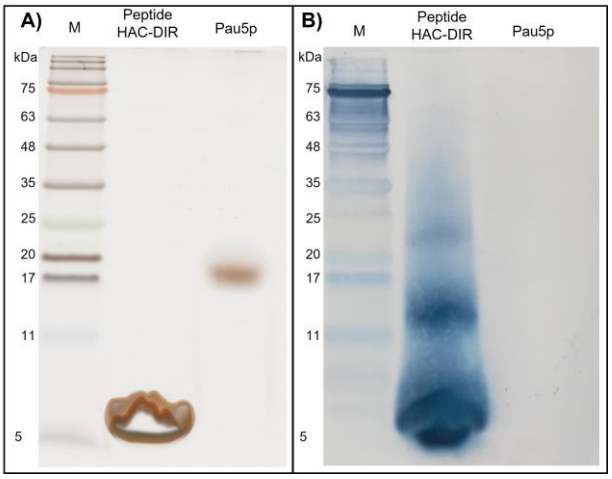

Figure 27: Western blot with the anti-HAC-DIR antibody
Pau5p was gained by collecting RP-HPLC Pau5p peaks from sparkling wine and applied to (**A**) SDS-PAGE with silver staining and (**B**) a Western blot. Anti-HAC-DIR-IgG served as primary antibody and Anti-hen IgY-AP as secondary antibody. Peptide HAC-DIR: Positive control. M: Marker (Serva Triple Color Protein Standard 3).

Several optimization efforts were conducted, including a variation of the NaCl concentration in the PBS and PBS-T buffers, variation of the BSA concentration in the blocking solution, using milk powder as blocking solution, using lyophilized sparkling wine or yeast supernatants as Pau5p sample (data not shown), or increasing the concentration of the primary antibody 10-fold and 100-fold (see Figure 28). Still, Pau5p could not be detected by the HAC-DIR antibody. Conversely, negative controls such as yeast cell lysates containing large amounts of various proteins showed unspecific staining.

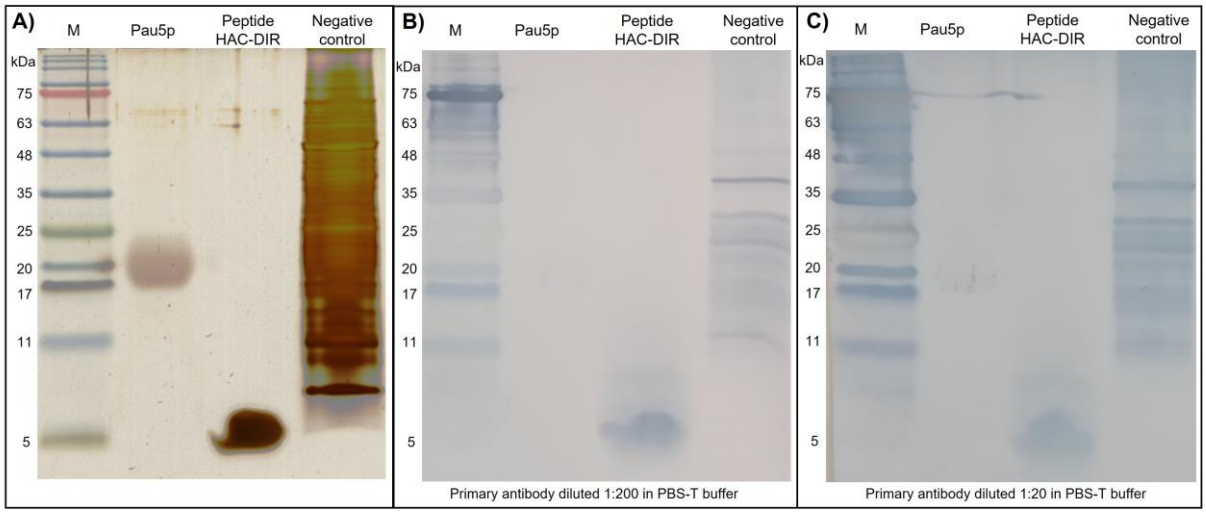

Figure 28: Western blot with higher concentrations of anti-HAC-DIR-IgG
An SDS-PAGE of Pau5p (collected via RP-HPLC), the peptide HAC-DIR as positive control and a yeast cell lysate as negative control was (**A**) silver stained and applied to a Western blot with subsequent immunostaining with Anti-HAC-DIR-IgG diluted (**B**) 1:200 or (**C**) 1:20 instead of 1:2000 in PBS-T buffer. M: Marker (Serva Triple Color Protein Standard 3).

Thus, a second peptide antibody against the peptide HAC-AAG (10 mg/mL, 1707.79 g/mol (MS), 73% (HPLC)) was ordered from Davids Biotechnologie GmbH. In this case, the fourth immunization boost was performed not only with the peptide but also with 65 collected and pooled RP-HPLC Pau5p peaks to improve the recognition of the native protein. The so gained antibody with a titer of 80 000 (0.69 mg/mL) could detect Pau5p, but the reaction was not specific (see Figure 29). Several attempts to increase the specificity of the Western blot did not reduce the unspecific staining of the negative controls (data not shown).

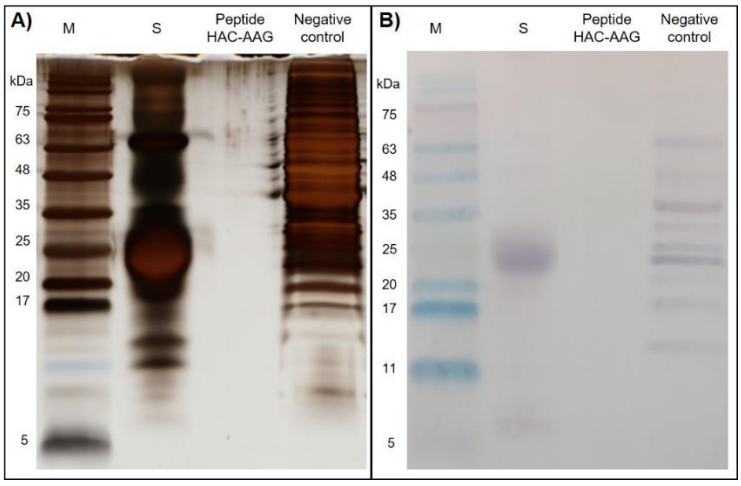

Figure 29: Western blot with the anti-HAC-AAG antibody
SDS-PAGE of purified sparkling wine (S) containing Pau5p was followed by a Western blot with the anti-HAC-AAG antibody. The positive control peptide HAC-AAG was too small and ran out of the bottom of the gel. *P. pastoris* wildtype (TMW 3.0177) cell lysate served as negative control and was unspecifically stained in the Western blot albeit an increased NaCl concentration in the PBS and PBS-T buffer of 150 mM. M: Marker (Serva Triple Color Protein Standard 3).

As Pau5p is a highly glycosylated protein, the epitopes of the peptide antibodies might be concealed by the sugar residues. Therefore, the RP-HPLC purified Pau5p as well as lyophilized sparkling wine were treated with two different enzymatic deglycosylation kits and

subsequently applied to a Western blot. As shown in Figure 30, complete deglycosylation could not be verified by SDS-PAGE, and subsequent Western blot did not lead to positive results.

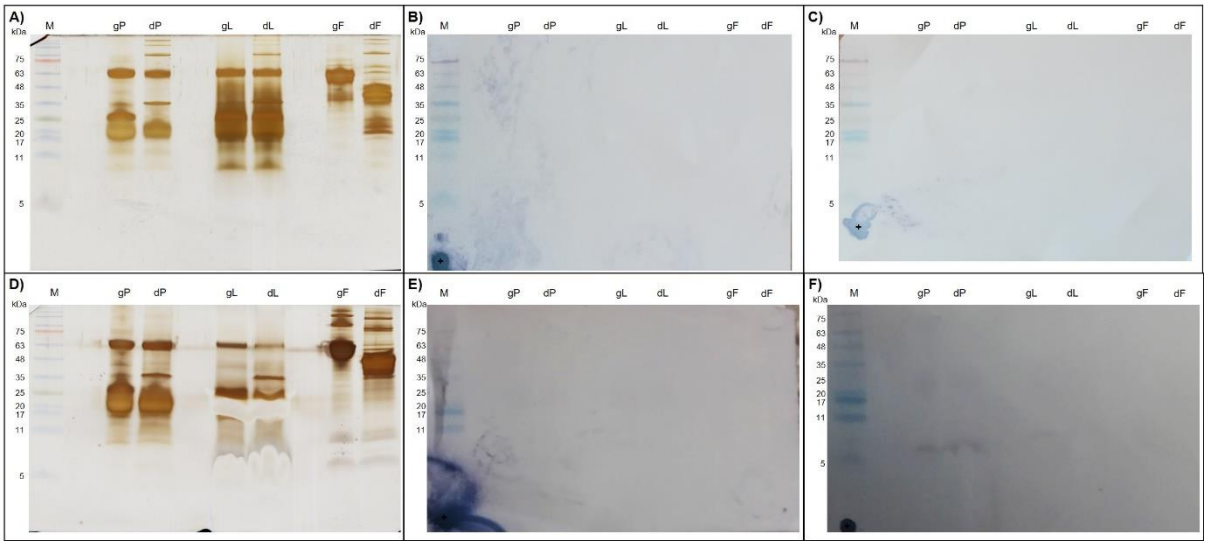

Figure 30: SDS-PAGE and Western blot of enzymatically deglycosylated Pau5p
A 12 % SDS-PAGE (**A+D**) and the corresponding Western blots with anti-HAC-DIR-IgG (**B+E**) as well as anti-HAC-AAG-IgG (**C+F**) are shown. Samples were deglycosylated with either the NEB kit (**A+B+C**) or with the Agilent Technologies kit (**D+E+F**). Of each sample, the enzymatically treated sample (d) and its native glycoslyated form (g) were applied. M: Marker (Serva Triple Color Protein Standard 3), P: purified sparkling wine, L: unpurified sparkling wine lyophilizate, F: fetulin provided as positive control of the declycosylation kits, +: Western blot positive controls (peptide HAC-DIR for **B+E** and peptide HAC-AAG for **C+E**).

The antibody development and verification were hence terminated and without specific anti-Pau5p antibody no immunochemical detection assay could be developed.

3.3.2 Reversed-phase high pressure liquid chromatography

RP-HPLC was elected as alternative relative quantification method for Pau5p. The principal method developed in the previous project AiF 18125 N was optimized in this study. First, the Phenex™-NY, 15 mm, 0.2 µm filters used for sample preparation were replaced by Phenex™-RC, 4 mm, 0.2 µm filters because it is possible that proteins stick to the nylon membrane. The new filters have a regenerated cellulose membrane suitable for protein filtration and are smaller in diameter to reduce losses of the small sample volumes. Next, the HPLC column was changed. The predecessors used the Aeris Peptide XB-C18 3.6 µm 250 x 2.1 mm column, which is recommended for small proteins or peptides up to 10 kDa. However, Pau5p has a molecular size of ~ 17 kDa and thus the column with the same properties but for proteins larger than 10 kDa (Aeris Widepore XB-C18 3.6 µm 250 x 2.1 mm) was applied. Finally, the gradient of the mobile phase was altered. Therefore, the acetonitrile gradient from 3-65% in 35 min was replaced by a gradient from 3-50% in 14 min. The overall time per sample was thus reduced from 60 min to 40 min allowing for a higher throughput. All those optimization steps also lead to a much-improved chromatogram of the Pau5p analysis,

as can be seen in Figure 31. This optimized method was used for relative Pau5p quantification of sparkling wine or yeast culture samples.

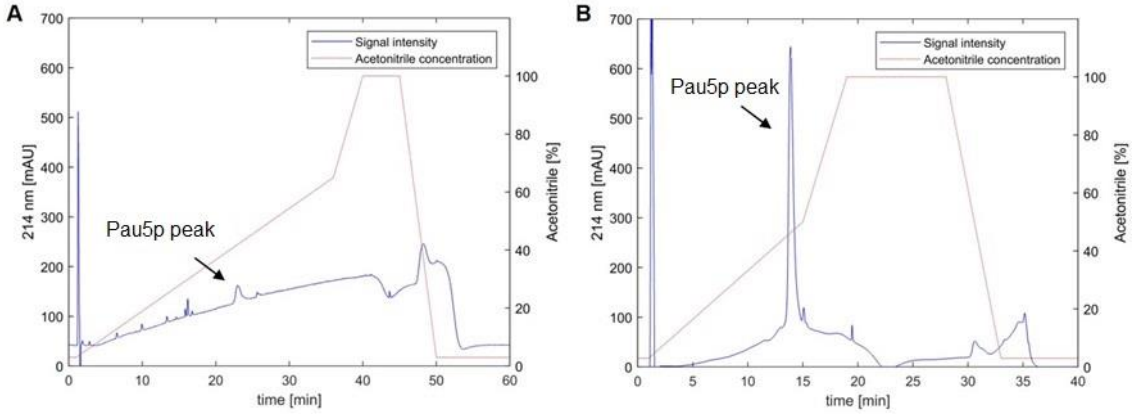

Figure 31: Pau5p RP-HPLC analysis optimization
The chromatograms of the RP-HPLC analysis of sparkling wine with the method developed in the project AiF 18125 N (**A**) and with the method optimized in this study (**B**) are shown.

3.4 Pau5p production potential of *Saccharomyces cerevisiae*

3.4.1 Screening of the Pau5p formation ability of different *S. cerevisiae* strains

To test whether different *S. cerevisiae* yeast strains produce different amounts of Pau5p, a screening of 32 yeast strains was conducted. Amongst those, 30 strains are industrially used for (sparkling) wine production and two are commercial beer fermenting yeasts. The yeasts were cultured in white grape juice for 4 d at 20 °C before the Pau5p content in the purified supernatant was measured with the previously mentioned RP-HPLC method (see 3.3.2) and normalized against the cell dry weight.

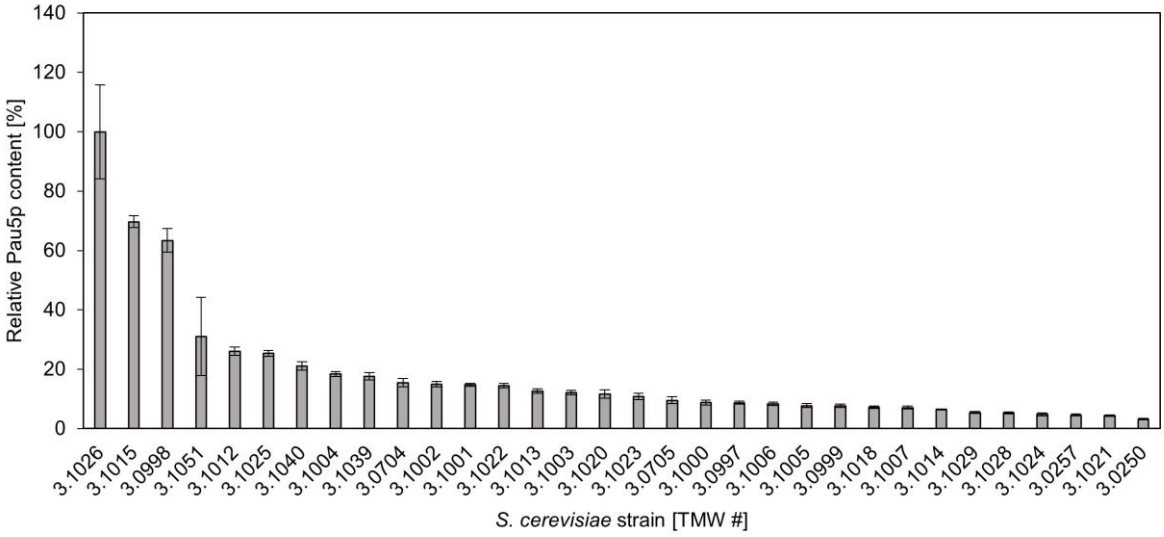

Figure 32: Strain-specific differences in the Pau5p production potential of 32 *S. cerevisiae* strains
The relative Pau5p production of 30 industrial (sparkling) wine yeast strains and two beer yeast strains (TMW 3.0250 and 3.0257) differs significantly. The strains were cultivated in white grape juice for 4 d at 20 °C. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the amount of the highest producer TMW 3.1026 was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

As shown in Figure 32, the screening revealed significant strain-specific differences in the Pau5p content of the supernatant after ANOVA analysis (p = 3.1 * 10^{-47}). The Pau5p production was low in most strains. The two additionally tested brewing yeasts (TMW 3.0250 and TMW 3.0257) were among the lowest producers. In contrast, strain TMW 3.1026 was identified as the most potent Pau5p producer in this set of strains under the given conditions, with significantly higher concentrations of the protein in the supernatant than all other tested strains. Thus, the Pau5p content of this strain was set to 100% and the remaining values were computed relative to it. TMW 3.1015 and TMW 3.0998 produced less Pau5p than TMW 3.1026 but still significantly more than the residual strains.

3.4.2 Fermentation conditions influencing the Pau5p production

Further experiments were conducted to analyze the influence of culture conditions on the Pau5p production. Strain *S. cerevisiae* TMW 3.1026 was chosen as the highest producer and TMW 3.1006 as a representative of the group of low Pau5p producers. All values for the relative Pau5p content given in the following experiments have been normalized to the control conditions (static incubation at 20 °C without daylight, inoculation OD_{600} = 0.5) of strain TMW 3.1026. The greatest percentual increase in Pau5p production was observed when the initial cell density of the cultures was reduced (see Figure 33).

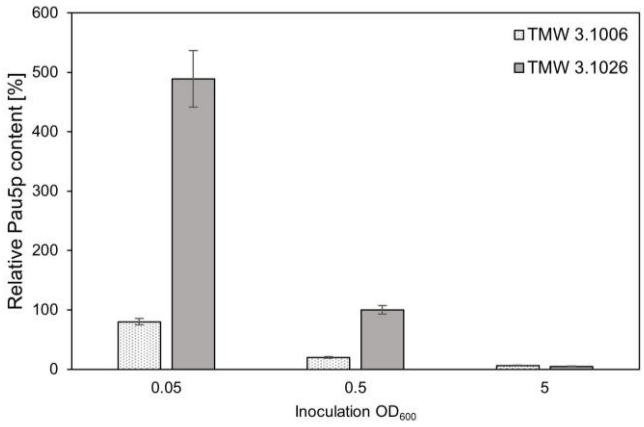

Figure 33: A reduced inoculation density increased the Pau5p production
Significantly increased Pau5p production was achieved with a decreased cell density in the inoculum. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, OD_{600} = 0.5) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

A highly significant (p = 2.4 * 10^{-8}) increase of 488.9% in Pau5p production relative to the control conditions was found in the supernatant of TMW 3.0126 cultures, when the initial OD_{600} was reduced 10-fold from 0.5 to 0.05. Inversely, the relative Pau5p content for this strain was only 4.5% of the control conditions when the initial cell density was raised 10-fold to OD_{600} = 5. Moreover, also strain TMW 3.1006 showed a significantly (p = 4.1 * 10^{-10}) increased Pau5p production relative to the control conditions after inoculation with a 10-fold reduced inoculum density.

Comparison of different incubation temperatures (10, 15, 20, 25, and 30 °C) revealed a significantly (p = 4.0 * 10^{-13}) higher Pau5p production by TMW 3.1026 with increasing temperature above 15 °C (see Figure 34). At 30 °C, this strain produced 191.8% Pau5p compared to the control condition at 20 °C. In contrast, the supernatants of TMW 3.1006 cultures contained most Pau5p at 15 °C, although the values for this strain were generally lower (maximum 39.9%).

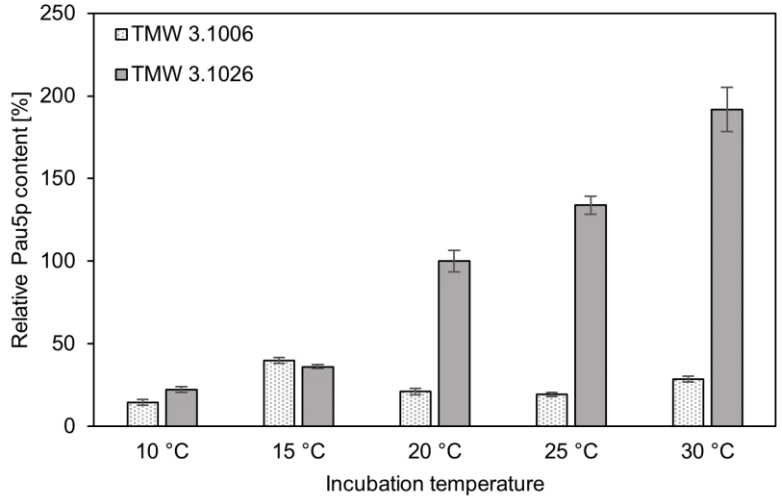

Figure 34: Different effects of the temperature on the Pau5p production by two yeast strains
S. cerevisiae TMW 3.1026 produced significantly more Pau5p with increasing temperature whereas *S. cerevisiae* TMW 3.1006 produced most Pau5p at 15 °C. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, 20 °C) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

Preliminary experiments suggested an influence of daylight on the Pau5p production in *S. cerevisiae*. Therefore, the two selected *S. cerevisiae* strains were cultivated at room temperature under conditions of diffuse daylight and in culture bottles that were darkened by wrapping with aluminum foil, respectively.

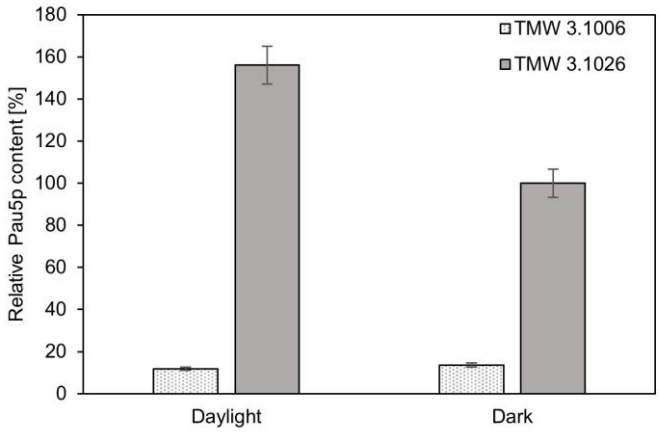

Figure 35: Varying Pau5p production by cultivation of yeasts under diffused daylight
Pau5p production was increased by cultivation under diffused daylight. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae*

TMW 3.1026, darkness) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

As a result, TMW 3.1026 produced significantly (p = 0.001) more Pau5p when exposed to diffuse daylight, whereas the difference in Pau5p content after incubation under darkness or daylight was not significant for strain TMW 3.1006 (see Figure 35).

In the case of additional 7% ethanol in the grape juice, a significant increase of Pau5p production was observed only for strain TMW 3.1006 ($p = 7.3 * 10^{-8}$), as shown in Figure 36. The difference was not significant for strain TMW 3.1026.

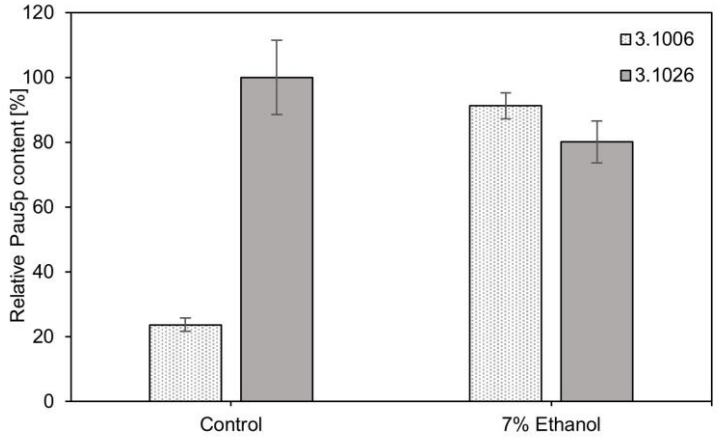

Figure 36: Influence of ethanol on the Pau5p production
The low Pau5p producing yeast strain TMW 3.1006 showed an increased Pau5p production by the addition of 7% ethanol to the medium. The high Pau5p production strain TMW 3.1026 produced slightly but not significantly less Pau5p in the presence of alcohol. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, control without ethanol) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

The addition of diammonium hydrogen phosphate (DAP) and three different commercially available nitrogen-containing nutrient preparations to the grape juice did not significantly alter the Pau5p production relative to the control conditions (see Figure 37).

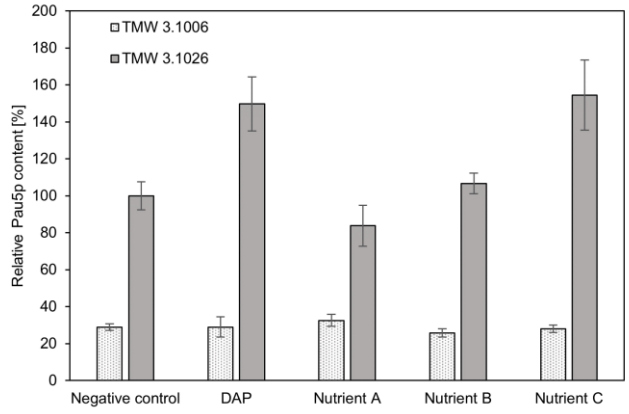

Figure 37: Effect of additional nutrients on the Pau5p formation
No significant effect of additional nutrients on the Pau5p content in the yeast cultures was measured. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, negative control) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

Decreasing the initial pH value to 2.5 compared to the natural pH of the grape juice of 3.5 significantly decreased Pau5p production for both strains (TMW 3.1006: p = 0.0299, TMW 3.1026: p = 0.001).

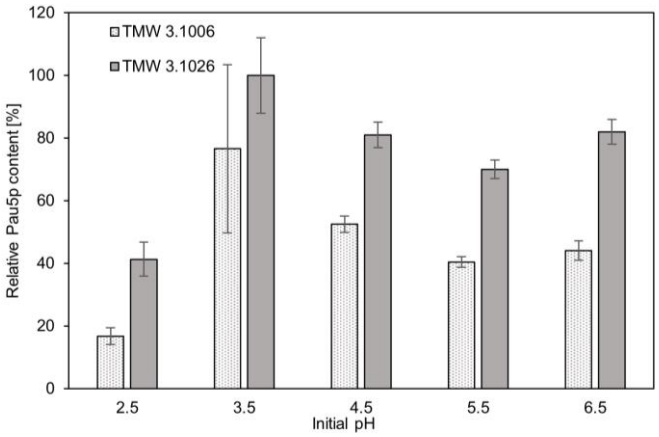

Figure 38: Effect of an altered initial pH value on the Pau5p production by *S. cerevisiae*
Pau5p production was repressed by pH 2.5 but not significantly affected by higher starting pH values. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, pH 3.5) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

In contrast, an initial increase of the pH value did not reveal a clearly altered pattern of the Pau5p production of both strains (see Figure 38). However, since the yeasts acidified the medium during cultivation to pH 4.6 or lower, the effect of the initially increased pH value might have been reduced.

Moreover, factors that reduce the Pau5p production by *S. cerevisiae* were found in this study. The first of those factors was the addition of 1 M sorbitol to the grape juice to increase the osmolarity, as can be seen in Figure 39.

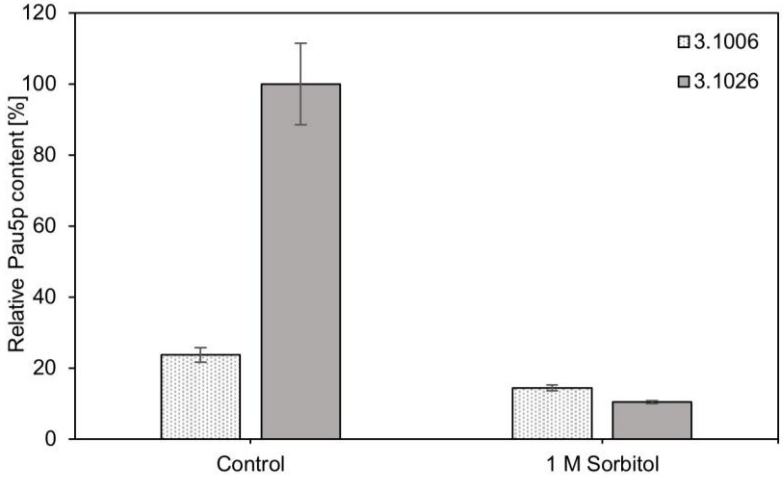

Figure 39: Decreased Pau5p production in grape juice due to a higher osmolarity
A reduced Pau5p formation was observed for both yeast strains when adding 1 M sorbitol to the white grape juice to increase the osmolarity. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, control without sorbitol) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

The sorbitol in the medium led to a significant decrease in the relative Pau5p content for both strains (TMW 3.1006: $p = 0.0031$; TMW 3.1026: $p = 3.2 * 10^{-5}$).

When *S. cerevisiae* TMW 3.1026 was co-cultivated with *Metschnikowia pulcherrima* (TMW 3.1016) or *Torulaspora delbrueckii* (TMW 3.1017), respectively, the Pau5p content was significantly decreased ($p = 0.0001$) (see Figure 40).

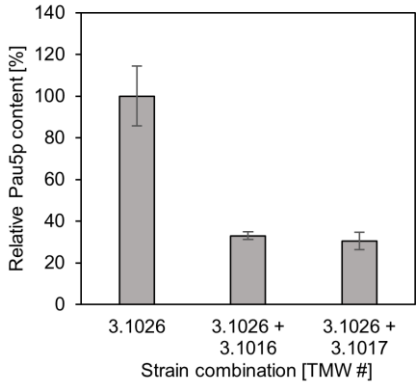

Figure 40: Significant reduction of Pau5p production in co-cultures with non-*Saccharomycetes*
Co-cultivation with *M. pulcherrima* (TMW 3.1016) or *T. delbrueckii* (TMW 3.1017) significantly decreased Pau5p production. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

Although *S. cerevisiae* showed similar cell counts after four days of incubation in all cultures, *M. pulcherrima* was underrepresented and *T. delbrueckii* was overrepresented. In this experiment, the Pau5p peaks were normalized to the number of colony forming units (cfu) of *S. cerevisiae*. As a control, strains TMW 3.1016 and TMW 3.1017 were also cultivated in pure cultures. No Pau5p was detectable in these cultures (data not shown).

Another experiment showed that when shaking the cultures in Erlenmeyer flasks, no Pau5p and when stirring them slowly, almost no Pau5p was produced (data not shown). To further elucidate the effect of agitation, an experiment was conducted under anaerobic conditions by closing the culture vessels with a septum and degassing the headspace with CO_2. A hollow needle (Ø 0.60 mm) was applied to avoid overpressure in the bottles.

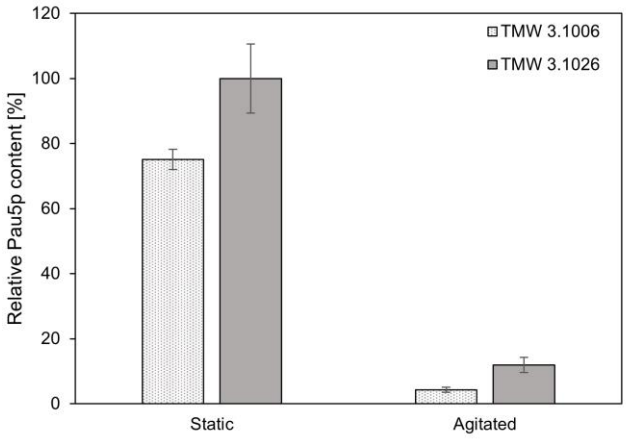

Figure 41: Significant decrease of Pau5p production by agitation of the yeast cultures
Agitation of yeast cells significantly decreased Pau5p production compared to static cultures. Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, static) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

The results showed that agitation of the cells significantly reduced the Pau5p content in the culture supernatant of both strains (TMW 3.1026: $p = 2.3 * 10^{-5}$, TMW 3.1006: $p = 2.2 * 10^{-9}$) (see Figure 41).

Finally, three Pau5p enhancing conditions (30 °C, daylight, inoculation OD_{600} = 0.05) were combined and compared with standard conditions (20 °C, darkness, inoculation OD_{600} = 0.5).

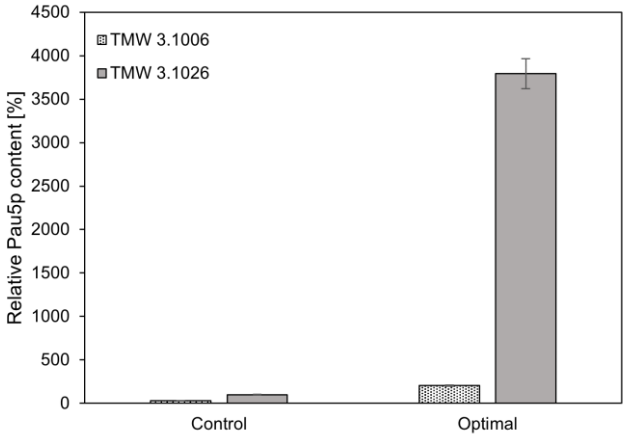

Figure 42: Highly increased Pau5p production by the combination of enhancing conditions
A combination of three Pau5p enhancing conditions, namely increased temperature (30 °C), incubation under diffused daylight, and a reduced inoculation OD_{600} (0.05), led to optimal Pau5p production compared to our standard condition (20 °C, darkness, OD_{600} = 0.5). Culture supernatants were purified and the Pau5p content was measured via RP-HPLC and normalized to the cell dry weight and the control (*S. cerevisiae* TMW 3.1026, control) was set to 100%. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard errors.

As shown in Figure 42, a significant ($p = 2.6 * 10^{-9}$) increase of 3792.4% was observed in the Pau5p content of the supernatant of strain TMW 3.1026 under the combined set of optimum conditions compared to the standard conditions. Even for the low producing strain TMW 3.1006, we achieved a significant ($p = 8.7 * 10^{-9}$) increase from 29.8% to 204.8%. For both

strains, these were the largest Pau5p peak areas of the whole experiment series, and the increase was more than the sum of the increase for the single experiments.

3.5 Pau5p formation under sparkling wine production conditions

3.5.1 Influence of the yeast strain and ammonium availability

To find out, whether Pau5p is also produced under the prevalent conditions of secondary fermentation and whether the results from the yeast screening can be transferred to sparkling wine production, a base wine was bottle fermented with nine different sparkling wine yeast strains under practical conditions in cooperation with the Hochschule Geisenheim University (HGU). The three high-Pau5p producing yeasts from the screening (TMW 3.1026, TMW 3.1015, and TMW 3.0998, see 3.4.1) as well as 5 medium or low producers (TMW 3.1004, TMW 3.1006, TMW 3.1012, TMW 3.1021, and TMW 3.1022) were selected. Furthermore, a standard yeast strain frequently used in the enological institute of the HGU was used, and supplemented base wine without inoculation served as negative control. The base wine, a 2018 Silvaner (~ 9% alcohol), was mixed with 24 g/L sugar and a combinatory vitamin preparation resulting in 32.5 mg/hL thiamin hydrochloride (vitamin B1) and 25 g/hL diammonium hydrogen phosphate (DAP). To elucidate, if the amount of yeast available nitrogen plays a role in Pau5p formation by *S. cerevisiae*, a second variant of base wine was prepared by adding extra DAP to a final concentration of 50 g/hL. The progress of the fermentation was tracked by measuring the CO_2 content spectroscopically and therewith calculating the internal pressure in the bottles. The resulting fermentation graphs are shown in Figure 43.

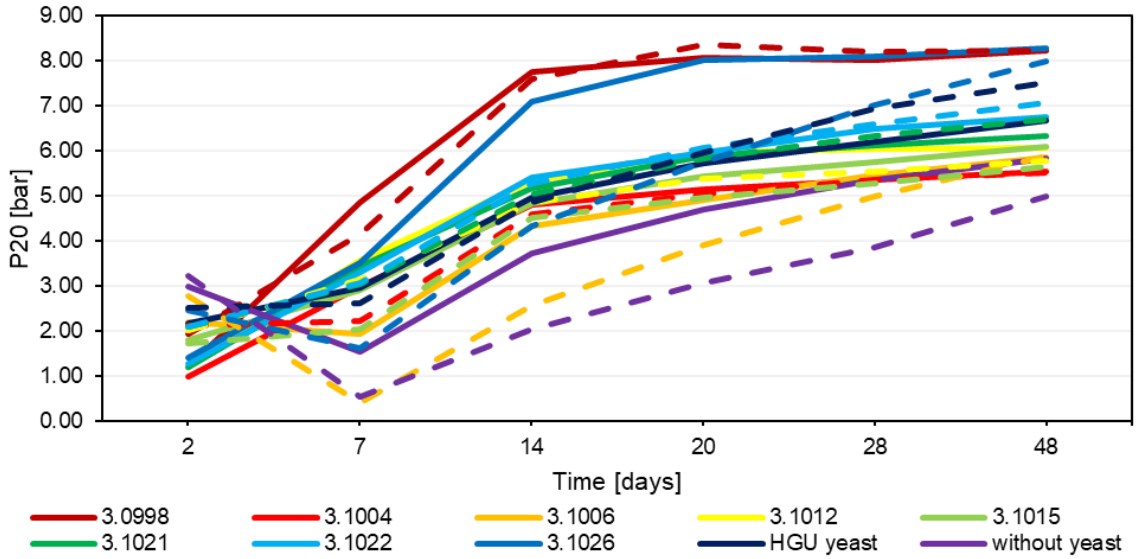

Figure 43: Fermentation graphs of nine different yeast strains in bottle fermentation
The internal bottle pressure at 20 °C (P20) was calculated from the spectroscopic CO2 measurement. Straight lines represent the wines with 50 g/hL DAP whereas the dashed lines display the wines with 25 g/hL DAP. Not all fermentations were completed after 7 weeks of fermentation, when the riddling started. The negative control underwent spontaneous fermentation. Each experiment was performed in triplicates and the mean of those is shown.

The bottles were manually riddled after 7 weeks of fermentation in the sparkling wine cellar. Not all fermentations were completed at that time. The fermentation appears to have been faster in some cases in the samples with 50 g/hL ammonium compared with the samples with 25 g/h, in other cases there was no difference. Only two strains achieved complete fermentation: TMW 3.0998 and TMW 3.1026. The negative control without inoculated yeast underwent spontaneous fermentation due to unsterile working conditions in the wine cellar. The fermentable residual sugar in the sparkling wines, measured via FTIR, confirms these findings (see Figure 44).

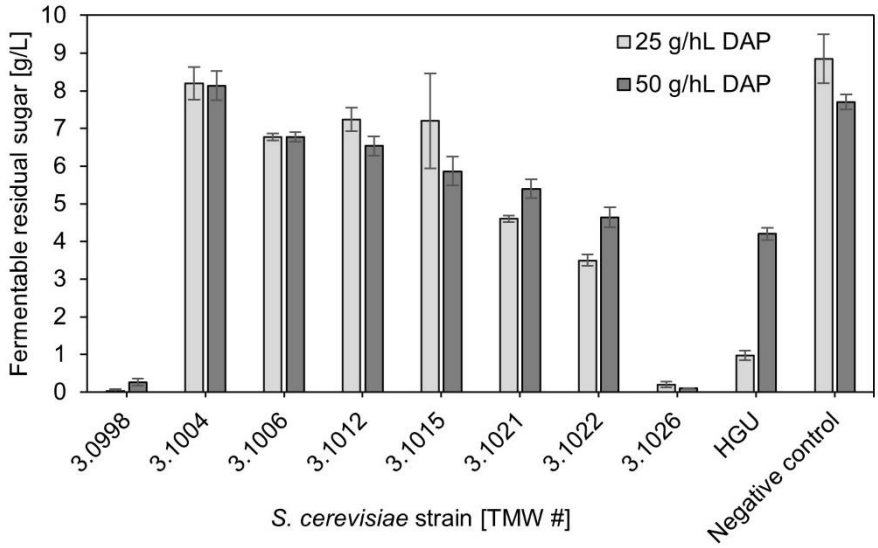

Figure 44: Fermentable residual sugar in sparkling wine bottle fermentation
The fermentable residual sugar in the bottles fermented with different yeast strains is shown. The base wine was supplemented with 24 g/hL sugar. Each experiment was performed in triplicates. The error bars indicate the standard error.

In accordance with the fermentation graphs, the residual sugar after disgorgement was nearly zero only for the two strains TMW 3.0998 and TMW 3.1026. However, no clear difference between wines with 25 g/hL or 50 g/hL DAP is visible in these results.

The measured ammonium concentration in the base wines was 0.07 g/L or 0.10 g/L for the wine supplemented with additional 25 g/hL (= 0.25 g/L) or 50 g/hL (= 0.5 g/L) DAP, respectively. The difference between the added DAP and the measured ammonium can be explained by the fact, that DAP reacts into two ammonium molecules and one hydrogen phosphate molecule if dissolved in water. The yeasts utilized almost none of those additional nutrients. In the low DAP wines, the average ammonium concentration was reduced to 0.06 g/L, but in the high DAP wines it was still at an average level of 0.10 g/L.

According to the NOPA analyses, the YAN of the base wine was 17 mg/L. After supplementation with DAP, the value was increased to 44 mg/L and 47 mg/L. The NOPA values of the fermented sparkling wine ranged from 42-57 mg/L with an average of 47 mg/L

and 52 mg/L for the samples supplemented with 25 g/hL and 50 g/hL DAP, respectively (data not shown).

The Pau5p contents were generally very low with values only slightly increased compared to the base wine before fermentation (see Figure 45).

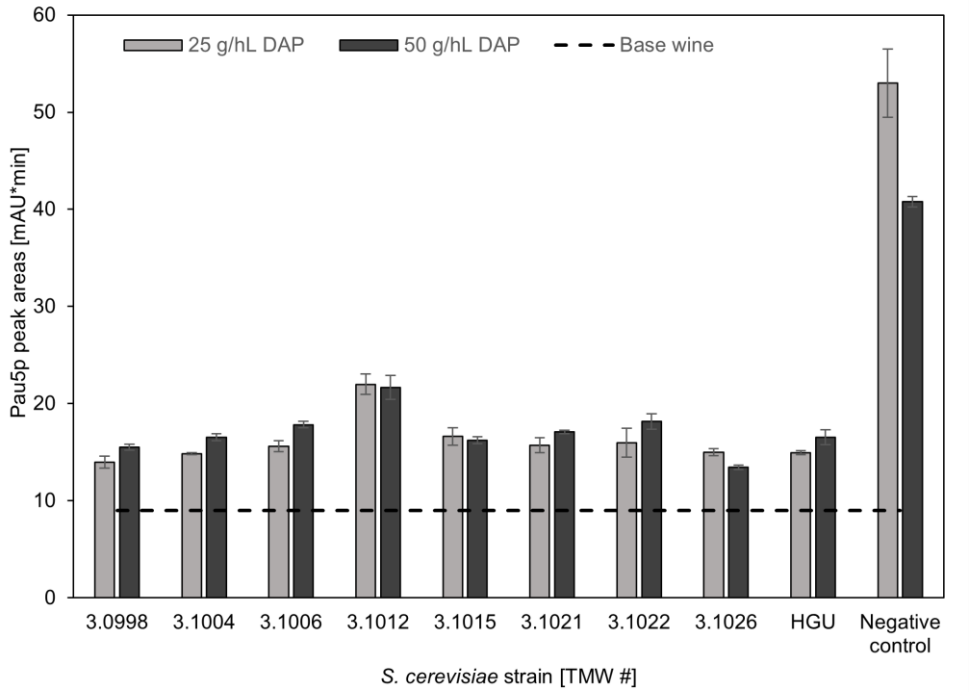

Figure 45: Pau5p content in sparkling wines fermented with nine different yeast strains
The Pau5p formation of nine different *S. cerevisiae* sparkling wine yeast strains after bottle fermentation of a base wine (Silvaner 2018) supplemented with 25 g/hL or 50 g/hL DAP is shown. The negative control was not inoculated on purpose but underwent spontaneous fermentation. For RP-HPLC, 50 mL samples were centrifuged, dialyzed and phenol extracted. Each experiment was performed in biological triplicates and technical duplicates. The error bars indicate the standard error.

The highest Pau5p values were measured in the negative control, which was not inoculated but underwent spontaneous fermentation due to unsterile process conditions in the practically oriented Geisenheim facility. There is no significant difference between the applied yeast strains or between the wines with varying amounts of DAP.

3.5.2 Effect of different amounts of yeast starter culture on the Pau5p content in sparkling wine bottle fermentation

Previous results in lab scale showed that a decreased initial cell density led to a significantly increased Pau5p formation by *S. cerevisiae* (see 3.4.2). To find out whether this knowledge can be applied to increase the Pau5p content in sparkling wine, bottle fermentations with different amounts of the yeast strains TMW 3.1026 and TMW 3.1021 were performed. The base wine, a 2019 Silvaner, had already large amounts of Pau5p.

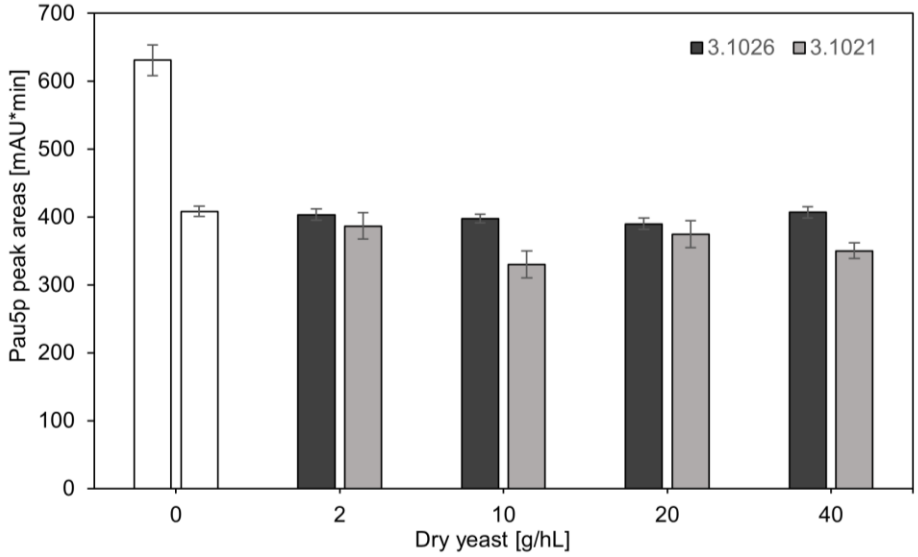

Figure 46: Varying amounts of yeast have no effect on the Pau5p content in bottle fermented sparkling wine
A 2019 Silvaner base wine was fermented with 2, 10, 20, or 40 g/hL dry yeast of a high-Pau5p producing yeast strain (TMW 3.1026) and a low-Pau5p producing yeast strain (TMW 3.1021). Of each sample, 50 mL were centrifuged, dialyzed, lyophilized, phenol extracted, and the Pau5p content was measured via RP-HPLC. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard error.

As shown in Figure 46, the amount of Pau5p after fermentation was much lower than in the base wine before fermentation suggesting an instability of Pau5p under fermentative conditions. The negative control without inoculation underwent spontaneous fermentation and has a similar level than the other samples. No difference between the varying amounts of applied active dry yeast was visible. The Pau5p contents in the samples fermented with the high-Pau5p producing yeast TMW 3.1026 were slightly higher than in those fermented with the low-Pau5p producing yeast strain TMW 3.1021, but this difference was not significant.

3.5.3 Effects of fining agents and lees aging on the Pau5p content

Another practical experiment was conducted to elucidate the influence of supplemented riddling aid and of the storage period on the lees after fermentation on the final Pau5p content in the product. The Silvaner 2018 base wine was fermented with the two yeast strains TMW 3.1026 and TMW 3.1021, a high-Pau5p producer and a low-Pau5p producer according to our screening, respectively. Different amounts of silicate-clarifying suspension as riddling aid were added (0, 35, or 70 mL/hL). Of each combination, 12 bottles were filled to enable disgorgement of triplicates at four different time points: Directly after 8 weeks of fermentation and 2, 4, and 6 months thereafter. The results of the subsequent RP-HPLC Pau5p analysis are shown in Figure 47.

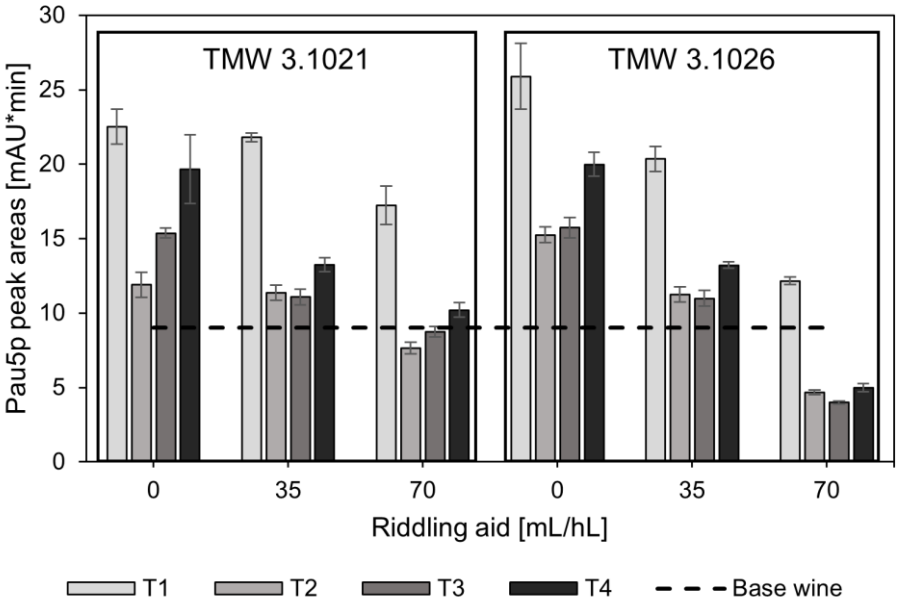

Figure 47: Effect of riddling aid and storage on the lees on the Pau5p concentration in sparkling wine
The addition of riddling aid as well as a longer storage on the lees reduces the final Pau5p content in sparkling wine. A 2018 Silvaner base wine was fermented with a high-Pau5p producing yeast strain (TMW 3.1026) and a low-Pau5p producing yeast strain (TMW 3.1021) with the addition of 0, 35, or 70 mL/hL riddling aid. Bottles were disgorged directly after fermentation (8 weeks) and 2, 4, or 6 months later (T1-T4 respectively). Of each sample, 50 mL were centrifuged, dialyzed, lyophilized, phenol extracted, and the Pau5p content was measured via RP-HPLC. All experiments were performed in biological triplicates and technical duplicates. The error bars indicate the standard error.

The presence of riddling aid reduced the Pau5p content regardless of the yeast strain used for fermentation and the time point of riddling. The latter however, had a great influence on the Pau5p content as well. Directly after fermentation, the Pau5p content was slightly increased compared to the base wine, but after storage for 2 months a significant drop in the Pau5p content was observed. Due to prolonged aging of 4-6 months, more protein of interest was again detectable. However, the values were still lower than that measured directly after fermentation. As in the previous practical experiments, no clear influence of the yeast strain on the Pau5p concentration was identified.

A final experiment was performed to test the power of bentonite fining agents to reduce the Pau5p content in base wines prior to secondary fermentation. In a first step, four different base wines were produced. Two different grape musts – a 2019 Riesling and a 2020 Chardonnay – were fermented with two different yeast strains – TMW 3.1021 and TMW 3.1026 – respectively. The HPLC analysis of the resulting base wines revealed that the fermented grape must has a greater influence on the Pau5p release by *S. cerevisiae* than the inoculated yeast strain, which can be seen in Table 24.

Table 24: Pau5p contents of four different base wines
Two different grape musts were fermented with two different yeast strains. The resulting four different base wines were analyzed via RP-HPLC. The measured Pau5p peak areas are shown in this table in mAU*min.

	Riesling 2019	Chardonnay 2020
TMW 3.1026	635	181
TMW 3.1021	1029	122

All musts were completely fermented, because the FTIR analyses showed almost no residual fermentable sugar (data not shown). Both Riesling wines had higher alcohol contents (10.4%) than the two Chardonnay wines (8.7%). The two Riesling base wines were chosen for the subsequent experiment due to their high Pau5p values. They were treated with three different commercial fining agents containing either sodium bentonite, calcium bentonite, or a commercial product which contains a mixture of both types.

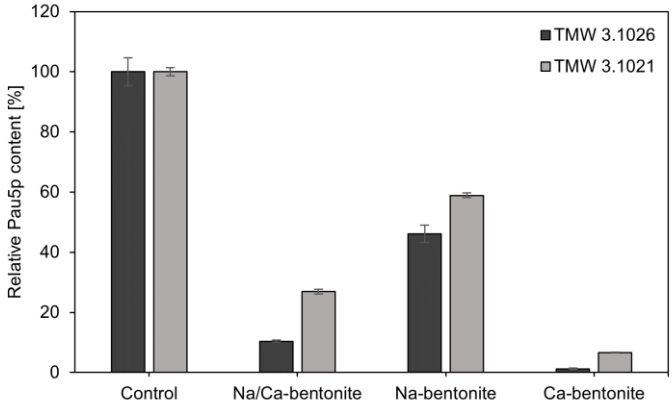

Figure 48: Bentonite fining agents reduce the Pau5p content in base wine
Two Riesling base wines produced by two different yeast strains (TMW 3.1026 and TMW 3.1021) were treated with three different bentonite fining agents. The Pau5p content of the untreated control for each base wine was set to 100%, and the treated samples were normalized to their control, respectively.

As shown in Figure 48, all fining agents reduced the amount of Pau5p drastically. But calcium bentonite was far more efficient in removing the yeast protein than sodium bentonite was, and the Pau5p adsorption capacity of mixed product was between that of Na- and Ca-bentonite.

4 Discussion

4.1 Proposed theses

While Pau5p is proposed as a gushing-reducing glycoprotein from *S. cerevisiae* in sparkling wines, it appears difficult to directly exploit this knowledge in wine making. This is apparently so, because Pau5p expression is highly sensitive to the growth stages of *S. cerevisiae* and upregulated under conditions, only part of which can be transferred to common sparkling wine making procedures. Furthermore, technological aids used in industrial (sparkling) wine production can reduce Pau5p upon production. Still, this work delivers basic insight in Pau5p production and regulation as well as exploitable approaches to support Pau5p formation and maintenance during the (sparkling) wine making process.

Consequently, from the results obtained during this study, the initial working hypotheses can be reformulated, and the following theses can be derived:

- The generation of a peptide-based antibody, which specifically detects the glycosylated protein Pau5p and could be used to control Pau5p in wine making, cannot be achieved by hen immunization.
- Different wine yeast strains of *S. cerevisiae* produce different amounts of Pau5p under certain conditions.
- Pau5p is regulated in a very complex and sensitive manner: Lower inoculation density, higher temperature, incubation under daylight, and the presence of ethanol can enhance Pau5p release by *S. cerevisiae* strains, while agitation of the culture, co-cultivation with non-*Saccharomycetes*, and a high osmolarity by elevated sorbitol concentrations can decrease the Pau5p release by *S. cerevisiae* strains. The addition of yeast nutrients or an alteration of the initial pH value have no influence on the Pau5p production by *S. cerevisiae* strains.
- Environmental conditions and use of technological aids in current (sparkling) wine can outweigh the effect of yeast strain specific Pau5p release. The Pau5p concentration in the final product is reduced by the application of bentonite fining agents or silicate based riddling aids in sparkling wine production.
- Pau5p is mainly produced during primary fermentation and only low amounts of the protein are released during secondary fermentation.
- Pau5p is unstable during secondary fermentation.

Transfer of these results in lab scale to practical (sparkling) wine production, requires a sophisticated selection of fermentative yeast strains, adjustment of the (sparkling) wine making process, and the use of beneficial technological aids. Unexpectedly, the application

of appropriate Pau5p-producing yeast strains may be promising namely in the primary fermentation. These theses will be discussed in detail in the following chapters.

4.2 *PAU5* cloning and antibody generation

4.2.1 Cloning and recombinant expression of *PAU5*

The yeast protein Pau5p is known to be highly glycosylated in its secreted and foam active state (Kupfer, 2018). Generally, *N*- and *O*-glycosylation can be distinguished. In case of the first-mentioned, sugars are attached to asparagine residues of the signal sequence Asn-X-Ser/Thr where X can be any amino acid but proline (Goto, 2007; Jigami, 2008). The amino acid sequence of Pau5p, however, does not contain such a signal sequence, which means that the protein is exclusively *O*-glycosylated (Luo & van Vuuren, 2008). The first step of this post-translational modification takes place in the endoplasmic reticulum, where a mannose molecule is linked with the ß-hydroxy group of a serin or threonine residue. Next, up to four further mannose molecules are attached in the Golgi apparatus (Goto, 2007; Kukuruzinska et al., 1987). Pau5p contains eleven serine and twelve threonine residues, hence in total 23 putative *O*-glycosylation sites. The glycosylation pattern varies widely between different species and for small peptides, a slight change in *O*-glycosylation can already have a great effect on the physic-chemical properties such as structure and function of small proteins (Goto, 2007; Jentoft, 1990; Lommel & Strahl, 2009). We therefore chose to express recombinant protein with the native mannosylation pattern and chose *S. cerevisiae* as host for homologous protein expression. The cloning and expression were successful, albeit not enough recombinant protein could be generated for purification. The *PAU5* gene is transferred to the host strain via a plasmid that does not integrate into the genome but stays episomal in the cytoplasm. It is known, that transformed *Saccharomyces* hosts often suffer from plasmid instability including plasmid losses or alterations (Zhang et al., 1996). In this study, plasmid losses were avoided by constant selective pressure with the antibiotic G418, but plasmid alterations are always possible, which might have had some effect on the promoter region of the gene of interest. Otherwise, it is also thinkable that the produced protein simply was highly unstable and degraded intracellularly or after its release. An indication for such a kind of incompatibility could be the observation, that the *PAU5* gene was frequently mutated in *E. coli*.

To possibly overcomer such drawbacks, we chose an additional approach by using *Pichia pastoris* as an alternative host. *Pichia* is a powerful tool for protein expression that has been successfully used in other studies at our department, and which has been well investigated and often reviewed (Baghban et al., 2018; Cregg, 1999; Cregg et al., 2009; Higgins, 1995; Juturu & Wu, 2017; Sreekrishna et al., 1997). Still, the glycosylation pattern of *Pichia* differs from that one of *Saccharomyces*. While both yeasts produce mainly *α*1-2Man$_{1\text{-}4}$ *O*-glycans, in

P. pastoris some of the *α*1-2Man$_4$ and *α*1-2Man$_3$ chains are replaced by a Man*β*1-2Man*β*1-2-disaccharide (Goto, 2007). Still, the heterologous expression system has the advantage of being commercially available with lots of different protocols and experience reports. Our *PAU5* cloning success in *Pichia* was, however, the same as in *Saccharomyces*. Correct transformants were generated and the protein was expressed, but at too low amounts. In this case, however, the vector was integrated into the genome at the *AOX1* locus. Based on the exact event of homologous recombination, different genotypes can occur. Namely, the gene of interest can replace the native *AOX1* gene resulting in a Mut^S strain. Those consequently lack the alcohol dehydrogenase AOX1, which is the main converter of methanol. The *AOX2* locus is still intact and thus those strains have a slow methanol utilization rate. The yeasts are poorly growing if fed with methanol as sole carbon source, which is necessary for induction of recombinant protein expression. Another possible recombination event is the insertion of the gene of interest, which leaves the native *AOX1* gene intact. The resulting genotype is called Mut$^+$ and the associated phenotype is fastly growing on methanol. A special scenario is the integration of several vectors into one genome resulting a gene cassette with several head-to-tail repeats of the gene of interest. Those strains can be either Mut^S or Mut$^+$ and they are desirable as they lead to drastically higher yields (Cregg et al., 2009; Macauley-Patrick et al., 2005; Romanos, 1995). Indeed, other studies at our department have shown, that those are the most promising candidates for successful protein purification (Frisch et al., 2021). A high-throughput expression screening of such strains could detect high-yield strains with optimal insertion location and copy numbers (Weis, 2019). On top, codon-optimized constructs could lead to higher recombinant protein levels (He et al., 2014; Hu et al., 2013; Öberg et al., 2011). In this work, we were only able to generate Mut^S strains with a single vector copy, again indicating some incompatibility of high amounts of Pau5p with the yeast's basic metabolic functions. The inability to express higher amount of Pau5p of these yeasts could also result from the fact that its non-glycosylated form is an integral membrane protein, which may disturb membrane functions in many ways.

4.2.2 Antibody generation

The most promising way to generate specific antibodies against a protein is the immunization of a host animal with the native protein. There was, however, no possibility to purify large amounts of native protein from sparkling wine or yeast cultures as part of the current study for a lack of a suitable fast detection method. Also, the recombinant expression experiments did not result in sufficient amounts of recombinant Pau5p for purification. As an alternative, Pau5p peptides were therefore synthesized and used for immunization of chickens by Davids Biotechnologie. However, the resulting peptide antibodies were unable to specifically detect native Pau5p. A steric hindrance caused by protein folding leading to an unfavorable protein structure is highly unlikely, as the antibody was tested under denaturing conditions in SDS-

PAGE. On the other hand, a possible explanation is the high glycosylation of the protein, which might sterically hinder the binding of the antibody to its epitope peptide (Huang et al., 1997; Lisowska, 2002). Two different deglycosylation kits have therefore been applied to the protein prior to antibody detection, both of which were based on an enzymatic elimination of sugar residues. The implied available enzymes mainly remove *N*-glycosylation, and only simple *O*-glycans are removed from glycoproteins. As Pau5p is solely and intensively *O*-glycosylated, it was probably still mannosylated to some extent after the treatment. This finding was supported by the SDS-PAGE pattern, which could not verify a complete success of the deglycosylation. Chemical deglycosylation with triflic acid might be an alternative for complete removal of all sugar residues from the protein (Edge, 2003). However, the antibody was developed as a basis for simple on-site immunochemical detection assays of Pau5p. Such a complex sample treatment involving hazardous substances did not appear as practicable for application in sparkling wine cellars. The peptide antibody generation in this study was performed with two different peptides covering 30% of the amino acid sequence of Pau5p. These two peptides were selected as the most promising ones with a peptide prediction tool by Davids Biotechnologie, but still their antigenicity was prognosed to be fairly "medium". Based on the above discussed considerations, we propose the following thesis: The generation of a peptide-based antibody which specifically detects the glycosylated protein Pau5p is not possible by hen immunization.

In future studies, a great amount of native protein would be necessary for immunization of a host animal to gain a selective polyclonal anti-Pau5p antibody instead of a peptide antibody. Furthermore, different host such as a rodents or goats instead of a hen might be immunized, as the choice of the host animal is a crucial decision in the development of high-affinity antisera (Hanly et al., 1995). However, those are very time-demanding approaches and in this study RP-HPLC has successfully been applied as alternative detection method for Pau5p to elucidate the factors influencing the content of this technically relevant mannoprotein in sparkling wine.

4.3 Factors influencing the Pau5p release by *S. cerevisiae*

4.3.1 Yeast strain

The results of the yeast screening (see chapter 3.4.1) demonstrated that there are great strain-specific differences regarding the Pau5p production by *S. cerevisiae*. This is in contrast to results presented by Rachidi et al. (2000b) who did not find differences on the transcriptional level of *PAU* genes when two different yeast strains were compared under standard conditions. However, the present study clearly shows that such differences can be found when Pau5p is quantified on the protein level and a larger set of strains is studied. To the best of our knowledge, this is the first study in which the Pau5p formation of a large

number of different *S. cerevisiae* strains was compared. As our study focused on problems in the sparkling wine industry, we mainly used yeast strains that are used for sparkling wine production. These included three potential Pau5p producing strains (TMW 3.1026, TMW 3.0998, TMW 3.1015). The utilization of such yeasts in base or sparkling wine production can possibly reduce the gushing potential of sparkling wine as compared to a product fermented with a weak Pau5p producing yeast strain, due to the gushing reducing effect of the glycoprotein Pau5p (Kupfer et al., 2017a). We also included *S. cerevisiae* strains applied for fruit-, white-, rosé-, and red wine production, which were all medium or low producers of Pau5p, in our screening. Moreover, both beer specific yeast strains produced almost no Pau5p in our experiments. This is interesting, because several studies reported that Pau5p expression is upregulated in wine strains under winemaking conditions (Luo & van Vuuren, 2008; Rachidi et al., 2000a) and it was suggested that PAU proteins are involved in sterol transport. A remodeling of the cell wall with sterols can enhance resistance to higher alcohol concentrations during winemaking (Di Gianvito et al., 2018; Rossignol et al., 2003; Wilcox et al., 2002). Thus, this might be an adaption of wine yeast strains to the stressful conditions of wine fermentation, which the two beer yeast strains appear to lack as a result of lower ethanol concentrations in beer fermentations.

4.3.2 Environmental conditions in lab scale

Further experiments with strains TMW 3.1026 and TMW 3.1006, revealed that the strain-specific differences were consistent under different fermentation conditions. All these experiments were performed in lab scale (100 mL cultures) with grape juice. Samples were taken after 4 days and the setup was more like primary fermentation than secondary fermentation, but should not be compared to practical wine making conditions. The strongest increase in Pau5p secretion was observed when the cultures were inoculated with a 10-fold reduced cell density. Given that all values were normalized to the cell dry weight of the cultures, our data indicate that Pau5p is mainly produced during cell growth. This can be explained by the fact that the non-glycosylated form of this protein belongs to the integral membrane proteins (Luo & van Vuuren, 2008), which are typically regulated in a cell-cycle dependent manner (Smits et al., 2001). The second-best improvement of Pau5p release was achieved by increasing the temperature up to 30 °C, at least for strain TMW 3.1026. This is in contrast to the findings of Luo and van Vuuren (2008), who found that *PAU5* is upregulated at 10 °C. However, in their study, they analyzed the intracellular Pau5p content after two hours of induction, whereas in our study the extracellular Pau5p level was measured after four days of fermentation. Moreover, those authors used a different *S. cerevisiae* strain, and our results suggest that the regulation is strain-specific, especially regarding temperature conditions. Apart from that, an induction of *PAU5* by temperature seems plausible, as seripauperins are related with the TIP (temperature inducible protein)/

SRP family including TIP1 – a protein inducible by cold shock as well as by heat shock (Kondo & Inouye, 1991; Rachidi et al., 2000b). The third improving condition was the incubation of the cells under diffuse daylight compared to darkness. Again, this effect was only apparent for strain TMW 3.1026 and not for TMW 3.1006. Although nothing is known about light perception in yeasts*, S. cerevisiae* does have a circadian rhythm (Eelderink-Chen et al., 2010) and for example two genes are involved in photoadaptation of *Neurospora crassa*, which are homologs of the *S. cerevisiae* Tup1–Ssn6 complex (Olmedo et al., 2010).

As already discussed in section 4.3.1, higher alcohol levels are suspected to increase the Pau5p formation by *S. cerevisiae*. This is also reflected in our results, where one of the two strains (TMW 3.1006) showed significantly increased Pau5p contents in the culture medium supplemented with ethanol. It is furthermore known, that high ethanol levels activate the high osmolarity (HOG) pathway and the cell wall integrity (CWI) pathway, which collaboratively regulate adaptive changes of the cell wall (Udom et al., 2019). The regulation of Pau5p as integral cell wall protein in response to increased alcohol levels therefore seems plausible. Then again, Luo and van Vuuren (2008) proposed that the *PAU5* promoter is not activated, but that instead the protein is stabilized by the presence of ethanol and that therefore increased levels of Pau5p can be measured under such conditions. As we were determining the relative protein concentration in the supernatant, we cannot make any statement regarding the gene regulation mechanism. But in either way, ethanol seems to have a positive effect on the Pau5p content in the final product.

In the experiment that combined three Pau5p increasing conditions, both *S. cerevisiae* strains reached normalized Pau5p peak areas that were larger than the sum of the values of the single parameters, meaning that there is probably a synergistic effect here. This is especially interesting for strain TMW 3.1006, which did not show an increased Pau5p secretion for higher temperature or incubation under diffused daylight in the single experiments and only achieved a 4-fold increase with the reduced cell density, whereas under "optimal" combined conditions the increase was almost 7-fold. A few fermentation conditions did not show a clear effect on the Pau5p production, including the addition of nutrients and an increased pH value. The addition of diammonium hydrogen phosphate slightly – but not significantly – increased the Pau5p production. Although the nitrogen requirements in the second fermentation during sparkling wine production are generally low (Martí-Raga et al., 2015), the effect of nitrogen addition should be greater in base wine than in grape juice as the latter has more yeast-available nitrogen. Therefore, the effect of nitrogen addition has been elucidated under practical conditions in secondary fermentation. This, however, confirmed the previous results and had no effect on Pau5p production (see

3.5.1 and 4.3.3). Regarding the pH value, Luo and van Vuuren (2008) found that acidity (pH 3.5) does not induce Pau5p expression, which is in accordance with our findings.

The addition of 1 M sorbitol to the culture medium significantly decreased the Pau5p production in both yeast strains tested. According to literature, this condition increases the Pau5p stability but has no effect on the gene expression (Luo & van Vuuren, 2008). High osmolarity has great influences on the cellular metabolism by activation of the HOG pathway, which mainly leads to an increased glycerol production. The contrary condition, namely low osmolarity, activates the CWI pathway, which leads to a remodeling of the cell wall to avoid swelling of the cells (Heinisch & Rodicio, 2017; Stefan Hohmann, 2009). As already discussed above for increased ethanol levels, this might well lead to an altered gene regulation of cell wall proteins such as Pau5p.

Rivero et al. (2015) state that Pau5p can lead to resistance against a killer toxin of other yeasts and is thus important for the co-existence of different yeasts in the vineyard. Tronchoni et al. (2017) even found out that in mixed starter cultures of *S. cerevisiae* and *T. delbrueckii, PAU* genes are upregulated after three to four hours of fermentation, albeit this induction disappeared after 12 h. In our experimental setting and after four days, the co-cultivation led to a decrease of the Pau5p content in the culture supernatant. Finally, we found out that agitation significantly decreased Pau5p production compared to static cultures. These data suggest that Pau5p might be involved in or co-regulated together with proteins that are important for cell–cell-contact or flocculation. Interestingly, the *FLO* genes – a gene family responsible for yeast flocculation – as well as most *PAU* genes are both located at the subtelomeric ends of their respective chromosomes. It has been demonstrated that *PAU* genes are differentially expressed in a yeast strain with a deleted *FLO5* gene compared to its flocculent wild type strain (Di Gianvito et al., 2018).

We conclude that different wine yeast strains of *S. cerevisiae* produce different amounts of Pau5p under certain conditions and that under our given conditions in the laboratory, lower inoculation density, higher temperature, incubation under daylight, and the presence of ethanol can enhance Pau5p release by *S. cerevisiae* strains. Agitation of the culture, co-cultivation with non-*Saccharomycetes*, and a high osmolarity by elevated sorbitol concentrations can decrease the Pau5p release, while the addition of yeast nutrients or an alteration of the initial pH value have no influence on the Pau5p production by *S. cerevisiae* strains.

4.3.3 Applicability to sparkling wine production

4.3.3.1 Influence of the yeast strain on the PAU5 content in sparkling wine

Considering all the results from the enological experiments, we suggest that Pau5p is mainly produced during primary fermentation and only low amounts of the protein are released during secondary fermentation. The results from the screening of the Pau5p production potential of different yeast strains are therefore not applicable to the secondary fermentation. Regarding the primary fermentation, strain-specific differences were observed, but they can be outweighed by environmental conditions such as different grape musts. These vary extensively between different grape musts and from winery to winery. Currently, these are not considered in the context of yeast choice and respective Pau5p production.

4.3.3.2 Applicability of lab-scale Pau5p influencers to sparkling wine production

In lab-scale fermentations of grape juice, the greatest influence on the Pau5p release was achieved by decreasing the inoculated yeast cell density. In the first practical experiment, the spontaneous fermented sparkling wine caused by contamination without deliberate inoculation had a significantly increased Pau5p content compared to the inoculated samples. We hence made a practical experiment with different amounts of starter culture. Both tested yeast strains produced similar levels of Pau5p even if the used amount of active dry yeast was decreased 20-fold compared to the highest inoculation. An effect might be obtained by further decreasing the inoculation size. This would not be appropriate in industrial sparkling wine production. To ensure successful fermentation, *S. cerevisiae* is usually inoculated at a final density of 1-3 x 10^6 cells/mL, while the indigenous yeast cell density ranges from 100-10 000 cells/mL (Romano et al., 2019). Nutrient competition with non-*Saccharomyces* yeasts can lead to sluggish or stuck fermentations (Medina et al., 2012). Furthermore, wine producers need to consider sensory aspects and the inoculum size effects the volatile composition of the wine and the glycerol production by *S. cerevisiae* (Carrau et al., 2010; Mateo et al., 2001; Yalçın & Özbaş, 2006).

The applicability of the other Pau5p enhancing conditions in our lab-scale experiments to sparkling wine production needs dedicated evaluation. While the ethanol concentration is increasing automatically during wine fermentation, the other parameters might be influenced directly. However, an alteration of the fermentation temperature has extensive consequences on the fermentation process and therefore on the final product quality. The temperature during primary fermentation is one of the most influencing factors on yeast species dynamics and lower temperature favors non-*Saccharomyces* yeasts. Also, the temperature impacts ethanol tolerance and biosynthetic pathways in wine yeasts and their production of volatile aroma compounds such as higher alcohols and esters (Fleet, 2007; Romano et al., 2019). The use of mixed starter cultures is seen as an interesting tool for higher flavor complexity

(Masneuf-Pomarede et al., 2016), but with regard to its repressing effect on Pau5p production, we discourage the co-cultivation of different yeasts to reduce the gushing potential of sparkling wine.

Incubation under daylight might be realized by implementing daylight lamps into the production plants in the case of tank fermentations. While the traditional method is always static, stirring of cells under exclusion of oxygenation is regularly used in industrial tank fermentations (Arntz, 1997). It might be considerable to interrupt this fermentation for a certain time period to let the yeast cells settle on the ground and enhance Pau5p formation. Of course, such measures always would need validation of their effectiveness on the Pau5p content in the product, of their impact on the sensorial quality of the wine, and of their economic profitability.

4.3.3.3 Effect of ammonium supplementation

Different amounts of supplemented ammonium had no effect on Pau5p production during secondary fermentation, which was in accordance with our earlier findings from the lab-scale experiments. Typical recommendations for YAN concentrations in wine production are 150-200mg/L (Waterhouse et al., 2016). To the best of our knowledge, there is no such proposition for the secondary fermentation in sparkling wine production. The applied amount of DAP in our experiments was thus based on the suggestion in the product data sheet (50-60 g/hL). The yeasts assimilated almost none of the inorganic nitrogen, which is possibly due to the elevated alcohol concentration in the base wine of approximately 9%. It is known that in the presence of ethanol, the yeast's ability to take up nitrogen via permeases diminishes (Sablayrolles et al., 1996). In sparkling wine production, it is favorable to supply the yeasts with nitrogen already during the rehydration phase by adding DAP to the acclimation medium. This has shown to positively influence secondary fermentation and has had a greater effect than the addition of nitrogen to base wine (Martí-Raga et al., 2016; Martí-Raga et al., 2015). An excess of ammonium nitrogen should also be avoided to avoid negative impacts on the sensorial character of the wine (D'Amato et al., 2006; Martínez-Moreno et al., 2014).

4.4 Factors influencing the Pau5p stability or persistence

Based on the results of this study, we propose that Pau5p is unstable during secondary fermentation and that its concentration in the final product is further reduced by the application of bentonite fining agents or silicate based riddling aids.

As described in the results part, we observed significant losses of Pau5p during secondary fermentation of a base wine with originally high Pau5p levels (chapter 3.5.2). Also, we have seen that the Pau5p content of another base wine increased during the secondary

fermentation, but two months thereafter decreased even below the initial value (chapter 3.5.3). Wine protein instability is a common problem, which describes the aggregation or flocculation of proteins and might lead to a reduced content of this specific protein. This process is influenced amongst others by the protein and polyphenol content and their ratios, the pH value of the wine, and the sulfate concentration (Cosme et al., 2020; Waterhouse et al., 2016). Aggregated proteins are not detectable with our analytical Pau5p quantification method. Other possible explanations for the protein loss are acid hydrolysis and degradation by proteases. While *S. cerevisiae* generally cannot secrete aspartic proteases, which would be active at low pH levels in the wine, intracellular aspartic proteases have been found in the extracellular matrix. Furthermore, non-*Saccharomyces* wine yeast strains, which are often present in winemaking environments, have been reported to secrete active proteases (Theron & Divol, 2014). Four to six months after fermentation the Pau5p content started to increase again in our experiment. This might be due to autolysis, a process by which amongst other substances mannoproteins from the yeast cell wall are released to the wine (Gnoinski et al., 2021; Martínez-Rodríguez & Pueyo, 2009). The Pau5p concentration six months after fermentation was still below the initial value, but sparkling wines are often aged on the lees for years to benefit from positive autolytic effects. In this work, we could not follow any effect of autolysis on the Pau5p content upon long-term aging.

The application of fining agents significantly reduced the Pau5p content. In the case of the silicate-clarifying suspension, a correlation between the applied amount and the Pau5p concentration reduction was observed. When testing different kinds of bentonite, we found that calcium bentonite has a greater Pau5p binding capacity than sodium bentonite. Generally, sodium bentonite has greater protein adsorption capacities than calcium bentonite (Blade & Boulton, 1988; Waterhouse et al., 2016). Therefore, the greater reduction of Pau5p by calcium bentonite must be due to selective effects. Such selective effects of bentonites have been reported in the literature. For example, Salazar et al. (2010) found that sodium bentonite selectively removes protein fractions of 20-30 kDa and 60 kDa. Partial selectivity of Na/Ca-bentonite on protein adsorption was reported in another study, but in contrast to our findings, the authors demonstrated that glycosylated proteins were not removed in notable amounts (Jaeckels et al., 2017). Early findings by Achaerandio et al. (2001) showed no adsorption selectivity of bentonite for distinct proteins, but they tested only three standard proteins (bovine serum albumin, ovalbumin, and lysozyme). To increase the Pau5p content in sparkling wine, Na-bentonite should be used instead of Ca-bentonite and the amount of fining agents should be kept as low as possible.

4.5 Conclusion

In the current study different ways were explored to monitor and increase the concentration of the gushing-reducing yeast mannoprotein Pau5p from *S. cerevisiae*. It can be concluded that this protein is regulated in a very complex manner. Besides the specific strain of *S. cerevisiae* used as starter culture, the environmental conditions play a major role in this regulation process. The most promising approach to increase the Pau5p content in sparkling wines is the use of base wines with high Pau5p contents. We found that the protein is mainly produced during primary fermentation and that its release during secondary fermentation is neglectable. The use of fining agents should be reduced to an unavoidable minimum and if bentonites are applied, Na-bentonite should be preferred over Ca-bentonite. Further promising approaches include the exposure to daylight or sedimentation due to stirring recesses during sparkling wine fermentation for their potential to increase the Pau5p secretion by *S. cerevisiae*.

5 Summary

Spontaneous excessive over-foaming of carbonated beverages upon opening of a bottle despite correct handling – a phenomenon called gushing – causes severe economic and reputational damages to the beverages industry. In previous studies, the yeast mannoprotein Pau5p has been identified as a negative gushing biomarker in sparkling wine. In the current study, approaches were explored to monitor and increase the concentration of the gushing-reducing protein Pau5p from *Saccharomyces cerevisiae*. Pau5p was therefore successfully cloned and expressed both in *S. cerevisiae* and in *Pichia pastoris*. The expression levels were too low to purify recombinant protein for antibody production. Two antibodies against Pau5p peptides were generated as third-party service. As none of the antibodies produced in chickens could specifically detect Pau5p, immunochemical detection assays could not be established. Instead, the RP-HPLC analysis for this protein was optimized and used for further analyses. A screening of 30 different wine yeast strains of *S. cerevisiae* revealed strain-specificity of Pau5p secretion and three high-producers of the protein were identified (TMW 3.1026, TMW 3.1015, TMW 3.0998). Under the given conditions in our laboratory, lower inoculation density, higher temperature, incubation under daylight, and the addition of ethanol to the medium could enhance Pau5p release by *S. cerevisiae* strains. Agitation of the culture, co-cultivation with non-*Saccharomycetes*, and a high osmolarity could decrease the Pau5p release, while the addition of yeast nutrients or an alteration of the initial pH value had no influence on the Pau5p production by *S. cerevisiae* strains. The applicability of such factors to sparkling wine production was tested under practical conditions in collaboration with the Hochschule Geisenheim University. The protein can be produced in high quantities during primary fermentation whereas the release during secondary fermentation is neglectable. Strain-specific differences therefore do not exist in during the latter. In base wine production, the Pau5p release is influenced by the yeast strain but this effect can be outweighed by the influence of changing environmental conditions such as the use of different grape musts. Based on the current findings the protein appears as unstable during secondary fermentation and lees aging. Fining agents such as silicate-clarifying suspensions efficiently remove Pau5p, and their use should therefore be minimized. Na-bentonite should be preferred over Ca-bentonite because the latter has a higher Pau5p adsorption capacity. Taken together, the current study generates new insight into the factors that affect the production of the gushing-reducing protein Pau5p by *S. cerevisiae.* The information generated during the current study is very valuable for the sparkling wine producing industry enabling a respective awareness of Pau5p towards yeast strain selection and process optimization, and finally helps to reduce commercial and image losses caused by gushing.

6 Zusammenfassung

Spontanes, extremes Überschäumen kohlensäurehaltiger Getränke nach dem Öffnen der Flaschen trotz korrekter Handhabung – ein als Gushing bekanntes Phänomen – sorgt für schwerwiegende wirtschaftliche Schäden sowie Imageprobleme in der Getränkeindustrie. Das Mannoprotein Pau5p aus *Saccharomyces cerevisiae* wurde in vorangegangenen Studien als negativer Biomarker für Gushing in Sekt identifiziert. In dieser Studie wurde an unterschiedliche Ansätze zur Verfolgung und Anreicherung des Gushing-reduzierenden Proteins Pau5p in Sekt geforscht. Dazu wurde *PAU5* erfolgreich in *S. cerevisiae* und *Pichia pastoris* kloniert und exprimiert. Die Expressionslevels waren allerdings zu gering, um ausreichend rekombinantes Protein für die Gewinnung von Antikörpern aufzureinigen. Zwei Antikörper wurden deswegen gegen Pau5p Peptide als Leistung Dritter über die Immunisierung von Hühnern generiert. Allerdings konnte keiner der Antikörper Pau5p spezifisch detektieren, sodass kein immunchemisches Nachweisverfahren zur Detektion von Pau5p entwickelt werden konnte. Alternativ wurde eine RP-HPLC Analytik für das Protein optimiert und für die folgenden Untersuchungen verwendet. Ein Screening von 30 verschiedenen *S. cerevisiae* Weinhefestämmen zeigte, dass Pau5p stammspezifisch sekretiert wird und drei starke Pau5p Produzenten konnten identifiziert werden (TMW 3.1026, TMW 3.1015, TMW 3.0998). Unter den in unserem Labor vorherrschenden Bedingungen konnten eine geringere Inokulationsdichte, eine erhöhte Temperatur, Inkubation bei Tageslicht und die Zugabe von Ethanol zum Medium die Pau5p Freisetzung durch *S. cerevisiae* Stämme fördern. Das Rühren der Kultur, eine Co-kultivierung mit nicht-*Saccharomyceten* sowie eine erhöhte Osmolarität konnten die Pau5p Freisetzung erniedrigen, während die Zugabe von Hefenährstoffen oder eine Änderung des initialen pH-Werts keinen Einfluss auf die Pau5p Produktion durch *S. cerevisiae* Stämme hatten. Die Anwendbarkeit solcher Faktoren auf die Sektherstellung wurde unter praktischen Bedingungen in Zusammenarbeit mit der Hochschule Geisenheim University untersucht. Das Protein kann in großen Mengen während der ersten Fermentation gebildet werden, wohingegen die Freisetzung während der zweiten Fermentation vernachlässigbar ist. Stamm-spezifische Unterschiede existieren daher während der sekundären Fermentation nicht. Während der primären Fermentation ist die Pau5p Freisetzung zwar durch den Hefestamm beeinflusst, dieser Effekt kann aber durch den Einfluss von geänderten Umweltbedingungen, zum Beispiel die Verwendung unterschiedlicher Moste, übertroffen werden. Nach dem gegenwärtigen Stand der Untersuchungen ist das Protein während der sekundären Fermentation und während der Reifung auf der Hefe unstabil. Schönungsmittel, wie zum Beispiel Silikatsuspensionen, entfernen Pau5p effizient und sollten daher nur so wenig wie möglich eingesetzt werden. Na-Bentonit sollte gegenüber Ca-Bentonit vorgezogen werden, da letzteres eine höhere Pau5p Adsorptionskapazität besitzt. Zusammenfassend

liefert diese Studie neue Einsichten in die Faktoren, welche die Produktion des Gushing-reduzierenden Proteins Pau5p durch *S. cerevisiae* beeinflussen. Die gewonnen Informationen sind sehr wertvoll für Sekt-herstellende Industrie, weil sie die Berücksichtigung von Aspekten der Hefestammwahl und des Pau5p Bildungs- und Prozessverhaltens in der Prozessoptimierung ermöglichen und so kommerzielle und imagebezogene Gushing-Schäden vermeiden helfen.

7 References

Abramova, N., Sertil, O., Mehta, S., & Lowry, C. V. (2001). Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. *Journal of bacteriology, 183*(9), 2881-2887.

Achaerandio, I., Pachova, V., Güell, C., & López, F. (2001). Protein adsorption by bentonite in a white wine model solution: Effect of protein molecular weight and ethanol concentration. *American journal of enology and viticulture, 52*(2), 122. Retrieved from http://www.ajevonline.org/content/52/2/122.abstract

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., . . . Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nature Biotechnology, 37*(4), 420-423. doi:10.1038/s41587-019-0036-z

Amaha, M., & Horiuchi, T. (1979). Das Biergushing und seine Ursache. *Nihon Jozo Kyokai-shi, 74*, 805-812.

Amaha, M., & Kitabatake, K. (1981). Gushing in beer. *Brewing science, 2*(1), 457-489.

Amaha, M., Kitabatake, K., Nakagawa, A., Yoshida, J., & Harada, T. (1973). *Gushing inducers produced by some mould strains.* Paper presented at the Proceedings of the European Brewery Convention Congress, Salzburg, Elsevier: Amsterdam.

Arntz, H. (1997). *Deutsches Sektlexikon* (Zweite, neubearbeitet Auflage ed.): Verband Deutscher Sektkellereien, Gesellschaft für Geschichte des Weines e.V. (GGW).

Bach, H. P. (2001). Das Wildwerden oder Überschäumen von Sekt. *Der Deutsche Weinbau*(5), 18-23.

Bach, H. P., Görtges, S., Burger, K., Schneider, R., & Portugall, L. (2001). Das Wildwerden oder Überschäumen (Gushing) des Sektes. *Der Deutsche Weinbau, 23*(16-17), 36-41.

Bach, H. P., Troost, G., & Rhein, O. H. (2010). *Sekt, Schaumwein, Perlwein* (3 ed.): Ulmer.

Baghban, R., Farajnia, S., Ghasemi, Y., Mortazavi, M., Zarghami, N., & Samadi, N. (2018). New developments in *Pichia pastoris* expression system, review and update. *Current pharmaceutical biotechnology, 19*(6), 451-467.

Barford, J. P., & Hall, R. J. (1979). An examination of the Crabtree effect in *Saccharomyces cerevisiae*: the role of respiratory adaptation. *Microbiology, 114*(2), 267-275.

Bauer, R., & Dicks, L. M. (2004). Control of malolactic fermentation in wine. A review. *South African Journal of Enology and Viticulture, 25*(2), 74-88.

Baumgartner, D., Bill, R., & Roth, I. (2001). Traubenmostanalyse mit Hilfe der FTIR-Spektroskopie. *Schweizer Zeitschrift für Obst-und Weinbau, 2*, 46-48.

Bayry, J., Aimanianda, V., Guijarro, J. I., Sunde, M., & Latgé, J.-P. (2012). Hydrophobins - unique fungal proteins. *PLOS Pathogens, 8*(5), e1002700. doi:10.1371/journal.ppat.1002700

Beattie, G. B. (1951). British views on the fobbing and gushing beer problem. *Wallerstein Laboratories Communications, 14*, 81-99.

Beyer, H. M., Gonschorek, P., Samodelov, S. L., Meier, M., Weber, W., & Zurbriggen, M. D. (2015). AQUA cloning: A versatile and simple enzyme-free cloning approach. *PLoS One, 10*(9), e0137652. doi:10.1371/journal.pone.0137652

Bilinski, T., Bylak, A., & Zadrag-Tecza, R. (2017). The budding yeast *Saccharomyces cerevisiae* as a model organism: possible implications for gerontological studies. *Biogerontology, 18*(4), 631-640. doi:10.1007/s10522-017-9712-x

Bioland-Verarbeitungsrichtlinien - Wein und Sekt vom 19.03.2019, (2019).

Bird, A. J., Gordon, M., Eide, D. J., & Winge, D. R. (2006). Repression of *ADH1* and *ADH3* during zinc deficiency by Zap1-induced intergenic RNA transcripts. *The EMBO Journal, 25*(24), 5726-5734. doi:https://doi.org/10.1038/sj.emboj.7601453

Blade, W. H., & Boulton, R. (1988). Adsorption of protein by bentonite in a model wine solution. *American journal of enology and viticulture, 39*(3), 193. Retrieved from http://www.ajevonline.org/content/39/3/193.abstract

Blasco, L., Viñas, M., & Villa, T. G. (2011). Proteins influencing foam formation in wine and beer: the role of yeast. *International Microbiology, 14*(2), 61-71. doi:10.2436/20.1501.01.136

Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis, 8*(2), 93-99.

Bowers, P. G., Hofstetter, C., Le Ngo, H., & Toomey, R. T. (1999). Temperature dependence of bubble nucleation limits for aqueous solutions of carbon dioxide, hydrogen, and oxygen. *Journal of colloid and interface science, 215*(2), 441-442.

Brenner, M. W. (1957a). *Gushing Beer II. Causes and some means of prevention.* Paper presented at the Proceedings EBC Congress, Copenhagen.

Brenner, M. W. (1957b). *Gushing beer. I. Nature, measurement, and prevalence.* Paper presented at the Proceedings. Annual meeting-American Society of Brewing Chemists.

Burger, M., & Becker, K. (1949). *Oxalate studies on beer.* Paper presented at the Proceedings. Annual meeting-American Society of Brewing Chemists.

Burger, M., Glenister, P. R., & Becker, K. (1956). *Oxalate studies in beer. II. Supplementary work and observations on calcium-oxalate relationships.* Paper presented at the Proceedings. Annual meeting-American Society of Brewing Chemists.

Buxaderas, S., & López-Tamames, E. (2012). Chapter 1 - Sparkling wines: Features and trends from tradition. In J. Henry (Ed.), *Advances in Food and Nutrition Research* (Vol. 66, pp. 1-45): Academic Press.

Caridi, A. (2006). Enological functions of parietal yeast mannoproteins. *Antonie van Leeuwenhoek, 89*(3), 417-422. doi:10.1007/s10482-005-9050-x

Carrau, F., Medina, K., Fariña, L., Boido, E., & Dellacassa, E. (2010). Effect of *Saccharomyces cerevisiae* inoculum size on wine fermentation aroma compounds and its relation with assimilable nitrogen content. *International Journal of Food Microbiology, 143*(1), 81-85. doi:https://doi.org/10.1016/j.ijfoodmicro.2010.07.024

Carrington, R., Collett, R. C., Dunkin, I. R., & Halek, G. (1972). Gushing promoters and suppressants in beer and hops. *Journal of the Institute of Brewing, 78*(3), 243-254.

Cilindre, C., Castro, A. J., Clément, C., Jeandet, P., & Marchal, R. (2007). Influence of *Botrytis cinerea* infection on Champagne wine proteins (characterized by two-dimensional electrophoresis/immunodetection) and wine foaming properties. *Food Chemistry, 103*(1), 139-149.

Cosme, F., Fernandes, C., Ribeiro, T., Filipe-Ribeiro, L., & Nunes, F. M. (2020). White wine protein instability: Mechanism, quality control and technological alternatives for wine stabilisation—an overview. *Beverages, 6*(1), 19.

Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumours. *Biochemical Journal, 23*(3), 536-545. doi:10.1042/bj0230536

Cregg, J. M. (1999). Expression in the methylotrophic yeast *Pichia pastoris*. *Gene expression systems*, 157-191.

Cregg, J. M., Tolstorukov, I., Kusari, A., Sunga, J., Madden, K., & Chappell, T. (2009). Expression in the yeast *Pichia pastoris*. *Methods in enzymology, 463*, 169-189.

Curtis, N. S., & Martindale, L. (1961). Studies on gushing I. Introduction. *Journal of the Institute of Brewing, 67*(5), 417-421.

D'Amato, D., Corbo, M. R., Nobile, M. A. D., & Sinigaglia, M. (2006). Effects of temperature, ammonium and glucose concentrations on yeast growth in a model wine system. *International journal of food science & technology, 41*(10), 1152-1157.

Dachs, E., & Nitschke, R. (1977). Fallstudie zum Problem “Gushing”. *Brauwelt, 117*(5), 129-131.

Dashko, S., Zhou, N., Compagno, C., & Piškur, J. (2014). Why, when, and how did yeast evolve alcoholic fermentation? *FEMS yeast research, 14*(6), 826-832. doi:10.1111/1567-1364.12161

Deckers, S. M., Gebruers, K., Baggerman, G., Lorgouilloux, Y., Delcour, J. A., Michiels, C., . . . Neven, H. (2010). CO2-hydrophobin structures acting as nano-bombs in beer. *Brewing science, 63*, 54-61.

Deckers, S. M., Vissers, L., Khalesi, M., Shokribousjein, Z., Verachtert, H., Gebruers, K., . . . Titze, J. (2013). Thermodynamic view of primary gushing. *Journal of the American Society of Brewing Chemists, 71*(3), 149-152.

Di Gianvito, P., Tesnière, C., Suzzi, G., Blondin, B., & Tofalo, R. (2018). Different genetic responses to oenological conditions between a flocculent wine yeast and its FLO5 deleted strain: Insights from the transcriptome. *Food research international, 114*, 178-186. doi:https://doi.org/10.1016/j.foodres.2018.07.061

Domizio, P., Liu, Y., Bisson, L. F., & Barile, D. (2014). Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiology, 43*, 5-15.

Duina, A. A., Miller, M. E., & Keeney, J. B. (2014). Budding yeast for budding geneticists: a primer on the *Saccharomyces cerevisiae* model system. *Genetics, 197*(1), 33-48.

Dukes, B. (2010). How do I measure yeast assimilable nitrogen (YAN)? In C. E. Butzke (Ed.), *Winemaking problems solved. Woodhead Publishing Series in Food Science, Technology and Nutrition.* (Vol. 193, pp. 65): Woodhead Publishing Limited und CRC Press LLC.

Dukes, B., & Butzke, C. E. (1998). Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. *American journal of enology and viticulture, 49*(2), 125-134. Retrieved from http://europepmc.org/abstract/AGR/IND21975056

Dupin, I. V. S., McKinnon, B. M., Ryan, C., Boulay, M., Markides, A. J., Jones, G. P., . . . Waters, E. J. (2000). *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: Their

release during fermentation and lees contact and a proposal for their mechanism of action. *Journal of Agricultural and Food Chemistry, 48*(8), 3098-3105. doi:10.1021/jf0002443
Edge, A. S. B. (2003). Deglycosylation of glycoproteins with trifluoromethanesulphonic acid: Elucidation of molecular structure and function. *Biochemical Journal, 376*(2), 339-350.
Eelderink-Chen, Z., Mazzotta, G., Sturre, M., Bosman, J., Roenneberg, T., & Merrow, M. (2010). A circadian clock in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, 107*(5), 2043-2047. doi:10.1073/pnas.0907902107
European Union. (2008). Official Journal of the European Union, L 148, 06 June 2008. 47-49.
European Union. (2019). *Official Journal of the European Union, L 149/1, 07 June 2019*.
Fabrizio, P., & Longo, V. D. (2003). The chronological life span of *Saccharomyces cerevisiae*. *Aging cell, 2*(2), 73-81.
Fleet, G. H. (2007). Wine. In *Food Microbiology: Fundamentals and Frontiers, Third Edition*: American Society of Microbiology.
Fleet, G. H. (2008). Wine yeasts for the future. *FEMS yeast research, 8*(7), 979-995. doi:10.1111/j.1567-1364.2008.00427.x
Frisch, L. M., Mann, M. A., Marek, D. N., Baudrexl, M., Vogel, R. F., & Niessen, L. (2021). Studies on the gushing potential of *Penicillium expansum*. *Food research international, 139*, 109915.
Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., . . . Verstrepen, K. J. (2016). Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. *Cell, 166*(6), 1397-1410.e1316. doi:https://doi.org/10.1016/j.cell.2016.08.020
Gardner, R. J. (1972). Surface viscosity and gushing. *Journal of the Institute of Brewing, 78*(5), 391-399.
Gardner, R. J., Laws, D. R. J., & McGuinness, J. D. (1973). The suppression of gushing by the use of hop oil. *Journal of the Institute of Brewing, 79*(3), 209-211.
Gastl, M., Zarnkow, M., & Back, W. (2008). Gushing – ein multikausales Problem! *Brauwelt, 148*(32), 896-899.
Gava, A., Borsato, D., & Ficagna, E. (2020). Effect of mixture of fining agents on the fermentation kinetics of base wine for sparkling wine production: Use of methodology for modeling. *LWT, 131*, 109660. doi:https://doi.org/10.1016/j.lwt.2020.109660
Gietz, R. D., & Schiestl, R. H. (2007). Frozen competent yeast cells that can be transformed with high efficiency using the LiAc/SS carrier DNA/PEG method. *Nat Protoc, 2*(1), 1-4. doi:10.1038/nprot.2007.17
Gjertsen, P. (1967). Gushing in beer: its nature, cause and prevention. *Brew. Dig, 42*(5), 80-84.
Gjertsen, P., Trolle, B., & Andersen, K. (1963). *Weathered barley as a contributory cause of gushing in beer.* Paper presented at the Proceedings of the European Brewery Convention Congress, Brussels, Elsevier: Amsterdam.
Gnoinski, G. B., Schmidt, S. A., Close, D. C., Goemann, K., Pinfold, T. L., & Kerslake, F. L. (2021). Novel methods to manipulate autolysis in sparkling wine: Effects on yeast. *Molecules, 26*(2), 387.
Goffeau, A., Barrell, B. G., Bussey, H., Davis, R., Dujon, B., Feldmann, H., . . . Johnston, M. (1996). Life with 6000 genes. *Science, 274*(5287), 546-567.
González-Ramos, D., Muñoz, A., Ortiz-Julien, A., Palacios, A. T., Heras, J. M., & González, R. (2010). A *Saccharomyces cerevisiae* wine yeast strain overproducing mannoproteins selected through classical genetic methods. *Oeno One, 44*(4), 243-249.
Gonzalez, R., Martínez-Rodriguez, A. J., & Carrascosa, A. V. (2003). Yeast autolytic mutants potentially useful for sparkling wine production. *International Journal of Food Microbiology, 84*(1), 21-26.
Goto, M. (2007). Protein *O*-Glycosylation in fungi: Diverse structures and multiple functions. *Bioscience, Biotechnology, and Biochemistry, 71*(6), 1415-1427. doi:10.1271/bbb.70080
Gray, P. P., & Stone, I. (1956). Metal-induced wildness in beers. *Proceedings. Annual meeting - American Society of Brewing Chemists, 14*(1), 83-108. doi:10.1080/00960845.1956.12006480
Gray, P. P., & Stone, I. (1957). Cobalt and wilderness in beer. *Journal of the Institute of Brewing, 63*(6), 512-516.
Gray, P. P., & Stone, I. (1960). Trace metal chelation in beer with EDTA. *Proceedings. Annual meeting - American Society of Brewing Chemists, 18*(1), 166-174. doi:10.1080/00960845.1960.12006915
Guggenberger, J., & Kleber, W. (1963). Über den Mechanismus des Wildwerdens von Bier. *Proc. Eur. Brew. Conv. Congr., Brussels, Elsevier Scientific: Amsterdam*, 299-319.
Gyllang, H., & Martinson, E. (1976). *Aspergillus fumigatus* and *Aspergillus amstelodami* as causes of gushing. *Journal of the Institute of Brewing, 82*(3), 182-183.

Habschied, K., Krstanović, V., Velić, N., Šantek, B., Novak, M., & Slačanac, V. (2014). Gushing potential of wheat malt infected with *Fusarium culmorum*. *Journal of Hygienic Engineering and Design, 6*, 166-170.

Hanly, W. C., Artwohl, J. E., & Bennett, B. T. (1995). Review of polyclonal antibody production procedures in mammals and poultry. *ILAR Journal, 37*(3), 93-118. doi:10.1093/ilar.37.3.93

He, M., Wu, D., Wu, J., & Chen, J. (2014). Enhanced expression of endoinulinase from *Aspergillus niger* by codon optimization in *Pichia pastoris* and its application in inulooligosaccharide production. *Journal of Industrial Microbiology and Biotechnology, 41*(1), 105-114.

Heinisch, J. J., & Rodicio, R. (2017). Stress responses in wine yeast. In *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 377-395): Springer.

Henning, K. (1963). Über das Heraussprudeln von Schaumwein beim Öffnen der Flaschen. *Weinberg und Keller*(10), 103 - 107.

Higgins, D. R. (1995). Overview of Protein Expression in *Pichia pastoris*. *Current Protocols in Protein Science, 2*(1), 5.7.1-5.7.18. doi:https://doi.org/10.1002/0471140864.ps0507s02

Hippeli, S., & Elstner, E. F. (2002). Are hydrophobins and/or non-specific lipid transfer proteins responsible for gushing in beer? New hypotheses on the chemical nature of gushing inducing factors. *Zeitschrift für Naturforschung, 57 c*(1-2), 1-8.

Hippeli, S., & Hecht, D. (2008). Die Rolle von ns-LTP1 und Proteasen bei der Entstehung des primären Gushing. *Brauwelt, 32*, 900-904.

Hohmann, S. (1996). Pyruvate decarboxylases. In F. K. Zimmerman & K. D. Entian (Eds.), *Yeast sugar metabolism: Biochemistry, genetics, biotechnology, and applications* (pp. 187–212): Boca Raton: CRC Press.

Hohmann, S. (2009). Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. *FEBS Letters, 583*(24), 4025-4029. doi:https://doi.org/10.1016/j.febslet.2009.10.069

Hu, H., Gao, J., He, J., Yu, B., Zheng, P., Huang, Z., . . . Chen, D. (2013). Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. *PLoS One, 8*(3), e58393.

Huang, X., Barchi, J. J., Lung, F.-D. T., Roller, P. P., Nara, P. L., Muschik, J., & Garrity, R. R. (1997). Glycosylation affects both the three-dimensional structure and antibody binding properties of the HIV-1IIIB GP120 peptide RP135. *Biochemistry, 36*(36), 10846-10856. doi:10.1021/bi9703655

Hudson, J. R. (1962). Some observations on the treatment of gushing beers with nylon powder. *Journal of the Institute of Brewing, 68*(6), 460-466.

Ivit, N. N., & Kemp, B. (2018). The impact of non-*Saccharomyces* yeast on traditional method sparkling wine. *Fermentation, 4*(3), 73.

Jaeckels, N., Tenzer, S., Meier, M., Will, F., Dietrich, H., Decker, H., & Fronk, P. (2017). Influence of bentonite fining on protein composition in wine. *LWT, 75*, 335-343. doi:https://doi.org/10.1016/j.lwt.2016.08.062

Jährig, A., & Schade, W. (1993). *Mikrobiologie der Gärungs-und Getränkeindustrie - Eine Einführung*: CENA-Verlag.

Jentoft, N. (1990). Why are proteins *O*-glycosylated? *Trends in biochemical sciences, 15*(8), 291-294.

Jigami, Y. (2008). Yeast glycobiology and its application. *Bioscience, Biotechnology, and Biochemistry, 72*(3), 637-648.

Jolly, N. P., Varela, C., & Pretorius, I. S. (2014). Not your ordinary yeast: non-Saccharomyces yeasts in wine production uncovered. *FEMS yeast research, 14*(2), 215-237. doi:10.1111/1567-1364.12111

Juturu, V., & Wu, J. C. (2017). Heterologous protein expression in *Pichia pastoris*: latest research progress and applications.

Karathia, H., Vilaprinyo, E., Sorribas, A., & Alves, R. (2011). *Saccharomyces cerevisiae* as a model organism: A comparative study. *PLoS One, 6*(2), e16015. doi:10.1371/journal.pone.0016015

Kieninger, H. (1976). Gushing des Flaschenbieres - derzeitiger Forschungsstand. *Brauwelt, 116*(48), 1600-1603.

Kitabatake, K., & Amaha, M. (1974). Production of the gushing factor by a *Nigrospora* sp. in liquid culture media. *Bulletin of Brewing Science, 20*, 1-8.

Kitabatake, K., & Amaha, M. (1976). Properties of the two gushing inducing factors of *Nigrospora* sp. no. 207 produced on barley grains. *Bulletin of brewing science*, 9-19.

Kleemola, T., Nakari-Setälä, T., Linder, M., Penttilä, M., Kotaviita, E., Olkku, J., & Haikara, A. (2001). *Characterization and detection of the gushing factors produced by fungi.* Paper presented at the Proceedings of the 28th EBC congress, Budapest 2001.

Kondo, K., & Inouye, M. (1991). TIP1, a cold shock-inducible gene of *Saccharomyces cerevisiae*. *J Biol Chem, 266*(26), 17537-17544.

Kukuruzinska, M. A., Bergh, M. L. E., & Jackson, B. J. (1987). Protein glycosylation in yeast. *Annual review of biochemistry, 56*(1), 915-944.

Kupfer, V. M. (2018). *The influence of plant-and yeast-derived proteins on gushing in sparkling wine.* Technische Universität München,

Kupfer, V. M., Vogt, E. I., Siebert, A. K., Meyer, M. L., Vogel, R. F., & Niessen, L. (2017a). Foam-stabilizing properties of the yeast protein PAU5 and evaluation of factors that can influence its concentration in must and wine. *Food research international, 102*, 111-118.

Kupfer, V. M., Vogt, E. I., Ziegler, T., Vogel, R. F., & Niessen, L. (2017b). Comparative protein profile analysis of wines made from *Botrytis cinerea* infected and healthy grapes reveals a novel biomarker for gushing in sparkling wine. *Food research international, 99*, 501-509.

Laitila, A., Sarlin, T., Kotaviita, E., Huttunen, T., Home, S., & Wilhelmson, A. (2007). Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors. *Journal of Industrial Microbiology and Biotechnology, 34*(11), 701-713.

Linck, A., Vu, X.-K., Essl, C., Hiesl, C., Boles, E., & Oreb, M. (2014). On the role of GAPDH isoenzymes during pentose fermentation in engineered *Saccharomyces cerevisiae*. *FEMS yeast research, 14*(3), 389-398. doi:https://doi.org/10.1111/1567-1364.12137

Linder, M. B. (2009). Hydrophobins: proteins that self assemble at interfaces. *Current Opinion in Colloid & Interface Science, 5*(14), 356-363.

Lisowska, E. (2002). The role of glycosylation in protein antigenic properties. *Cellular and Molecular Life Sciences CMLS, 59*(3), 445-455. doi:10.1007/s00018-002-8437-3

Lochbühler, B., Manteau, S., Morge, C., Caillet, M.-M., Charpentier, C., Schnell, S., . . . Rauhut, D. (2015). Yeast protein extracts: an alternative fining agent for red wines. *European Food Research and Technology, 240*(4), 689-699. doi:10.1007/s00217-014-2373-y

Lombardi, S. J., De Leonardis, A., Lustrato, G., Testa, B., & Iorizzo, M. (2015). Yeast autolysis in sparkling wine aging: Use of killer and sensitive *Saccharomyces cerevisiae* strains in co-culture. *Recent Pat Biotechnol, 9*(3), 223-230. doi:10.2174/1872208310666160414102015

Lommel, M., & Strahl, S. (2009). Protein *O*-mannosylation: conserved from bacteria to humans. *Glycobiology, 19*(8), 816-828.

Lonvaud-Funel, A. (1995). Microbiology of the malolactic fermentation: Molecular aspects. *FEMS Microbiology Letters, 126*(3), 209-214. doi:10.1111/j.1574-6968.1995.tb07420.x

Luo, Z., & van Vuuren, H. J. (2008). Stress-induced production, processing and stability of a seripauperin protein, Pau5p, in *Saccharomyces cerevisiae*. *FEMS yeast research, 8*(3), 374-385.

Luo, Z., & van Vuuren, H. J. (2009). Functional analyses of PAU genes in *Saccharomyces cerevisiae*. *Microbiology, 155*(12), 4036-4049.

Lusk, L. T. (2016). Controlling beer foam and gushing. In *Brewing Materials and Processes* (pp. 175-198): Elsevier.

Lutterschmid, G., Muranyi, M., Stübner, M., Vogel, R. F., & Niessen, L. (2011). Heterologous expression of surface-active proteins from barley and filamentous fungi in *Pichia pastoris* and characterization of their contribution to beer gushing. *International Journal of Food Microbiology, 147*, 17-25.

Lutterschmid, G., Stübner, M., Vogel, R. F., & Niessen, L. (2010). Induction of gushing with recombinant Class II hydrophobin FcHyd5p from *Fusarium culmorum* and the impact of hop compounds on its gushing potential. *Journal of the Institute of Brewing, 116*(4), 339-347. doi:https://doi.org/10.1002/j.2050-0416.2010.tb00784.x

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., & Harvey, L. M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast, 22*(4), 249-270.

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., . . . Lopez, R. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research, 47*(W1), W636-W641. doi:10.1093/nar/gkz268

Marangon, M., Vincenzi, S., & Curioni, A. (2019). Wine fining with plant proteins. *Molecules, 24*(11), 2186.

Marchal, R., & Jeandet, P. (2009). Chapter 5 - Use of enological additives for colloid and tartrate salt stabilization in white wines and for improvement of sparkling wine foaming properties. In M. V. Moreno-Arribas & M. C. Polo (Eds.), *Wine chemistry and biochemistry* (Vol. 735). New York, NY, USA: Springer

Marchal, R., Salmon, T., Gonzalez, R., Kemp, B., Vrigneau, C., Williams, P., & Doco, T. (2020). Impact of *Botrytis cinerea* contamination on the characteristics and foamability of yeast macromolecules released during the alcoholic fermentation of a model grape juice. *Molecules, 25*(3), 472. doi:10.3390/molecules25030472

Marchal, R., Tabary, I., Valade, M., Moncomble, D., Viaux, L., Robillard, B., & Jeandet, P. (2001). Effects of *Botrytis cinerea* infection on Champagne wine foaming properties. *Journal of the Science of Food and Agriculture, 81*(14), 1371-1378. doi:10.1002/jsfa.941

Martí-Raga, M., Marullo, P., Beltran, G., & Mas, A. (2016). Nitrogen modulation of yeast fitness and viability during sparkling wine production. *Food Microbiology, 54*, 106-114.

Martí-Raga, M., Sancho, M., Guillamón, J. M., Mas, A., & Beltran, G. (2015). The effect of nitrogen addition on the fermentative performance during sparkling wine production. *Food research international, 67*, 126-135. doi:https://doi.org/10.1016/j.foodres.2014.10.033

Martí-Raga, M., Martín, V., Gil, M., Sancho, M., Zamora, F., Mas, A., & Beltran, G. (2016). Contribution of yeast and base wine supplementation to sparkling wine composition. *Journal of the Science of Food and Agriculture, 96*(15), 4962-4972.

Martínez-Moreno, R., Quirós, M., Morales, P., & Gonzalez, R. (2014). New insights into the advantages of ammonium as a winemaking nutrient. *International Journal of Food Microbiology, 177*, 128-135. doi:https://doi.org/10.1016/j.ijfoodmicro.2014.02.020

Martínez-Rodríguez, A. J., & Pueyo, E. (2009). Sparkling wines and yeast autolysis. In *Wine chemistry and biochemistry* (pp. 61-80): Springer.

Martínez, J. M., Cebrián, G., Álvarez, I., & Raso, J. (2016). Release of mannoproteins during *Saccharomyces cerevisiae* autolysis induced by pulsed electric field. *Frontiers in Microbiology, 7*(1435). doi:10.3389/fmicb.2016.01435

Masneuf-Pomarede, I., Bely, M., Marullo, P., & Albertin, W. (2016). The genetics of non-conventional wine yeasts: Current knowledge and future challenges. *Frontiers in Microbiology, 6*(1563). doi:10.3389/fmicb.2015.01563

Mateo, J. J., Jiménez, M., Pastor, A., & Huerta, T. (2001). Yeast starter cultures affecting wine fermentation and volatiles. *Food research international, 34*(4), 307-314. doi:https://doi.org/10.1016/S0963-9969(00)00168-X

Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2012). Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *International Journal of Food Microbiology, 157*(2), 245-250.

Mendes-Ferreira, A., Mendes-Faia, A., & Leão, C. (2004). Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. *Journal of applied microbiology, 97*(3), 540-545. doi:https://doi.org/10.1111/j.1365-2672.2004.02331.x

Menezes, R., Tenreiro, S., Macedo, D., Santos, C. N., & Outeiro, T. F. (2015). From the baker to the bedside: yeast models of Parkinson's disease. *Microbial cell, 2*(8), 262.

Müller, M. P., Schmid, F., Becker, T., & Gastl, M. (2010). Impact of different hop compounds on the overfoaming volume of beer caused by primary gushing. *Journal of the Institute of Brewing, 116*(4), 459-463.

Müller, V., Besier, A., & Fröhlich, J. (2013). Überschäumen vermindern. *Brauindustrie, 1*, 18-21.

Murtey, M. D., & Ramasamy, P. (2016). Sample preparations for scanning electron microscopy – life sciences. In *Modern electron microscopy in physical and life sciences*: IntechOpen.

Narziß, L., Back, W., Reicheneder, E., Simon, A., & Grandl, R. (1990). Untersuchungen zum Gushing-Problem. *Monatsschrift für Brauwissenschaft, 9*, 296-305.

Nielsen, J. (2019). Yeast systems biology: model organism and cell factory. *Biotechnology journal, 14*(9), 1800421.

Niessen, L., Donhauser, S., Weideneder, A., Geiger, E., & Vogel, H. (1992). Mykologische Untersuchungen an Cerealien und Malzen im Zusammenhang mit dem Wildwerden (Gushing) des Bieres. *Brauwelt, 16/17*, 702-714.

Northeast Winemaking. (2021). Retrieved from https://northeastwinemaking.com/pages/helpful-charts-and-graphs

Öberg, F., Sjöhamn, J., Conner, M. T., Bill, R. M., & Hedfalk, K. (2011). Improving recombinant eukaryotic membrane protein yields in *Pichia pastoris*: the importance of codon optimization and clone selection. *Molecular membrane biology, 28*(6), 398-411.

Oldenburg, K. R., Vo, K. T., Michaelis, S., & Paddon, C. (1997). Recombination-mediated PCR-directed plasmid construction *in vivo* in yeast. *Nucleic Acids Research, 25*(2), 451-452. doi:10.1093/nar/25.2.451

Olmedo, M., Navarro-Sampedro, L., Ruger-Herreros, C., Kim, S.-R., Jeong, B.-K., Lee, B.-U., & Corrochano, L. M. (2010). A role in the regulation of transcription by light for RCO-1 and RCM-1, the *Neurospora* homologs of the yeast Tup1–Ssn6 repressor. *Fungal Genetics and Biology, 47*(11), 939-952. doi:https://doi.org/10.1016/j.fgb.2010.08.001

Parapouli, M., Vasileiadis, A., Afendra, A.-S., & Hatziloukas, E. (2020). *Saccharomyces cerevisiae* and its industrial applications. *AIMS microbiology, 6*(1), 1-31. doi:10.3934/microbiol.2020001

Patz, C.-D., David, A., Thente, K., Kürbel, P., & Dietrich, H. (1999). Wine analysis with FTIR spectrometry. *DWW. Die Wein-Wissenschaft, 54*(2-3), 80-87.

Pérez-Serradilla, J. A., & de Castro, M. D. L. (2008). Role of lees in wine production: A review. *Food Chemistry, 111*(2), 447-456. doi:https://doi.org/10.1016/j.foodchem.2008.04.019

Pérez-Través, L., Querol, A., & Pérez-Torrado, R. (2016). Increased mannoprotein content in wines produced by *Saccharomyces kudriavzevii* × *Saccharomyces cerevisiae* hybrids. *International Journal of Food Microbiology, 237*, 35-38.

Postulkova, M., Riveros-Galan, D., Cordova-Agiular, K., Zitkova, K., Verachtert, H., Derdelinckx, G., . . . Branyik, T. (2016). Technological possibilities to prevent and suppress primary gushing of beer. *Trends in Food Science & Technology, 49*, 64-73.

Pozo-Bayón, M. Á., Andújar-Ortiz, I., & Moreno-Arribas, M. V. (2009). Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. *Food research international, 42*(7), 754-761.

Puig-Deu, M., López-Tamames, E., Buxaderas, S., & Torre-Boronat, M. C. (1999). Quality of base and sparkling wines as influenced by the type of fining agent added pre-fermentation. *Food Chemistry, 66*(1), 35-42.

Quirós, M., Gonzalez-Ramos, D., Tabera, L., & Gonzalez, R. (2010). A new methodology to obtain wine yeast strains overproducing mannoproteins. *International Journal of Food Microbiology, 139*(1), 9-14. doi:https://doi.org/10.1016/j.ijfoodmicro.2010.02.014

Rachidi, N., Barre, P., & Blondin, B. (2000a). Examination of the transcriptional specificity of an enological yeast. A pilot experiment on the chromosome-III right arm. *Current Genetics, 37*(1), 1-11. doi:10.1007/s002940050001

Rachidi, N., Martinez, M. J., Barre, P., & Blondin, B. (2000b). *Saccharomyces cerevisiae* PAU genes are induced by anaerobiosis. *Molecular microbiology, 35*(6), 1421-1430.

Rankine, B. (1977). Gushing in sparkling wine. *Australian Grapegrower and Winemaker*, 26.

Rivero, D., Berná, L., Stefanini, I., Baruffini, E., Bergerat, A., Csikász-Nagy, A., . . . Cavalieri, D. (2015). Hsp12p and PAU genes are involved in ecological interactions between natural yeast strains. *Environ Microbiol, 17*(8), 3069-3081. doi:10.1111/1462-2920.12950

Riveros G., D., Shokribousjein, Z., Losada-Pérez, P., Rheza Khalesi, M., Cordova, K., Michiels, C., . . . Derdelinckx, G. (2015). Comparison of structure, sequence, physical interactions and its effects on primary gushing among several class II hydrophobins. *Brewing science, 68*, 38-45.

Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.-M., . . . Mouret, J.-R. (2015). Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. *Applied microbiology and biotechnology, 99*(5), 2291-2304.

Romano, P., Ciani, M., & Fleet, G. H. (2019). *Yeasts in the production of wine*: Springer.

Romanos, M. (1995). Advances in the use of *Pichia pastoris* for high-level gene expression. *Current Opinion in biotechnology, 6*(5), 527-533.

Rossignol, T., Dulau, L., Julien, A., & Blondin, B. (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast, 20*(16), 1369-1385. doi:10.1002/yea.1046

Rudin, A. D., & Hudson, J. R. (1958). Significance of isohumulone and certain metals in gushing beers. *Journal of the Institute of Brewing, 64*(4), 317-318.

Sablayrolles, J.-M. (2009). Control of alcoholic fermentation in winemaking: Current situation and prospect. *Food research international, 42*(4), 418-424. doi:https://doi.org/10.1016/j.foodres.2008.12.016

Sablayrolles, J.-M., Dubois, C., Manginot, C., Roustan, J.-L., & Barre, P. (1996). Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck wine fermentations. *Journal of fermentation and bioengineering, 82*(4), 377-381.

Salazar, F. N., Zamora, F., Canals, J. M., & López, F. (2010). Protein stabilization in sparkling base wine using zirconia and bentonite: Influence on the foam parameters and protein fractions. *Journal International Des Sciences De La Vigne Et Du Vin, 44*(Special Issue Macrowine), 51-58.

Sandra, P., Claus, H., & Verzele, M. (1973). Fatty acids in isomerized hop extracts and gushing. *Journal of the Institute of Brewing, 79*(2), 142-147.

Sarlin, T., Kivioja, T., Kalkkinen, N., Linder, M. B., & Nakari-Setälä, T. (2012). Identification and characterization of gushing-active hydrophobins from *Fusarium graminearum* and related species. *Journal of basic microbiology, 52*(2), 184-194.

Sarlin, T., Nakari-Setälä, T., Linder, M., Penttilä, M., & Haikara, A. (2005). Fungal hydrophobins as predictors of the gushing activity of malt. *Journal of the Institute of Brewing, 111*(2), 105-111.

Schägger, H. (2006). Tricine–SDS-PAGE. *Nature protocols, 1*(1), 16.

Schanderl, H. (1964). Sogenannte Überschäumer beim Sekt. *Das Weinblatt, 58*, 361.

Schildbach, R. (1987). Auswuchs der Gerste und Überschäumen des Bieres. *Brauwelt, 36*, 1559-1567.

Schumacher, T. (2002). Gushing in Fruchtsaftschorlen. *Flussiges Obst, 69*(5), 304-310.

Schur, F., Anderegg, P., Senften, H., & Pfenninger, H. (1980). Brautechnologische Bedeutung von Oxalat. *Brauerei-Rundschau, 91*, 201-220.

Shokribousjein, Z., Philippaerts, A., Riveros, D., Titze, J., Ford, Y., Deckers, S. M., . . . Verachtert, H. (2014). A curative method for primary gushing of beer and carbonated beverages: Characterization and application of antifoam based on hop oils. *Journal of the American Society of Brewing Chemists, 72*(1), 12-21.

Sloey, W., & Prentice, N. (1962). *Effects of Fusarium isolates applied during malting on properties of malt.* Paper presented at the Proceedings. Annual meeting-American Society of Brewing Chemists.

Smits, G. J., van den Ende, H., & Klis, F. M. (2001). Differential regulation of cell wall biogenesis during growth and development in yeast. *Microbiology, 147*(4), 781-794. doi:https://doi.org/10.1099/00221287-147-4-781

Specker, C., Niessen, L., & Vogel, R. F. (2014). In vitro studies on the main beer protein Z4 of *Hordeum vulgare* concerning heat stability, protease inhibition and gushing. *Journal of the Institute of Brewing, 120*(2), 85-92.

Sreekrishna, K., Brankamp, R. G., Kropp, K. E., Blankenship, D. T., Tsay, J.-T., Smith, P. L., . . . Birkenberger, L. A. (1997). Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene, 190*(1), 55-62.

Stanislava, G. (2010). A Review: The role of barley seed pathogenesis-related proteins (PRs) in beer production. *Journal of the Institute of Brewing, 116*(2), 111-124.

Steidl, R. (2004). *Schönung und Stabilisierung: Zeitpunkt, Anforderungen, Maßnahmen*: Eugen Ulmer GmbH & Co.

Stone, I., & Gray, P. P. (1955). Traces of nickel in beers and brewing. *Proceedings. Annual meeting - American Society of Brewing Chemists, 13*(1), 145-158. doi:10.1080/00960845.1955.12006458

Stothard, P. (2000). The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques, 28*(6), 1102-1104.

Stübner, M., Lutterschmid, G., Vogel, R. F., & Niessen, L. (2010). Heterologous expression of the hydrophobin FcHyd5p from *Fusarium culmorum* in *Pichia pastoris* and evaluation of its surface activity and contribution to gushing of carbonated beverages. *International Journal of Food Microbiology, 141*(1), 110-115. doi:https://doi.org/10.1016/j.ijfoodmicro.2010.03.003

Taillandier, P., Portugal, F. R., Fuster, A., & Strehaiano, P. (2007). Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. *Food Microbiology, 24*(1), 95-100.

Theron, L. W., & Divol, B. (2014). Microbial aspartic proteases: current and potential applications in industry. *Applied microbiology and biotechnology, 98*(21), 8853-8868.

Thorne, R. S. W., & Helm, E. (1957). Contribution to the study of overfoaming beer. *Journal of the Institute of Brewing, 63*(5), 415-435.

Torresi, S., Frangipane, M. T., & Anelli, G. (2011). Biotechnologies in sparkling wine production. Interesting approaches for quality improvement: A review. *Food Chemistry, 129*(3), 1232-1241. doi:https://doi.org/10.1016/j.foodchem.2011.05.006

Tronchoni, J., Curiel, J. A., Morales, P., Torres-Pérez, R., & Gonzalez, R. (2017). Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. *International Journal of Food Microbiology, 241*, 60-68.

Udom, N., Chansongkrow, P., Charoensawan, V., & Auesukaree, C. (2019). Coordination of the cell wall integrity and high-osmolarity glycerol pathways in response to ethanol stress in *Saccharomyces cerevisiae*. *Applied and environmental microbiology, 85*(15).

Vejarano, R. (2020). Non-Saccharomyces in winemaking: Source of mannoproteins, nitrogen, enzymes, and antimicrobial compounds. *Fermentation, 6*(3), 76.

Viswanathan, M., Muthukumar, G., Cong, Y.-S., & Lenard, J. (1994). Seripauperins of *Saccharomyces cerevisiae:* a new multigene family encoding serine-poor relatives of serine-rich proteins. *Gene, 148*(1), 149-153.

Vogt, E. I., Kupfer, V. M., Vogel, R. F., & Niessen, L. (2016). A novel preparation technique of red (sparkling) wine for protein analysis. *EuPA open proteomics, 11*, 16-19.

Vogt, E. I., Kupfer, V. M., Vogel, R. F., & Niessen, L. (2017). Evidence of gushing induction by *Penicillium oxalicum* proteins. *Journal of applied microbiology, 122*(3), 708-718.

Waterhouse, A. L., Sacks, G. L., & Jeffery, D. W. (2016). *Understanding wine chemistry*: John Wiley & Sons.

Waters, E. J., Alexander, G., Muhlack, R., Pocock, K., Colby, C., O'neill, B., . . . Jones, P. (2005). Preventing protein haze in bottled white wine. *Australian Journal of Grape and Wine Research, 11*(2), 215-225.

Weis, R. (2019). High-throughput screening and selection of *Pichia pastoris* strains. In *Recombinant Protein Production in Yeast* (pp. 169-185): Springer.

Wershofen, T. (2004). Gushing - Ein überschäumend spritziges Erlebnis. *Brauwelt, 144*(35), 1061-1063.

Wilcox, L. J., Balderes, D. A., Wharton, B., Tinkelenberg, A. H., Rao, G., & Sturley, S. L. (2002). Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. *Journal of Biological Chemistry, 277*(36), 32466-32472. doi:10.1074/jbc.m204707200

Windisch, W. (1923a). "Wildes" Flaschenbier. *Wochenschrift für Brauerei, 40*(21), 121-123.

Windisch, W. (1923b). "Wildes" Flaschenbier. *Wochenschrift für Brauerei, 40*(12), 70-71.

Yalçın, S. K., & Özbaş, Z. Y. (2006). Production of glycerol by two endogenic wine yeast strains at different inoculum size. *Food Technology & Biotechnology, 44*(4).

Yoshida, J., Nakagawa, A., Eto, M., Kitabatake, K., & Amaha, M. (1975). Fungal flora of barley grains and the beer-gushing inducing potentials of the mold isolated. *Journal of Fermentation Technology, 53*(3), 184-188.

Zamora, F. (2009). Biochemistry of alcoholic fermentation. In *Wine chemistry and biochemistry* (pp. 3-26): Springer.

Zapf, M. W., Theisen, S., Vogel, R. F., Hecht, D., Elstner, E. F., & Niessen, L. (2005). *Characterization of surface active proteins from Fusarium and grain affecting foam stability.* Paper presented at the Proceedings of the 30th EBC Congress.

Zapf, M. W., Theisen, S., Vogel, R. F., & Niessen, L. (2006). Cloning of wheat LTP1500 and two *Fusarium culmorum* hydrophobins in *Saccharomyces cerevisiae* and assessment of their gushing inducing potential in experimental wort fermentation. *Journal of the Institute of Brewing, 112*(3), 237-245.

Zarnkow, M., & Back, W. (2001). Neue Erkenntnisse über gushingauslösende Substanzen. *Brauwelt, 141*(9/10), 363-367.

Zepf, M., & Geiger, E. (1999). Gushingproblematik durch Calciumoxalat, Teil 1. *Brauwelt, 139*(48), 2302-2304.

Zhang, Z., Moo-Young, M., & Chisti, Y. (1996). Plasmid stability in recombinant *Saccharomyces cerevisiae*. *Biotechnology advances, 14*(4), 401-435.

Zoecklein, B. (1988). *Bentonite fining of juice and wine*: Virginia Cooperative Extension Service.

Zoecklein, B. (1998). A review of méthode champenoise production. *Virginia Cooperative Extension, Virginia State University*(463-017).

Zoecklein, B., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (2013). *Wine analysis and production*: Springer Science & Business Media.

8 Abbreviations

Abbreviation	Explanation
ACN	Acetonitrile
APS	Ammonium persulfate
B. cinerea	*Botrytis cinerea*
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
DAP	Diammonium hydrogen phosphate
ddH_2O	HPLC-grade water
dH_2O	Demineralized water
DMF	N,N-Dimethylformamide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EAN	Easily-assimilable nitrogen
EDTA	Ethylenediaminetetraacetic acid
FCC	Frozen competent cells
FTIR	Fourier-transform infrared spectroscopy
GRAVY	Grand average of hydropathy
HGU	*Hochschule Geisenheim University*
HOG	High osmolarity glycerol
HPLC	High pressure liquid chromatography
kb	Kilobase
KLH	Keyhole limpet hemocyanin
M. pulcherrima	*Metchnikowia pulcherrima*
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MS	Mass spectrometry
MWCO	Molecular weight cut off
NBT	Nitro blue tetrazolium chloride
NOPA	Nitrogen by OPA
P. pastoris	*Pichia pastoris*
P20	Pressure at 20 °C
PB	Internal bottle pressure
PMSF	Phenylmethylsulfonyl flouride
PTM4	*Pichia* trace minerals 4
RC	Regenerated cellulose
RP-HPLC	Reversed phase high pressure liquid chromatography
rpm	Rounds per minute
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Temperature
T. delbrueckii	*Torulaspora delbrueckii*
TEMED	Tetramethylethylenediamine
TFA	Trifluoro acetic acid
TMW	*Technische Mikrobiologie Weihenstephan*
YAN	Yeast assimilable nitrogen

9 List of figures

10 List of tables

11 Publications and student projects

11.1 Peer-reviewed publications

Mann, M. A., Frisch, L. M., Vogel, R. F. & Niessen, L. (2021). Influence of fermentation conditions on the secretion of seripauperin 5 (PAU5) by industrial sparkling wine strains of *Saccharomyces cerevisiae*. *Food Research International*, *139*, 109912.

Frisch, L. M., **Mann, M. A.**, Marek, D. N., Baudrexl, M., Vogel, R. F. & Niessen, L. (2021). Studies on the gushing potential of *Penicillium expansum*. *Food Research International*, *139*, 109915.

Frisch, L. M., **Mann, M. A.**, Marek, D. N. & Niessen, L. (2021). Development and optimization of a loop-mediated isothermal amplification (LAMP) assay for the species-specific detection of *Penicillium expansum*. *Food Microbiology*, *95*, 103681.

Protzko, R. J., Hach, C. A., Coradetti, S. T., **Hackhofer, M. A.**, Magosch, S., Thieme, N., ... & Benz, J. P. (2019). Genomewide and enzymatic analysis reveals efficient D-galacturonic acid metabolism in the basidiomycete yeast Rhodosporidium toruloides. *MSystems*, *4*(6), e00389-19.

11.2 Articles

Frisch, L. M., **Hackhofer, M. A.** & Niessen, L. (2019). Modern statt klassisch - Entwicklung von LAMP Assays zur Detektion von Schimmelpilzen in Lebensmitteln. *LABO.*

11.3 Oral presentations

Hackhofer, M. A., Kupfer, V., Vogel, R. F. & Niessen, L. (09.05.2019). The role of PAU5 in gushing of sparkling wine. *Science and Wine World Congress*, Porto, Portugal

11.4 Student projects

The listed projects were supervised by the author and the results were partially incorporated into this work with permission of the respective students.

Brunner, A., Einsatz von Schönungsmitteln in der Weinherstellung und deren Einfluss auf die Proteinzusammensetzung, literature research project, 2021

Fazio, L., Investigation of the influence of the yeast strain and the fermentation conditions on the secretion of Seripauperin 5 (PAU5) by *Saccharomyces cerevisiae,* international research internship (6 months), 2019

Mai, K., Unterschiede in der Zellwandstruktur lebensmittelrelevanter Hefen, literature research project, 2021

Vadkertiova, M., Molekulare Differenzierung der Sekretion des Gushing-reduzierenden Proteins Seripauperin 5 durch verschiedene Wein- und Sekthefen (*Saccharomyces cerevisiae*), Bachelor's thesis, 2019

12 Curriculum vitae

Magdalena Agnes Mann
Born on 29 May 1994 in Munich, Germany
Married, maiden name: Hackhofer

Professional experience

04/2018-04/2021	Chair of Technical Microbiology, TUM School of Life Sciences, Technical University of Munich, Freising, Germany ***PhD student*** Topic: The role of Pau5p from *Saccharomyces cerevisiae* in gushing of sparkling wine
11/2017-01/2018	Chair of Wood Bioprocesses, TUM School of Life Sciences, Technical University of Munich, Freising, Germany ***Student assistant***
03/2017-09/2017	Chair of Wood Bioprocesses, TUM School of Life Sciences, Technical University of Munich, Freising, Germany ***Master thesis*** Topic: Molecular and Biochemical Characterization of the Pectinolytic Potential of Two Basidiomycete Red Yeasts: *Rhodotorula mucilaginosa* and *Rhodosporidium toruloides*
04/2015-07/2015	Microbial Ecology, ZIEL - Institute for Food & Health, TUM School of Life Sciences, Technical University of Munich, Freising, Germany ***Bachelor thesis*** Topic: Transcriptome sequencing of small cell populations in *Escherichia coli* O157:H7 str. EDL933 (EHEC)
01/2014-07/2017	Center for Human Genetics and Laboratory Diagnostics (AHC) - Dr. Klein, Dr. Rost and Colleagues, Martinsried, Germany ***Student assistant***

Education

10/2015-11/2017	**Master of Science** in Molecular Biotechnology, Technical University of Munich, Germany
10/2012-07/2015	**Bachelor of Science** in Molecular Biotechnology, Technical University of Munich, Germany
09/2004-06/2012	**Higher Education Entrance Qualification**, Gymnasium Markt Indersdorf, Germany

13 Appendix

13.1 DNA Sequences

The *PAU5* gene sequence was obtained from the NCBI database, the sequenced strain is named *Saccharomyces cerevisiae* S288C and the gene ID is as follows: 850524.

Relevant DNA sequences generated (at least *in silico*) during this study by cloning are listed below in FASTA format:

> pRS62K *PAU5* native (if sequenced with primers P15 + P16)

TAATCAAAAAGTTAACATGCATCACCATCACCATCACGAAAACCTGTATTTTCAGAGCGT
CAAATTAACTTCAATCGCCGCTGGTGTCGCCGCCATTGCCGCTGGTGCCTCCGCTGCA
GCAACCACTACACTATCTCAATCTGACGAAAGAGTTAATTTGGTTGAATTAGGTGTCTAT
GTTTCCGATATCAGAGCTCATTTGGCTGAATACTACTCCTTCCAAGCTGCCCACCCAACT
GAAACTTATCCAGTTGAAATTGCAGAAGCTGTTTTCAACTACGGTGATTTCACCACCATG
TTGACTGGTATTCCCGCCGACCAAGTTACCAGAGTTATCACTGGTGTTCCATGGTACTC
TAGCAGATTAAAGCCAGCTATCTCTAGCGCTCTATCCGCAGACGGTATCTACACTATTG
CAAATTAGTCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCC

> pRS62K *PAU5* HisTEV (if sequenced with primers P14 + P16)

GTCAAATTAACTTCAATCGCCGCTGGTGTCGCCGCCATTGCCGCTGGTGCCTCCGCTG
CAGCAACCACTACACTATCTCAATCTGACGAAAGAGTTAATTTGGTTGAATTAGGTGTCT
ATGTTTCCGATATCAGAGCTCATTTGGCTGAATACTACTCCTTCCAAGCTGCCCACCCAA
CTGAAACTTATCCAGTTGAAATTGCAGAAGCTGTTTTCAACTACGGTGATTTCACCACCA
TGTTGACTGGTATTCCCGCCGACCAAGTTACCAGAGTTATCACTGGTGTTCCATGGTAC
TCTAGCAGATTAAAGCCAGCTATCTCTAGCGCTCTATCCGCAGACGGTATCTACACTATT
GCAAATTAGTCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCC

> pPICZαA *PAU5* native (if sequenced with primers P17 + P19)

GACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGAT
CAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATT
CGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCAC
AAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCT
GTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCA
GCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTgaattcAT
GGTCAAATTAACTTCAATCGCCGCTGGTGTCGCCGCCATTGCCGCTGGTGCCTCCGCT
GCAGCAACCACTACACTATCTCAATCTGACGAAAGAGTTAATTTGGTTGAATTAGGTGTC
TATGTTTCCGATATCAGAGCTCATTTGGCTGAATACTACTCCTTCCAAGCTGCCCACCCA
ACTGAAACTTATCCAGTTGAAATTGCAGAAGCTGTTTTCAACTACGGTGATTTCACCACC
ATGTTGACTGGTATTCCCGCCGACCAAGTTACCAGAGTTATCACTGGTGTTCCATGGTA
CTCTAGCAGATTAAAGCCAGCTATCTCTAGCGCTCTATCCGCAGACGGTATCTACACTAT
TGCAAATTAGctctagaACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACC
ATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTG
GGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCAT
TTGC

> pPICZαA *PAU5* C-terminal His (if sequenced with primers P17 + P19)

GACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGAT
CAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATT
CGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCAC
AAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCT
GTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCA
GCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTgaattcAT
GGTCAAATTAACTTCAATCGCCGCTGGTGTCGCCGCCATTGCCGCTGGTGCCTCCGCT
GCAGCAACCACTACACTATCTCAATCTGACGAAAGAGTTAATTTGGTTGAATTAGGTGTC
TATGTTTCCGATATCAGAGCTCATTTGGCTGAATACTACTCCTTCCAAGCTGCCCACCCA
ACTGAAACTTATCCAGTTGAAATTGCAGAAGCTGTTTTCAACTACGGTGATTTCACCACC
ATGTTGACTGGTATTCCCGCCGACCAAGTTACCAGAGTTATCACTGGTGTTCCATGGTA
CTCTAGCAGATTAAAGCCAGCTATCTCTAGCGCTCTATCCGCAGACGGTATCTACACTAT
TGCAAATgctctagaACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATC
ATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGC
ACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTG
C

> pPICZαA *PAU5* HisTEV (if sequenced with primers P17 + P19)

GACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAGAT
CAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTTTACTGCTGTTTTATT
CGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCAC
AAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCT
GTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCA
GCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTCATCAT
CATCATCATCATGAAAACCTGTATTTTCAGAGCATGGTCAAATTAACTTCAATCGCCGCT
GGTGTCGCCGCCATTGCCGCTGGTGCCTCCGCTGCAGCAACCACTACACTATCTCAAT
CTGACGAAAGAGTTAATTTGGTTGAATTAGGTGTCTATGTTTCCGATATCAGAGCTCATT
TGGCTGAATACTACTCCTTCCAAGCTGCCCACCCAACTGAAACTTATCCAGTTGAAATTG
CAGAAGCTGTTTTCAACTACGGTGATTTCACCACCATGTTGACTGGTATTCCCGCCGAC
CAAGTTACCAGAGTTATCACTGGTGTTCCATGGTACTCTAGCAGATTAAAGCCAGCTATC
TCTAGCGCTCTATCCGCAGACGGTATCTACACTATTGCAAATTAGGAATTCACGTGGCC
CAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAAC
AAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATT
GAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGAC
CGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGC