TECHNISCHE UNIVERSITÄT MÜNCHEN

TUM School of Life Sciences

Monitoring of filamentous fungi and their proteins involved in gushing of sparkling wine

Lisa Maria Frisch

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.

Prüfungsvorsitzender:	Prof. Dr. J. Philipp Benz
Prüfer der Dissertation:	1. apl. Prof. Dr. Ludwig Niessen
	2. Prof. Dr. Wilfried Schwab

Die Dissertation wurde am 10.11.2021 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 31.01.2022 angenommen.

In den kleinsten Dingen zeigt die Natur die allergrößten Wunder.

— nach Carl von Linné

Danksagung

Ganz herzlich bedanke ich mich bei meinem Doktorvater Herrn apl. Prof. Dr. Ludwig Niessen für die exzellente Betreuung meiner Arbeit sowie das entgegengebrachte Vertrauen. Neben einer stets offenen Tür bei allen möglichen Fragen, der gegebenen Freiheit und gleichzeitigen Unterstützung, den vielen interessanten Diskussionen und dem musikalischen Austausch gilt mein besonderer Dank der vermittelten Begeisterung für die wunderschöne Welt der Schimmelpilze.

Meinem Mentor Herrn Prof. Dr. Rudi F. Vogel danke ich herzlich für die Möglichkeit, meine Dissertation an seinem Lehrstuhl für Technische Mikrobiologie anzufertigen. Vielen Dank für die vielen konstruktiven und zum Weiterdenken anregenden Diskussionen sowie die stete Unterstützung.

Mein Dank geht an Herrn Prof. Dr. Wilfried Schwab für die Begutachtung meiner Arbeit sowie an Herrn Prof. Dr. J. Philipp Benz für die Übernahme des Prüfungsvorsitzes.

Des Weiteren bedanke ich mich herzlich bei Frau Prof. Dr. Doris Rauhut und ihrem Team von der Hochschule Geisenheim University für die gute Zusammenarbeit. Dank geht hierbei auch an Herrn Dipl.-Oen. Michael Wallbraun sowie an Herrn Dr. Maximilian Freund für die Hilfe bei önologischen Fragen. Ich danke dem Verband Deutscher Sektkellereien e.V. für die Unterstützung dieser Arbeit. Hierbei gilt mein Dank auch allen Projektausschussmitgliedern für die Bereitstellung von Proben und den Einblick in die Welt des Sektes durch viele spannende Diskussionen. Zudem danke ich allen Winzern und Forschungseinrichtungen für die Bereitstellung von Probenmaterial.

Ganz besonderer Dank geht an meine Projektpartnerin und Bürokollegin Magdalena Mann. Zusammen haben wir die drei Jahre mit Höhen und Tiefen gemeistert, viel diskutiert, uns motiviert, schöne Erinnerungen und Erfahrungen gesammelt sowie wahre Freundschaft geschlossen. Unsere Kondoktorandinnenzeit werde ich nie vergessen.

Des Weiteren bedanke ich mich bei den verbliebenen Bürokolleginnen aus dem unvergesslichen Büro 36 – Esther Rogalski und Sandra Kolbeck – für die wunderbare Zeit. Ich werde immer gerne an unsere Büromeetings, Diskussionen, die vielen Unternehmungen, aber vor allem an unsere gegenseitige Unterstützung denken.

Danken möchte ich auch allen Kolleginnen und Kollegen am Lehrstuhl für Technische Mikrobiologie für die konstruktive Arbeitsatmosphäre und Hilfsbereitschaft. Besonderer Dank geht hierbei an die Pilzarbeitsgruppe sowie an Julia Bockwoldt für die stundenlangen Diskussionen über ELISA.

Weiterer Dank geht an all meine betreuten Studentinnen und Studenten für die gute und verlässliche Zusammenarbeit sowie die erbrachten Ergebnisse.

Ich möchte mich bei Kai Büchner herzlich für die zahlreichen Diskussionen über *Pichia* bedanken und bin froh, ein Teil der *Pichia*-Selbsthilfegruppe gewesen zu sein. Auch Melanie Baudrexl danke ich für die große Unterstützung in der Proteinaufreinigung mittels ÄKTA.

Großer Dank geht an meine liebe Familie und wunderbaren Freunde, besonders an meine Eltern, die mich während meines gesamten Studiums und meiner Promotion immer unterstützt, motiviert und an mich geglaubt haben.

Mein größter Dank gilt Jan. Für seine stete Unterstützung, seine Gelassenheit, sein Verständnis und seine Liebe bin ich von ganzem Herzen dankbar.

Diese Arbeit wurde im Rahmen eines Projekts des Forschungskreises der Ernährungsindustrie e.V. über die Arbeitsgemeinschaft industrieller Forschungsvereinigungen (AiF 19952 N) im Rahmen des Programms zur Förderung der Industriellen Gemeinschaftsforschung vom Bundesministerium für Wirtschaft und Energie gefördert. Die Geldgeber hatten keinen Einfluss auf das Design, die Ergebnisse oder die Darstellung dieser Arbeit.

Table of Contents

1 Introduction 1
1.1 Sparkling wine 1
1.2 The phenomenon of gushing 2
1.2.1 Bubble formation and stabilization 2
1.3 Gushing in beer 3
1.3.1 Proteins from filamentous fungi in beer 3
1.3.2 Proteins from *Hordeum vulgare* in beer 5
1.3.3 Secondary gushing in beer 6
1.4 Gushing in sparkling wine 6
1.4.1 Proteins from yeasts in sparkling wine 7
1.4.2 Proteins from *Vitis vinifera* in sparkling wine 7
1.4.3 Filamentous fungi on grapes and their proteins 8
1.4.4 Secondary gushing in sparkling wine 13
1.5 Analysis of gushing-related proteins and their producing organisms for gushing prediction 14
1.5.1 Loop-mediated isothermal amplification (LAMP) 14
1.5.2 Enzyme-linked immunosorbent assay (ELISA) 17
1.5.3 *Pichia pastoris* expression system 19
1.6 Aim of the study 21
2 Materials and methods 22
2.1 Materials 22
2.1.1 Equipment 22
2.1.2 Consumables 25
2.1.3 Chemicals 27
2.1.4 Media and buffers 31
2.1.5 Primers 38
2.1.6 Antibodies 41
2.1.7 Kits 42
2.1.8 Enzymes 42
2.1.9 Plasmids 43
2.1.10 Software and databases 43
2.1.11 Sparkling wines, base wines, and musts 44
2.1.12 Organisms 46
2.2 Methods 47
2.2.1 Microbiological methods 47

2.2.2 Protein-chemical methods 49
2.2.3 Immunochemical methods 53
2.2.4 Moleculobiological methods 56
2.2.5 Bioinformatical methods 66
3 Results 67
3.1 Cloning and heterologous expression of gushing-inducing proteins in *Pichia pastoris* 67
3.1.1 Production of *Pichia pastoris* transformants 67
3.1.2 Production and purification of recombinant proteins 74
3.2 Effect of recombinant proteins on gushing in sparkling wine 81
3.3 Development of immunochemical methods for the detection of gushing-inducing proteins 83
3.3.1 Antibody production and testing 83
3.3.2 Development of an ELISA for the detection of PEX2_044840 85
3.3.3 Dot blot for immunochemical detection of PDE_07106 94
3.4 Detection of gushing-relevant fungi on sample materials from European vineyards 95
3.4.1 Development of a LAMP assay for the detection of *P. expansum* 95
3.4.2 Optimization of LAMP assays for *P. oxalicum* and *B. cinerea* 105
3.4.3 Screening of samples from the harvests 2018, 2019, and 2020 110
3.5 Investigations of the role of ns-LTP1 *Vv* in gushing of sparkling wine 117
3.5.1 Relative quantification of ns-LTP1 *Vv* in sparkling wines via ELISA against ns-LTP1 from *H. vulgare* 117
3.5.2 Development of an ELISA for the detection of ns-LTP1 *Vv* 119
4 Discussion 125
4.1 Heterologous expression of gushing-inducing proteins in *Pichia pastoris* and their purification 126
4.2 Gushing potential of the purified proteins 131
4.3 Immunochemical detection of the gushing-inducing proteins 134
4.3.1 ELISA for the detection of PEX2_044840 from *P. expansum* 134
4.3.2 Detection of *P. oxalicum* proteins 137
4.4 LAMP assays as rapid diagnostic tools for early detection of gushing-relevant fungi on sample materials 138
4.4.1 LAMP assay for the detection of *P. expansum* 138
4.4.2 Detection of gushing-relevant fungi on grape, soil, and must samples from European vineyards 141
4.5 Quantification of ns-LTP1 *Vv* in sparkling wines and evaluation of its influence on the occurrence of gushing 149

4.6 Conclusion 152
5 Summary 153
6 Zusammenfassung 155
7 List of Abbreviations 157
8 List of Figures 161
9 List of Tables 163
10 References 165
11 Appendix 185
11.1 Sequences 185
11.2 Vector maps 191
11.3 Screening results 194
12 List of publications and student projects 215

1 Introduction

1.1 Sparkling wine

Sparkling wines are defined according to the International Organisation of Vine and Wine (2015) as special wines characterized by the production of persistent effervescence on uncorking that results from released carbon dioxide. The high carbon dioxide content which causes bubbling distinguishes sparkling wine from still wine (Troost et al., 1995).

Over the last years, the sparkling wine market has expanded with an increase of the global production of +57 % from 2002 to 2018 equivalent to a production of 20 million hectoliters in 2018 (International Organisation of Vine and Wine, 2020). Not only the supply, but also the sparkling wine demand has been rising. While in earlier times, sparkling wine was mostly consumed only at end-of-year celebrations, nowadays it commonly serves as aperitif or is bought for celebrations during the year. Furthermore, the supply became more diversified with a broader range of product prices (International Organisation of Vine and Wine, 2020).

Sparkling wines are produced worldwide, with Italy, France, Germany, and Spain as the leading producing countries (International Organisation of Vine and Wine, 2020). In 2018, the share of sparkling wine in total wine production was 28 % in Germany and hereby the largest among all producing countries (International Organisation of Vine and Wine, 2020). According to the Federal Statistical Office of Germany (Statistisches Bundesamt, 2021a), the number of sparkling wine producers in Germany increased from 794 in the year 1991 to 1086 in 2020. Between 1998 and 2018, the average annual production in Germany was above 3 million hectoliters and in 2018, Germany ranked third behind Italy (5.3 million hectoliters) and France (4.4 million hectoliters) regarding total sparkling wine production (International Organisation of Vine and Wine, 2020). In 2020, 2.7 million hectoliters of sparkling wine corresponding to 3.3 liters per capita were consumed in Germany (Statistisches Bundesamt, 2021a, b), making it one of the countries with the highest total consumption per year worldwide (International Organisation of Vine and Wine, 2020). In 2020, the German sparkling wine industry was able to generate a revenue of 1.65 billion € compared to 3.02 billion € generated by the German still wine industry, underlining the importance of sparkling wine for German winemakers (preliminary numbers of Bundesverband der Deutschen Spirituosen-Industrie und -Importeure e. V., 2021).

Effervescence is the main characteristic of sparkling wines (Liger-Belair, 2005). After opening of a bottle, the pressure releases and small condensed bubbles rise slowly to the surface of the liquid forming a foam (Kemp et al., 2018; Liger-Belair, 2005). On occasion, however, the pressure release results in an uncontrolled over-foaming of the liquid – a phenomenon known as gushing (Kemp et al., 2018).

1.2 The phenomenon of gushing

Gushing describes the spontaneous excessive over-foaming of carbonated beverages after opening of non-agitated bottles. This phenomenon can occur in different kinds of carbonated beverages such as sparkling wine (see Figure 1), beer, spritzer, or cider (Bach, 2001; Gjertsen, 1967; Schumacher, 2002; Wilson, 1999) causing considerable economic losses and reputational damages to the affected beverage companies.

Figure 1: Gushing-positive sparkling wine
A gushing-positive sparkling wine bottle was opened after standing upright for 2 h.

Gushing is caused by a complex interaction of different factors. According to the Carlsberg Research group, it can be differentiated into primary gushing and secondary gushing (Gjertsen et al., 1963): Primary gushing is defined as a periodically and locally restricted occurrence of over-foaming that is caused by the used raw materials, while secondary gushing is caused by technological failure such as particles that can be traced back to the production process.

1.2.1 Bubble formation and stabilization

Gushing occurs in carbonated beverages containing high levels of carbon dioxide. By opening of a bottle of carbonated liquid, the carbon dioxide changes from a dissolved to a gaseous form due to the resulting pressure change. Usually, this process happens slowly without over-foaming. In gushing-affected bottles, however, the carbon dioxide release is abrupt leading to over-foaming of the liquid. Here, stabilized microbubbles present in the solution were suggested to act as nucleation sites at which gaseous carbon dioxide can be released from the solution (Lutterschmid, 2011).

The stability of a gas bubble in a liquid is dependent on its dimension. If the radius of a bubble is smaller than a critical value, it will dissolve due to surface tension and ambient pressure (Yount, 1982). If the radius is greater than a critical value, the bubble will expand indefinitely leading to degassing of the solution. The critical value of the bubble radius depends on the carbon dioxide saturation of the liquid, with a smaller critical value for higher levels of supersaturation (Gardner, 1973).

Surface-active molecules can stabilize bubbles when they agglomerate at the gas-liquid interface of a bubble by minimizing their surface tension and gas permeability (Deckers et al., 2010; Deckers et al., 2012; Pellaud, 2002). Such molecules form a layer around the microbubbles and prevent breakdown of the bubbles under the pressure conditions in a closed bottle (Draeger, 1996; Pellaud, 2002). After opening of a bottle, there is a pressure release leading to uncontrolled growth of the formerly stabilized bubbles due to carbon dioxide diffusion into them (Pellaud, 2002). The radius of microbubbles in gushing beverages exceeds the critical value and the uncontrolled growth of the bubbles leads to an explosive release of carbon dioxide filled bubbles to the surface (Deckers et al., 2010).

Therefore, one key factor in understanding the underlying mechanisms of gushing in carbonated beverages are surface-active molecules that stabilize microbubbles.

1.3 Gushing in beer

Gushing is most noted in beer on which the majority of research has been focused in the past decades. The phenomenon is known in beer since the beverage was filled in bottles in the middle of the 16th century (Beattie, 1951). Kastner (1909) was the first scientist to describe over-foaming after opening of a beer bottle despite correct handling. Since then, many researchers investigated the causes for beer gushing.

1.3.1 Proteins from filamentous fungi in beer

Over-foaming of beer was frequently observed after wet years with high precipitation, so fungal infestation of the raw material was suspected as the causal factor early on (Gjertsen et al., 1963). Research on primary gushing in beer revealed an infection of malt with *Fusarium* (*F.*) spp. (Niessen et al., 1992; Sarlin et al., 2005a; Schwarz, 1996) or other fungal genera such as *Nigrospora*, *Stemphylium*, and *Penicillium* (Amaha et al., 1973; Kitabatake and Amaha, 1974) as major inducing factor since the occurrence of gushing was closely correlated with the quality of the used barley. Gushing-inducing factors that are produced by fungi were described to be stable polypeptides or peptide-containing substances consisting of hydrophobic amino acids (Amaha et al., 1973; Kitabatake, 1978). Further studies indicated that a group of small commonly secreted fungal proteins (10 kDa) – the so-called hydrophobins – can induce gushing in beer (Lutterschmid et al., 2010; Sarlin et al., 2005b). These proteins are

ubiquitous in filamentous fungi (Kershaw and Talbot, 1998; Wessels, 1996) and have multiple functions in the fungal life cycle such as fungal growth and dissemination, morphogenesis, or pathogenesis (Wessels, 1994; Wösten and Wessels, 1997).

Hydrophobins are highly surface-active amphiphilic proteins characterized by eight cysteine residues at conserved positions (Linder et al., 2005; Schuren and Wessels, 1990; Wessels et al., 1991). Formation of four disulfide bridges between cysteine residues results in four loops and efficient cross-linking of the protein (Kershaw and Talbot, 1998; Linder et al., 2005; Yaguchi et al., 1993). They self-assemble at hydrophilic/hydrophobic interfaces forming amphipathic membranes (Wösten et al., 1994; Wösten et al., 1993) and changing hydrophobicity or hydrophilicity of a surface (Wösten and Wessels, 1997). Surface-activity of hydrophobins by lowering of the surface tension depends on a conformational change of the molecules (van der Vegt et al., 1996). Wessels (1994) proposed to distinguish between two classes of hydrophobins due to differences in hydropathy patterns and biophysical properties: class I and class II hydrophobins. Class I hydrophobins form membranes that are highly insoluble (Wessels et al., 1991), while class II hydrophobins form membranes that are less stable (Russo et al., 1982). The addition of hydrophobins from barley fungal pathogens to bottled beer induced gushing in several studies (Lutterschmid et al., 2011; Lutterschmid et al., 2010; Sarlin, 2012; Sarlin et al., 2005b; Stübner et al., 2010). Zapf (2006) produced beer with transgenic *Saccharomyces* (*S*.) *cerevisiae* strains that contained hydrophobin genes of different classes from *F. culmorum*. In the experiment, gushing occurred in the beer that was produced with a yeast secreting a class II hydrophobin but not in the beer with a yeast secreting a class I hydrophobin. The author suggested that mainly class II hydrophobins are responsible for gushing and not class I hydrophobins due to their extreme insolubility. Also, Lutterschmid et al. (2011) observed a gushing-inducing effect only for the class II hydrophobin Hfb2 and not for the class I hydrophobin FcHyd3p.

The mechanism behind gushing induction by hydrophobins is assumed to be the agglomeration of hydrophobins at the gas/liquid interphase of carbon dioxide bubbles and their stabilization by forming a layer of amphiphilic proteins (Deckers et al., 2010). This layer prevents a breakdown of the bubbles under pressure in a closed bottle. After opening, the pressure in the bottle drops, the bubbles grow uncontrolled, and subsequently explode leading to over-foaming (Deckers et al., 2012; Mastanjević et al., 2017; Sarlin et al., 2005b) (see also section 1.2.1).

Another class of surface-active proteins are the so-called fungispumins (Zapf et al., 2007). These proteins are produced by gushing-inducing fungi such as *F. culmorum* and exhibit some hydrophobin characteristics. Zapf et al. (2007) demonstrated that the fungispumin alkaline foam protein A (AfpA) forms stable foam and enhances gushing in moderately gushing-positive

beer but does not induce it. These results indicate that fungispumins are not a primary cause for gushing but might contribute to it. Lutterschmid et al. (2011) also studied the effect of AfpA on gushing but did not see any gushing-enhancing or gushing-inducing effect or foam stabilizing properties. Instead, this protein showed a gushing-reducing effect when added to beer previously treated with a class II hydrophobin.

1.3.2 Proteins from *Hordeum vulgare* in beer

Protein Z and the non-specific lipid transfer protein 1 (ns-LTP1) from *Hordeum* (*H.*) *vulgare* are predominant beer proteins (Hejgaard and Kaersgaard, 1983; Kaersgaard and Hejgaard, 1979; Sørensen et al., 1993).

The form Z4 of protein Z is the most abundant type in beer (Evans and Hejgaard, 1999) and characterized by major foam-forming properties (Evans et al., 1999). Specker et al. (2014) cloned the gene coding for the Z4 protein from barley in *Pichia pastoris* and analyzed the effect of the recombinant protein on beer gushing. When added to gushing-negative beer, it did not induce gushing. However, when added to beer previously treated with a class II hydrophobin, it decreased the gushing volume considerably.

Ns-LTP1 was shown to be a major factor involved in beer foam formation when the protein was isolated from beer (Sørensen et al., 1993). In contrast, when the protein was extracted from barley, it was much less effective in foam formation. Sørensen et al. (1993) concluded that ns-LTP1 undergoes structural modification during malting and brewing, whereby the modifications on the ns-LTP1 protein are responsible for its much higher potential in beer foam formation. Jégou et al. (2000) found out that ns-LTP1 is glycated by Maillard reaction and completely unfolded due to cleavage of disulfide bonds enabling an easy adsorption of hydrophobic groups and amphipathic domains at air-water interfaces of beer foam. Moreover, the same authors supposed that glycation leads to increased amphiphilicity and solubility and therefore to stronger surface properties. Hippeli and Elstner (2002) hypothesized that the content of modified ns-LTP1 in beer needs to be below a threshold level in order to form the desirable beer foam. According to their hypothesis, exceeding of the threshold level will lead to gushing. The authors suggested an infection of barley during growth, ripening, harvesting, or storage with microorganisms as hypothetical reason for an oversupply of ns-LTP1 because in response to the infestation, the gene coding for ns-LTP1 is known to be upregulated (Gorjanović, 2007). Another suspected reason was metabolic activity of microorganisms on the grains that leads to a release of cell wall bound ns-LTP1. The same authors hypothesized higher ns-LTP1 contents in gushing-positive beer than in gushing-negative beer to be responsible for gushing (Hippeli and Hecht, 2008). However, when they tested gushing-positive beer, less to non ns-LTP1 was detected. From their findings, their alternative hypothesis was that not ns-LTP1 itself, but glycated peptides generated by proteolytic

fragmentation of modified ns-LTP1 by *Fusarium* proteases might induce gushing in beer. They assumed that during the brewing process, naturally present protease inhibitors in wheat kernels may become inactivated by heat, while heat-stable proteases from *Fusarium* maintain their activity leading to a degradation of ns-LTP1. Although, the authors did not prove their assumption of the presence of such fragments in beer. Lutterschmid et al. (2011) investigated the effect of recombinant ns-LTP1 from *H. vulgare* on gushing of beer. The authors found no gushing-inducing potential of this protein when added to beer. Instead, they demonstrated a gushing-reducing effect of the protein when added to beer that was previously treated with class II hydrophobins. The same effect was observed for naturally gushing-positive beer after addition of heat-treated ns-LTP1. Therefore, they suggested beer with a low content of ns-LTP1 to be more prone to gushing than beer with a normal content of this protein. In contrast to Hippeli and Hecht (2008), the authors did not assume degraded ns-LTP1 fragments to be the cause for gushing induction but rather a change in the proportions of gushing-inducing hydrophobins and ns-LTP1 in beer in favor of the hydrophobins.

1.3.3 Secondary gushing in beer

In contrast to primary gushing, secondary gushing is caused by failures in the production process (Gjertsen et al., 1963) and can be prevented by applying good manufacturing practices (Mastanjević et al., 2017). Agents for secondary gushing induction in beer can be metal ions (Guggenberger and Kleber, 1963; Rudin and Hudson, 1958), calcium oxalate crystals (Zepf and Geiger, 2000), cleaning compounds (Dachs and Nitschke, 1977), high air content in the headspace of bottles (Dachs and Nitschke, 1977), haze, or impurities from the bottles (Sarlin et al., 2005b) that can act as nucleation sites for the release of carbon dioxide (Draeger, 1996; Zarnkow and Back, 2001). Secondary gushing typically affects only occasional bottles within a lot, while primary gushing often concerns a whole beer batch (Sarlin, 2012).

1.4 Gushing in sparkling wine

Even though major attention in research has been given to beer gushing, sparkling wines can also be affected by the phenomenon. Especially red sparkling wines are prone to excessive over-foaming (Bach, 2001; Hennig, 1963). According to the association of German sparkling wine cellars (Verband Deutscher Sektkellereien e.V., personal communication, 12.09.2017), up to 2 % of the annual production of sparkling wine bottles can be affected by gushing in particular years leading to recalls and disposal of affected batches. However, the causes for primary gushing in sparkling wine have hardly been investigated so far. Yet, the importance of (surface-active) proteins influencing foaming properties of sparkling wines has been highlighted in previous research (Blasco et al., 2011; Brissonnet and Maujean, 1993; Kemp et al., 2018; Kupfer et al., 2017a; Martínez-Lapuente et al., 2018; Vogt et al., 2017b). Proteins in wine and sparkling wine can originate from the grape vine *Vitis* (*V.*) *vinifera*, from yeasts, or

from filamentous fungi and bacteria. They have molecular weights between approximately 9-65 kDa, with the majority between 20-30 kDa (Brissonnet and Maujean, 1993; Hsu and Heatherbell, 1987), and their presence in wine depends on several factors such as grape variety, vintage, or vinification method (Bayly and Berg, 1967; Dizy and Bisson, 1999).

1.4.1 Proteins from yeasts in sparkling wine

Yeast proteins found in wines and sparkling wines have been identified as cell wall proteins such as mannoproteins, and invertases (Cilindre et al., 2008; Dambrouck et al., 2003; Kwon, 2004; Waters et al., 1994).

Mannoproteins are glycoproteins released from the yeast in sparkling wine during fermentation and aging on lees due to autolysis (Martínez et al., 2016). These proteins were shown to be foam-active and to improve foam properties in sparkling wine due to their amphiphilic structure (Blasco et al., 2011; Núñez et al., 2006; Núñez et al., 2005; Vincenzi et al., 2014). They have hydrophobic and hydrophilic domains that facilitate their adsorption at the gas/liquid interface which leads to a stable foam (Blasco et al., 2011; Martínez-Lapuente et al., 2018).

Bach et al. (2001) showed that mannoproteins have a gushing-reducing effect in sparkling wine. When they added mannoproteins to gushing-positive sparkling wine, gushing was prevented. Kupfer et al. (2017b) demonstrated that the protein seripauperin 5 (Pau5p) from *S. cerevisiae* can be a negative marker for gushing in sparkling wine. *PAU5* belongs to the largest multigene family in *S. cerevisiae* – the *PAU* genes (Luo and van Vuuren, 2008; Viswanathan et al., 1994). These genes share homology with the *TIR* and *DAN* gene families that code for cell wall mannoproteins (Abramova et al., 2001). *O*-mannosylation of Pau5p was observed and can enhance the protein's stability (Luo and van Vuuren, 2008). In their studies, Kupfer et al. (2017b) showed that absence of Pau5p was positively correlated with the occurrence of gushing in sparkling wine. Moreover, Kupfer et al. (2017a) revealed foam-stabilizing properties of the glycosylated protein in grape juice. Due to the enhancement of amphiphilicity by glycosylation, the authors assumed Pau5p to act in a similar way as foam-stabilizing mannoproteins in gushing of sparkling wine and to prevent the occurrence of gushing similar to ns-LTP1 in beer (see section 1.3.2).

1.4.2 Proteins from *Vitis vinifera* in sparkling wine

Proteins from the grape vine *V. vinifera* found in wines and sparkling wines were mostly proteins that are involved in the sugar metabolism such as vacuolar invertases, or pathogenesis-related (PR) proteins such as chitinases, thaumatin-like proteins, osmotin-like proteins, or non-specific lipid transfer proteins (Cilindre et al., 2014; Cilindre et al., 2008; Kwon, 2004; Okuda et al., 2006; Wigand et al., 2009).

Grape invertases are *N*-glycoproteins with high hydrophobicity which indicates that they lead to enhanced foam properties in sparkling wine (Dambrouck et al., 2005; Hovasse et al., 2016; Marchal et al., 1996).

Among PR-proteins, non-specific lipid transfer (ns-LTP) proteins are small basic glycoproteins that are widely distributed in plants. They are characterized by strong structural homologies with eight conserved cysteine residues forming four disulfide bridges (Kader, 1996). Due to a hydrophobic cavity, they are able to bind and transport lipids *in vitro* (Kader, 1996; Salminen et al., 2016; Scheurer and Schülke, 2018). Ns-LTPs from *V. vinifera* were found extracellularly during somatic embryogenesis indicating a role in the plant development mechanism (Coutos-Thevenot et al., 1993). Furthermore, ns-LTPs are involved in plant defense and the adaption of plants to abiotic and biotic stress (Scheurer and Schülke, 2018).

Ns-LTP1 from *H. vulgare* was shown to be involved in foam formation in beer (see section 1.3.2) and thus, an involvement of ns-LTPs from *V. vinifera* in foam formation in sparkling wine was hypothesized (Cilindre et al., 2014). Moreover, a gushing-reducing effect of ns-LTP1 from *H. vulgare* in beer was found (see section 1.3.2). Therefore, Kupfer (2018) investigated the amount of ns-LTP1 in gushing-negative and gushing-positive sparkling wines. The results revealed the highest ns-LTP1 content in a gushing-negative sparkling wine and the lowest in a gushing-positive one. However, no significant difference was found by comparing the mean ns-LTP1 contents of the tested sparkling wines. A possible gushing-reducing effect of this protein in sparkling wine therefore needs to be further investigated.

1.4.3 Filamentous fungi on grapes and their proteins

The mycobiota on grapes differs widely from the fungi growing on the raw materials used for beer making. Instead of the typical field fungi found on cereals such as *Fusarium* spp., species of *Penicillium* (*P.*), *Aspergillus*, *Alternaria*, *Cladosporium*, or *Botrytis* (*B.*) *cinerea* are most abundant on grapes (Abrunhosa et al., 2001; Bellí et al., 2006; Sage et al., 2002; Serra et al., 2005). The three fungi *B. cinerea*, *P. oxalicum*, and *P. expansum* (see Figure 2) were shown to be involved in the quality of wine and sparkling wine and were also related to gushing in previous research. They will therefore be described in more detail in the following sections.

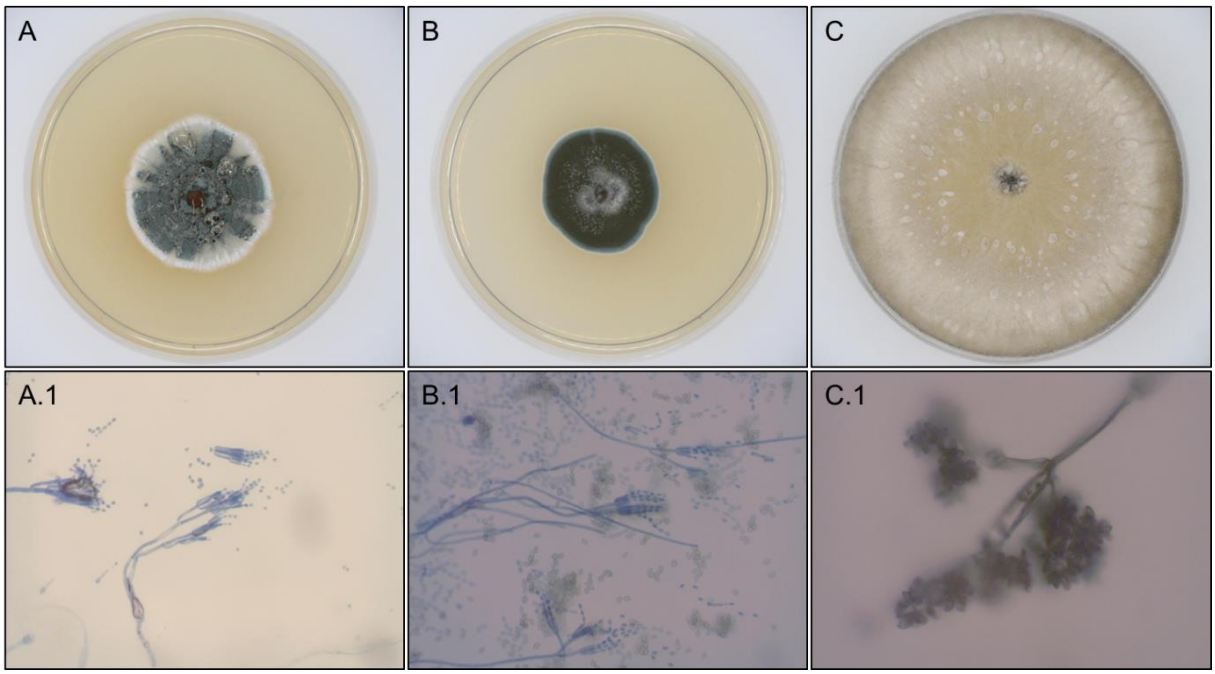


Figure 2: *P. expansum*, *P. oxalicum*, and *B. cinerea*
Colonies of the filamentous fungi *P. expansum* TMW 4.2808 (A), *P. oxalicum* TMW 4.2553 (B), and *B. cinerea* TMW 4.2743 (C) were photographed after 7 days of incubation on ME agar at AT. A.1-C.1 show conidiophores and conidia of the corresponding fungi in 40x magnification (light microscopy of cotton blue stained preparations).

1.4.3.1 *Botrytis cinerea*

B. cinerea Persoon (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a plant pathogen that is ubiquitous with a broad range of host plants. It occurs mainly in humid temperate and subtropical regions with a growth range between -2 °C and 33 °C and an optimum at 22-25 °C (Panasenko, 1967; Samson et al., 2019). Growth occurs at water activities between a_w 0.92 and a_w 1.0 (Panasenko, 1967). In viticulture, this fungus can lead to serious yield losses but also to quality enhancement of wines made from infected berries depending on the weather conditions and ripening stage of the berries during which infection occurs (König et al., 2009): An infection of the grape berry at an early ripening stage during long lasting wet weather causes berry decay and bunch rot. Hereby, *B. cinerea* produces laccases in infected berries that can be detected in must and wine and lead to color loss especially in red wines which eventually turn brownish. On the other hand, late infection of ripe grape berries during dry and warm weather conditions leads to a concentration of berry ingredients enhancing the quality of the resulting wine (e.g., dessert wines like ‘Trockenbeerenauslese’), causing noble rot. Hereby, *B. cinerea* produces gluconic acid that gives a distinct tastiness to the wine. The fungus produces sclerotia for long-term survival and over-winters as mycelium and sclerotium on leaf litter on the ground (König et al., 2009). The conidia produced are spread by rain dispersal, wind, and insects. They germinate rapidly after inoculation on the host plant surface. The fungus penetrates the host by wounds and natural openings due to its production of enzymes that facilitate penetration of the epidermis. Successful invasion and infection by the

fungus is triggered by effector proteins that act as pathogenicity factors and by the induction of programmed cell death (König et al., 2009).

B. cinerea occurs frequently on grapes: In Europe, it was found in different countries such as Spain (Bau et al., 2005), Portugal (Abrunhosa et al., 2001; Serra et al., 2005; Serra et al., 2006), France (Cilindre et al., 2008; Diguta et al., 2011; La Guerche et al., 2005), Italy (Lorenzini et al., 2016), Hungary (Varga et al., 2007), Slovakia (Felšöciová et al., 2015; Mikusová et al., 2010), or Germany (Becker et al., 2011; Kretschmer and Hahn, 2008; Leroch et al., 2011; Lopez Pinar et al., 2017).

Marchal et al. (2001) investigated the effect of a *B. cinerea* infection of grapes on the foaming properties of Champagne. They observed a considerable decrease of sparkling wine foamability when infected grapes were used. The same effect of reduced foamability was observed by Cilindre et al. (2007) when they tested botrytized champenois base wine. Moreover, the authors found an alteration in the wine protein composition due to the presence of *B. cinerea*: some proteins that were present in the healthy wine were absent or degraded in the infected wine indicating a proteolytic activity of proteins secreted by *B. cinerea* (ten Have et al., 2004). In a following study, the same authors revealed that especially those wine proteins with a molecular weight below 23 kDa disappeared in botrytized wine, while pectinolytic proteins from *B. cinerea* were present (Cilindre et al., 2008). Also, Marchal et al. (1998) showed a degradation of grape proteins in musts obtained from grapes infected with *B. cinerea* by proteins secreted from that fungus. Further studies of Marchal et al. (2006) demonstrated protease activity of proteins secreted by *B. cinerea* in a model wine and a relationship with the corresponding decrease in wine foaming properties. Proteases secreted by *B. cinerea* were also found to be responsible for the degradation of high and medium molecular weight molecules secreted by *S. cerevisiae* during alcoholic fermentation of a model grape juice leading to a considerable decrease in foamability (Marchal et al., 2020).

As the infection of grapes with *B. cinerea* was shown to cause degradation of proteins and therefore negatively influences the foaming properties of wine, Kupfer et al. (2017b) investigated the effect of protein alterations in botrytized wine on the occurrence of gushing in sparkling wine. The authors found out that especially the protein fraction with a molecular weight below 35 kDa (PR-proteins) was decreased in wine made from infected grapes and in gushing-positive sparkling wine. Moreover, a laccase originating from *B. cinerea* was detected in gushing-positive sparkling wine indicating the absence of specific proteins in gushing wines to be related to an infection of the used grapes with this fungus. Furthermore, they showed that the concentration of Pau5p from *S. cerevisiae* decreased in wine made from botrytized grapes. An absence of Pau5p was suggested as a marker for the occurrence of gushing (see

section 1.4.1). Therefore, the authors assumed *B. cinerea* to be indirectly involved in gushing induction of sparkling wine.

Furthermore, Kupfer (2018) showed that grapes infected with *B. cinerea* as well as wines made from these infected grapes had lower amounts of ns-LTP1 than healthy grapes and wine made from healthy grapes. Ns-LTP1 is speculated to be involved in foam formation and to have a gushing-reducing effect in sparkling wine (see section 1.4.2). The author assumed a degradation of the protein by fungal proteases, similar to the assumption of Hippeli and Hecht (2008) who suggested the degradation of barley ns-LTP1 by proteases secreted from *Fusarium* spp. in beer as a cause for gushing.

1.4.3.2 *Penicillium oxalicum*

P. oxalicum Currie & Thom is a fungus with a growth range between 8 °C and 35 °C showing optimal growth at 30 °C (Mislivec and Tuite, 1970b). The fungus can tolerate low pH values (Pitt and Hocking, 2009) and needs a water activity above a_w 0.86 for germination and sporulation (Mislivec and Tuite, 1970b). It is described as typical soil fungus and phytopathogen in temperate, subtropical, and tropical regions (Pitt and Hocking, 2009). *P. oxalicum* was detected on corn kernels (Mislivec and Tuite, 1970a), cucumber (O'Neill et al., 1991), tomatoes (Kwon et al., 2008; Umemoto et al., 2009), yam (Okigbo and Ogbonnaya, 2006), or grapes (Bau et al., 2005), but also in indoor air (Vesper et al., 2005). The fungus owes its name to its production of oxalic acid (Currie and Thom, 1915), the calcium salts of which are known to cause secondary gushing in beer (Zepf and Geiger, 2000). Besides, it produces the mycotoxin secalonic acid D that leads to toxicity in animals (Ciegler et al., 1980; Ehrlich et al., 1982; Steyn, 1970).

P. oxalicum occurs rather rarely on grapes. In Europe, it was mainly found in Mediterranean wine growing regions such as Spain (Bau et al., 2005), France (Bejaoui et al., 2006; Diguta et al., 2011; Sage et al., 2002), Italy (Lorenzini et al., 2016; Vogt et al., 2017a), Portugal (Serra et al., 2005; Serra et al., 2006; Vogt et al., 2017a), but also on grapes from Slovakia (Felšöciová et al., 2015).

Vogt et al. (2017b) found a gushing-inducing effect of proteins from *P. oxalicum* in sparkling wine. Culture supernatants of this fungus were able to create stable foam during foam fractionation by enriching surface-active molecules at the gas/liquid interphase resulting in stabilization of gas bubbles similar to the mechanism postulated for hydrophobins (see section 1.3.1). Inoculation of sparkling wines with culture supernatants resulted in severe gushing (Vogt et al., 2017b). Analysis of the gushing-inducing supernatant revealed a high concentration of the proteins PDE_04519 and PDE_07106 from *P. oxalicum*. These proteins showed similar characteristics as hydrophobins but had a higher molecular weight (~14 kDa

and ~20 kDa) and only four cysteine residues. The authors suggested that these surface-active proteins from *P. oxalicum* belong to a protein class involved in gushing induction in sparkling wine similar to the mechanism described for hydrophobins in beer gushing. Therefore, gushing in sparkling wine could occur when grapes infected with *P. oxalicum* were used for base wine production.

1.4.3.3 *Penicillium expansum*

P. expansum Link is a ubiquitous soil fungus and a typical airborne plant pathogen. It is very common in temperate regions due to its broad temperature range (-3 °C to 35 °C) for conidia germination, mycelial growth, and sporulation with an optimum temperature near 25 °C (König et al., 2009; Panasenko, 1967). It can tolerate low pH values (Samson et al., 2019) and the minimum water activity for its growth was found to be a_w 0.86 (Mislivec and Tuite, 1970b). The fungus is pathogenic on a broad variety of fruits. It was found to be the major cause of spoilage on pomaceous fruits such as pears and apples (Snowdon, 1990), but also occurs on strawberries, tomatoes, mangos, avocados, and grapes (Pitt and Hocking, 1997; Snowdon, 1990). Due to its easy dissemination by wind or insects, an infection with this fungus can lead to massive yield losses in orchards or vineyards (Kück, 2009). Moreover, *P. expansum* acts as a typical storage fungus. Isolations are regularly reported from corn, rice, wheat, and barley (Aziz et al., 2006), but also from indoor air (Samson et al., 2019). The fungus is the principal agent of one of the economically most important postharvest diseases known as Blue Mold Decay (Vico et al., 2014). This decay leads to considerable economic losses during fruit storage (Vico et al., 2014) due to the fungus' ability to grow at 0 °C and its low requirement for oxygen (Golding, 1945; Mislivec and Tuite, 1970b).

Besides its economic impact as food spoilage organism, *P. expansum* is also a health hazard due to its ability to produce mycotoxins (Morales et al., 2007). Mycotoxins are toxic secondary metabolites of filamentous fungi presenting a potential hazard in regard to food safety. They are also called "insidious poisons" as the toxins are ingested in low doses (Serra et al., 2005). *P. expansum* can produce the mycotoxins patulin and citrinin in rotting apples and other food commodities (Ciegler et al., 1977; Harwig et al., 1973) and is considered as the main source of patulin in food (Morales et al., 2007). Patulin has considerable effects on public health leading to gastrointestinal disturbances, nausea, and emesis in humans (Drusch and Ragab, 2003). Because of these harmful effects and its occurrence on apples, that are highly consumed by children, many countries worldwide have set legal limits for the patulin content in food products particularly for baby food (European Commission, 2006).

P. expansum is the main agent of a secondary disease of grapes called Green Mold that occurs in warm and humid years (König et al., 2009). The fungus infects berries that enter the ripening stage early, primarily through wounds or co-infection with *B. cinerea*. Large cracks in the berry

skin and pads of mycelium on the wounds are symptoms of an infection. The berries soften due to enzymatic maceration, decay, and shrink. Due to the fungus' enormous sporulation, there is an increased pressure of infection on surrounding healthy berries (König et al., 2009; Sommer et al., 2002). Co-infection with *B. cinerea* leads to the production of the volatile metabolite (–)-geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) which causes quality problems in wine making as it is responsible for unwanted earthy off-flavors in wine made from rotten grapes (La Guerche et al., 2005).

Proteins of *P. oxalicum* were shown to induce gushing in sparkling wine (see section 1.4.3.2). As gushing also occurs in sparkling wines produced from grapes grown in temperate regions like Germany and the temperature optimum of *P. oxalicum* at 30 °C seems too high for optimal growth of the fungus there, it can be assumed that other fungi might be responsible for gushing induction in temperate wine growing regions. To identify other filamentous fungi potentially involved in gushing induction, the amino acid sequence of the protein PDE_04519 from *P. oxalicum* was aligned with published sequences of *Penicillium* spp. in preliminary studies at the Chair of Technical Microbiology (TUM, Freising, Germany) revealing several proteins with high homology. Among them, *P. expansum* was the one frequently occurring on grapes. In Europe, it was reported in Spain (Bragulat et al., 2008), Italy (Lorenzini et al., 2016), Portugal (Abrunhosa et al., 2001; Serra, 2003; Serra et al., 2005; Serra et al., 2006), France (Bejaoui et al., 2006; Diguta et al., 2011; La Guerche et al., 2005; Sage et al., 2002), Slovakia (Felšöciová et al., 2021; Felšöciová et al., 2015; Mikusová et al., 2010; Tančinová et al., 2015), Hungary and Czech Republic (Ostrý et al., 2007; Varga et al., 2007), and Germany (Walter, 2008). In previous studies of the author of the current study, *P. expansum* was found to produce surface-active proteins in culture supernatant leading to stable foam and gushing in sparkling wine (Frisch, 2018). Analysis of proteins in gushing-inducing culture supernatant revealed the protein PEX2_044840 with a molecular weight of 20 kDa with characteristics similar to PDE_04519. Together with its co-occurrence with *B. cinerea*, which is known to reduce the amount of gushing-reducing substances like Pau5p (see section 1.4.3.1), it was assumed that this fungus is involved in the gushing mechanism in sparkling wine, especially in those produced from grapes in temperate wine growing regions. Though, the exact effect of the PEX2_044840 protein was not yet investigated.

1.4.4 Secondary gushing in sparkling wine

Secondary gushing in sparkling wine can be caused by particles that act as nucleation sites for the release of carbon dioxide. Such particles are tannins or tartrates adhering on a rough surface of the inner glass wall of a bottle (Hennig, 1963) or fungal spores and bacteria originating from the used cork (Schanderl, 1964). Furthermore, metal ions (Schanderl, 1964), filtration aids (Rankine, 1977), excess carbon dioxide, or residual air in the headspace of the

bottle (Rankine, 1977) were described to provoke gushing in sparkling wine. The causes are similar to those found in secondary beer gushing and can likewise be positively influenced by applying good manufacturing practice.

1.5 Analysis of gushing-related proteins and their producing organisms for gushing prediction

Proteins from filamentous fungi were shown to be involved in the induction of primary gushing in sparkling wine when rotten grapes were used. Therefore, the use of raw materials without gushing potential is essential for sparkling wine producers. An analysis of the grapes or the base wines for the presence of filamentous fungi and gushing-inducing proteins produced by them is necessary to determine the gushing risk and therefore to make decisions about further processing of a lot.

1.5.1 Loop-mediated isothermal amplification (LAMP)

Vogt et al. (2017a) developed a DNA-based assay for the detection of the gushing-inducing fungus *P. oxalicum* on grapes. They used a technique called loop-mediated isothermal amplification (LAMP) (Notomi, 2000). LAMP facilitates direct amplification of specific DNA sequences under isothermal conditions with high sensitivity, specificity, and rapidity. Due to its independence from costly and highly dedicated laboratory equipment as well as its simple handling by evaluation of the assay result by visual in-tube detection, this method is ideally suited for on-site investigations. In contrast to assays based on the polymerase chain reaction (PCR), no time-consuming agarose gel electrophoresis is needed for signal detection (Tanner et al., 2015). For the reaction, the thermophilic *Bst* DNA polymerase with high strand displacement activity and a set of four primers (FIP = Forward Inner Primer, BIP = Backward Inner Primer, F3 = Forward Outer Primer, B3 = Backward Outer Primer) specific for six distinct sequences on the target DNA are used (Notomi, 2000). The binding sites of the primers are shown schematically in Figure 3.

The two inner primers (FIP and BIP) comprise two parts (F1c/F2 and B1c/B2), respectively, one for priming early in the reaction and one for self-priming later. The two outer primers (F3 and B3) are needed in the beginning of the reaction to peel off the first generated product strand from the genomic DNA matrix (Notomi, 2000). Additionally, two loop primers (LF = loop primer forward, LB = loop primer backward) can be added that accelerate the reaction by generating additional starting points for DNA synthesis (Nagamine et al., 2002). Their positions are the region between F2 and F1 in the direction of F1 to F2, respectively, and between B1 and B2 in the direction of B1 to B2.

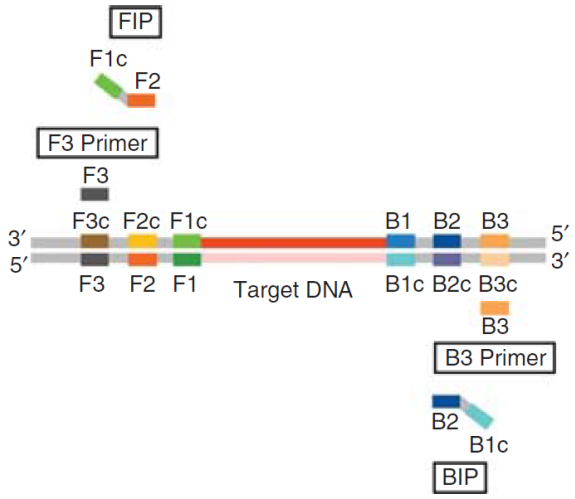


Figure 3: LAMP primer binding sites
The binding sites of the LAMP primers on the target DNA are shown (Tomita et al., 2008). FIP = Forward Inner Primer, BIP = Backward Inner Primer, F3 = Forward Outer Primer, B3 = Backward Outer Primer.

The LAMP reaction consists of two parts and will be described here only for one of the DNA strands. However, reactions run on both DNA strands in parallel. In the first part of the reaction, an initial DNA starting structure is produced with all four primers involved. The second part is for autocycling DNA amplification involving only the two inner primers (Notomi, 2000; Tomita et al., 2008). The step that produces the initial structure starts with binding of the F2 part of primer FIP and binding of primer F3 to their respective target sequence. This annealing initiates DNA synthesis by the *Bst* DNA polymerase and displacement of the newly synthesized strand releasing a FIP-linked complementary strand. The product forms a loop structure by backfolding of the F1c part of the FIP primer to the F1 region in the LAMP product. On this strand, the B2 part of primer BIP and primer B3 bind for initiation of DNA synthesis and strand displacement which results in the production of a dumbbell like double-looped DNA structure (see Figure 4, position 5). This DNA is converted to a stem-loop DNA structure by self-primed DNA synthesis serving as starting material for the autocycling DNA amplification step. As the reaction commences, more FIP and BIP primers, respectively, hybridize to the loop in the stem-loop DNA and prime strand displacement DNA synthesis releasing elongated concatemeric DNA structures as shown in Figure 4, positions 11 and 12 (Notomi, 2000; Tomita et al., 2008).

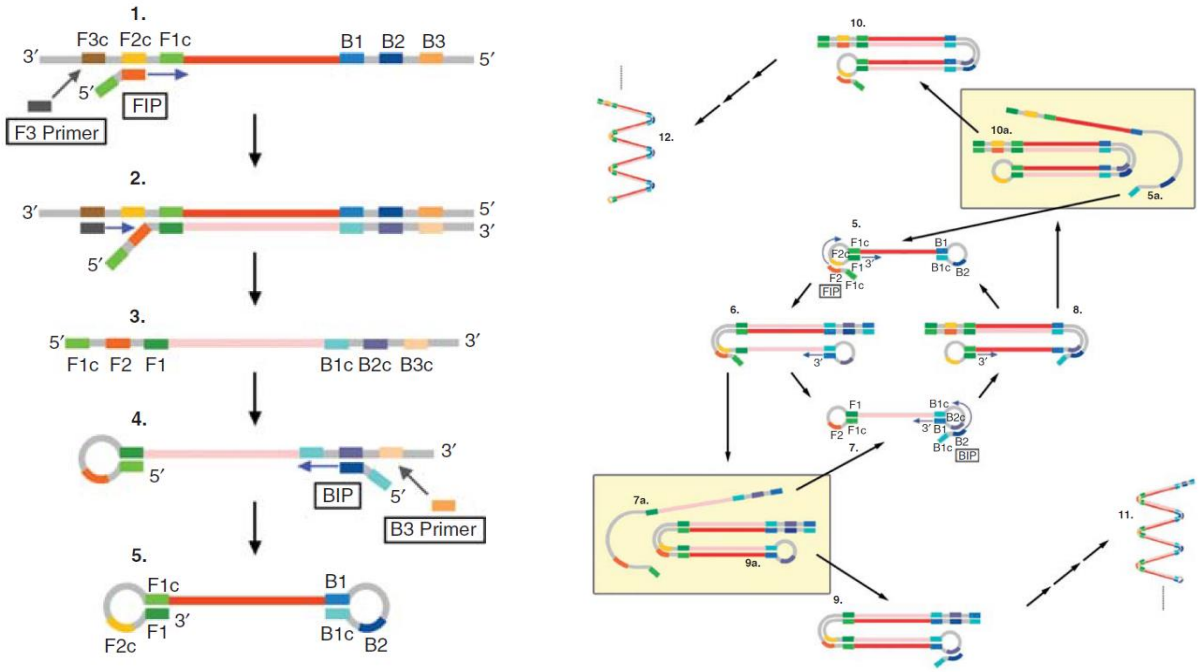


Figure 4: LAMP mechanism
The different steps of the LAMP mechanism are shown (Tomita et al., 2008). See Figure 3 for primer explanations.

Different methods were applied to visualize LAMP products which are summarized in the review of Zhang et al. (2014). Besides gel electrophoresis and electro- or immunochemical methods, in-tube detection can be performed. DNA-binding dyes such as SYBR Green (Noble and Fuhrman, 1998), Hoechst 33258 (Latt et al., 1975), or EvaGreen (Wang et al., 2006) have been used due to the occurrence of fluorescence upon binding of these dyes to double-stranded product DNA. Other authors used pyrophosphate ions that are released as by-product of DNA synthesis and form a complex with magnesium ions present in the LAMP reaction which precipitate and result in turbidity in a concentration-dependent manner (Mori et al., 2004; Mori et al., 2001). The use of a turbidimeter facilitates to measure magnesium pyrophosphate turbidity as a result of DNA synthesis in LAMP reactions in real-time. Indirect colorimetric indicators such as calcein were also used (Tomita et al., 2008). The fluorescence of calcein is quenched by the binding of manganese ions before the amplification reaction starts. Pyrophosphate ions that are produced during LAMP amplifications deprive manganese ions from the calcein complex resulting in a bright green fluorescence of calcein as a result of DNA synthesis during LAMP reactions. Another dye which was applied is the metal indicator hydroxy naphtol blue (Goto et al., 2009). The production of pyrophosphate ions during DNA amplification leads to a depletion of magnesium ions in the solution which causes a color change of the hydroxy naphtol blue indicator dye from violet to blue. Alternatively, pH-sensitive dyes such as neutral red or cresol red were used (Tanner et al., 2015). During DNA amplification, protons are released and the pH in the reaction changes to acidic. As a result, positive LAMP reactions containing neutral red are indicated by a color change from orange to pink, or pink to yellow with cresol red.

Niessen (2015) provided a comprehensive review about the application of LAMP assays for the detection and identification of fungi.

As mentioned previously, Vogt et al. (2017a) developed a LAMP assay for the detection of *P. oxalicum* with primers based on the coding gene for the gushing-inducing protein PDE_07106. The authors used calcein or neutral red as indicators and applied the assay on artificially and naturally infected grape samples.

LAMP assays for the detection of *B. cinerea* were developed by Duan et al. (2014) and Tomlinson et al. (2010). Duan et al. (2014) developed an assay using hydroxy naphtol blue as indicator dye. The authors optimized the assay for the detection of the fungus on inoculated tomato and strawberry petals and also tested diseased tissues from celery and cucumber. Tomlinson et al. (2010) developed a real-time LAMP assay and detected the fungus on infected rose petals and pelargonium leaves. Recently, Si Ammour et al. (2020) used a real-time LAMP assay kit for the detection of *B. cinerea* in grapevine bunch trash, immature and ripening berries. Moreover, several authors developed LAMP assays for the detection of fungicide-resistant *B. cinerea* isolates (Duan et al., 2018a; Duan et al., 2018b; Fan et al., 2019; Fan et al., 2018; Hu et al., 2017; Liu et al., 2019).

Two LAMP assays have been published that detect *P. expansum* together with other fungi but not exclusively: Tone et al. (2017) used melting curve analysis in conjunction with LAMP to discriminate *P. expansum* and three other fungal species, while Frisch and Niessen (2019) developed a LAMP assay using neutral red as indicator dye to detect *P. expansum* together with other patulin-producing fungi in a group-specific manner.

LAMP assays for the detection of gushing-relevant fungi have also been established for fungi involved in beer gushing. *Fusarium* spp. producing the hydrophobin Hyd5p were detected in cereal grains and malt using this amplification method (Denschlag et al., 2012, 2013). Niessen and Vogel (2010) developed a LAMP assay for the detection of the gushing-inducing fungus *F. graminearum* in barley and wheat in a species-specific manner.

1.5.2 Enzyme-linked immunosorbent assay (ELISA)

Detection of gushing-influencing organisms has some limitations since not the gushing-inducing factor itself is analyzed but rather its producer. Studies in beer gushing showed that the *Fusarium* level of barley or malt was an unreliable predictor of gushing tendency (Munar and Sebree, 1997). Therefore, direct analysis of gushing-influencing proteins is considered to provide the most reliable gushing prediction (Sarlin, 2012). In beer gushing, a competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of hydrophobins in order to predict the gushing potential of cereal raw materials (Sarlin et al., 2005b; Sarlin et al., 2007). Hereby, an antibody against a hydrophobin of *F. poae* was used.

In addition, Specker (2014) developed a competitive ELISA for the relative quantification of the hydrophobin FcHyd5p from *F. culmorum* in grain and malt as well as in brewing and beer samples. Moreover, the same author developed an ELISA for the detection of ns-LTP1 from barley as did several other authors (Evans et al., 1999; Murakami-Yamaguchi et al., 2009; Murakami-Yamaguchi et al., 2012). Using the antibody against barley ns-LTP1 from the study of Specker (2014), Kupfer (2018) optimized the competitive ELISA to detect ns-LTP1 from *V. vinifera*.

ELISA is one of the most frequently used quantitative immunoassays due to its high sensitivity and specificity (Luttmann et al., 2014). To perform the assay, antibody or antigen are adsorbed to a solid phase and the antigen concentration can be determined by an enzyme-substrate reaction that can be measured photometrically. For the generation of antibodies, purified proteins, cell fragments, or synthetic peptides can be used to trigger the immune system of animals such as rabbits, chickens, or mice. Different ELISA formats are available, with the sandwich and competitive ELISA as the most commonly applied (Luttmann et al., 2014).

The procedure of a competitive ELISA is schematically shown in Figure 5.

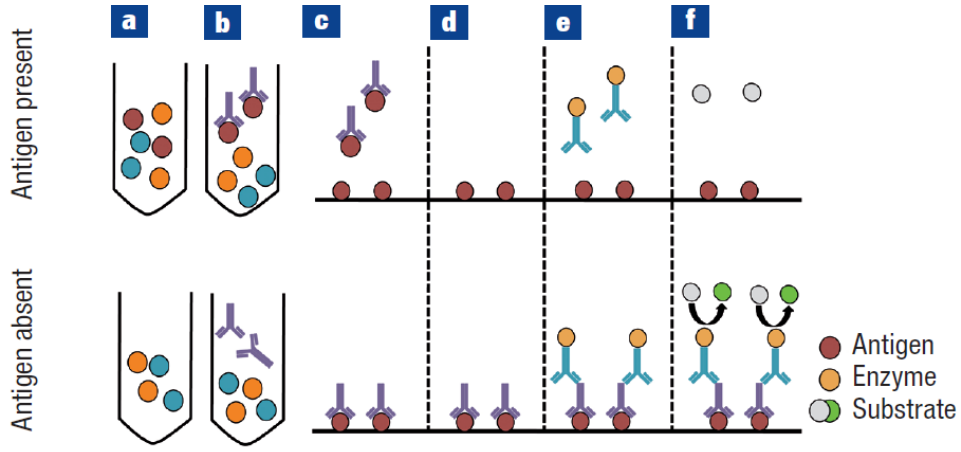


Figure 5: Competitive ELISA scheme
Steps of a competitive ELISA with present and absent antigen are shown (Shah and Maghsoudlou, 2016).

The primary antibody is incubated with a sample (see Figure 5, a) and binds to the antigen in case it is present in the sample (b). This mix is then transferred to a microtiter plate of which the solid phase is coated with antigen. Unbound antibodies can bind to the antigen on the solid phase (c). The more antibodies have already formed a complex with antigen present in the sample, the fewer antibodies are free to bind to the antigen on the solid phase: Thus, there is a competition between the antigen in the sample and the adhered antigen on the solid phase for the antibodies. After incubation, unbound antibodies are removed (d). The secondary antibody that is enzyme-marked binds to the primary antibody (e) and an added substrate leads to a color reaction detectable by a microplate reader (f). Due to the assay format, the measured extinction values are inversely proportional to the antigen concentration present in the sample (Shah and Maghsoudlou, 2016).

1.5.3 *Pichia pastoris* expression system

The development of an ELISA requires a standard for the creation of a calibration curve which is needed for the quantification of target protein in a sample. The standard can be obtained in high amounts by cloning and expression of the specific protein in an expression system. One suitable and commercially available system is the *Pichia pastoris* expression system. It is commonly used due to an easy-controlled expression process and high yields of recombinant protein. *Pichia pastoris* (*Komagataella phaffii* (Kurtzman, 2009)) is a methylotrophic yeast capable of metabolizing methanol (Cregg et al., 2000). The enzyme alcohol oxidase (AOX) catalyzes the first step in the methanol metabolism and is coded by the *AOX1* and *AOX2* genes, while the *AOX1* gene is responsible for the majority of alcohol oxidase activity (Cregg et al., 1989). The promoter derived from the *AOX1* gene drives expression of heterologous genes. This process is both methanol-induced and -regulated (Cregg et al., 2000; Tschopp et al., 1987). Heterologous proteins can be expressed intracellularly or secreted into the medium which requires a signal sequence to be included in the foreign protein. This signal sequence is frequently the α-factor secretion signal from *S. cerevisiae* (Cregg et al., 2000).

Several expression vectors are available including the pPICZα series (see Figure 6).

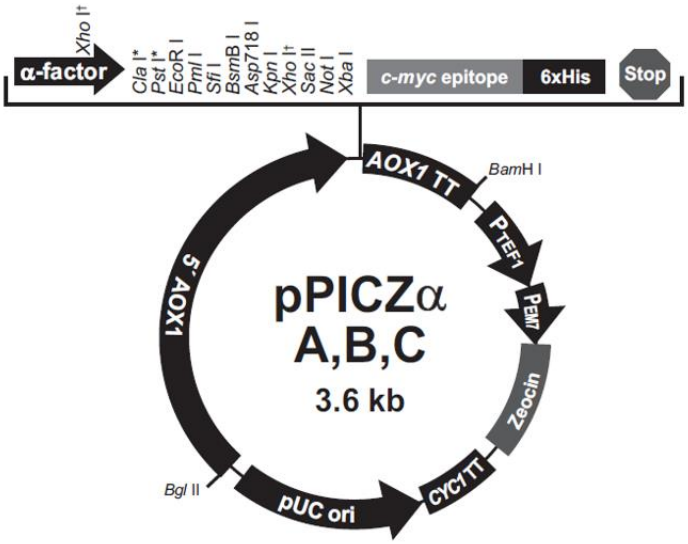


Figure 6: Map of pPICZα vector
The main features of the pPICZα vector series are shown (Invitrogen, 2010b).

These vectors contain the 5' *AOX1* promoter for methanol-inducible expression of the gene of interest. Between the promoter and the *AOX1* transcription termination region there is a multiple cloning site for integration of the gene of interest. The α-factor secretion signal allowing secretion of proteins into the medium is located upstream of the target gene. A *c-myc* epitope and a C-terminal 6xHis-tag can be used for detection and purification of the recombinant protein, respectively. Furthermore, the vector contains the *Sh ble* gene from *Streptoalloteichus hindustanus* conferring resistance to the antibiotic Zeocin™ for selection of transformants (Gatignol et al., 1988). The *TEF1* promoter and the EM7 promoter drive expression of the

Zeocin™ resistance gene in *Pichia pastoris* and *Escherichia* (*E*.) *coli*, respectively. The *CYC1* transcription termination region allows mRNA processing of the Zeocin™ resistance gene and a pUC origin leads to replication and maintenance of the plasmid in *E. coli*. Moreover, several restriction sites for integration are included (Invitrogen, 2010b).

Integration of the expression cassette into the *Pichia pastoris* genome is achieved by homologous recombination that requires the linearization of the vector prior to transformation (Cregg et al., 1985; Jansohn and Rothhämel, 2012).

Pichia pastoris has been used in various studies for successful expression of gushing-relevant proteins for tests regarding their gushing-inducing or -reducing potential. Using *Pichia pastoris* transformation, Lutterschmid et al. (2011) showed that the recombinant class II hydrophobin Hfb2 from *Trichoderma reesei* induced gushing in beer, while the recombinant class I hydrophobin FcHyd3p from *F. culmorum* did not. The recombinant fungispumin AfpA from *F. graminearum* and ns-LTP1 from *H. vulgare* reduced gushing. Also, the class II hydrophobins FcHyd5p from *F. culmorum* and Hfb1 from *Trichoderma reesei* were successfully expressed in *Pichia pastoris* and were shown to induce gushing (Niu et al., 2012; Stübner et al., 2010). Moreover, Specker et al. (2014) used *Pichia pastoris* to produce recombinant protein Z4 from barley and demonstrated its gushing-reducing effect.

1.6 Aim of the study

Gushing is an unwanted phenomenon leading to considerable economic losses and reputational damages in the beverage industry. In sparkling wine, proteins from filamentous fungi have been found to be involved in gushing induction, whereby the exact contribution of the specific proteins has not yet been assessed. In contrast to these fungal proteins, the protein ns-LTP1 from the grape itself has been assumed to have a gushing-reducing effect in sparkling wine. Early detection of the relevant fungi and their proteins in samples from vineyards as well as in musts or base wines can help to reduce the risk of gushing in sparkling wine. Therefore, the aim of this study was to develop and apply monitoring systems for filamentous fungi and their proteins that are involved in gushing of sparkling wine as well as to clarify the role of the protein ns-LTP1 from *V. vinifera* in the phenomenon.

The working hypotheses and anticipated experimental approaches underlying this aim were the following:

- Surface-active proteins from *P. expansum* and *P. oxalicum* are involved in gushing induction in sparkling wine.
- These proteins can be generated in sufficient amounts by cloning and heterologous expression to test their gushing-inducing potential in sparkling wine.
- Gushing-inducing fungal proteins can be detected and quantified in base wines by immunochemical assays using the recombinant proteins as calibration standards.
- The presence of the gushing-relevant fungi *P. expansum*, *P. oxalicum*, and *B. cinerea* can be monitored in sample materials from vineyards using rapid LAMP-based diagnostic assays.
- The protein ns-LTP1 from *V. vinifera* has a gushing-reducing effect in sparkling wine similar to the effect of ns-LTP1 from *H. vulgare* in beer.
- Ns-LTP1 *Vv* can be monitored in sparkling wine with an immunochemical assay.

2 Materials and methods

2.1 Materials

2.1.1 Equipment

The equipment that was used in the current study is listed in Table 1.

Table 1: Equipment
The used equipment and its model and manufacturer are listed.

Equipment	Model	Manufacturer
Autoclave	Systec VX-150	Systec GmbH, Linden, Germany
Bottle capper	Emily Ø 26-29 mm	Ferrari® Group, Parma, Italy
Camera (inside UV cabinet)		Intas Science Imaging Instruments GmbH, Göttingen, Germany
Camera Axio Cam	ICc 1	Carl Zeiss AG, Oberkochen, Germany
Camera Colony Doc it™		Ultra Violet Products Ltd, Upland, Canada
Camera mobile phone	Pixel 3a	Google LLC, Mountain View, CA, USA
Centrifuge	Rotina 380 R; Sigma 6-16K; Sigma 1-14	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
Computer		ASUSTeK COMPUTER INC., Taipei, Taiwan
Counting chamber	Thoma, depth 0.1 mm	BRAND GmbH, Wertheim, Germany
Dot blot apparatus		Stratagene, La Jolla, CA, USA
Electroporator	Gene Pulser® II Apparatus	Bio-Rad Laboratories, Inc., Hercules, CA, USA
FPLC fraction collector	F9-R	Cytiva, Marlborough, MA, USA
FPLC system	ÄKTApure 25L1	Cytiva, Marlborough, MA, USA
Freeze dry system	FreeZone 2.5	Labconco Corporation, Kansas City, MO, USA
Freezer	Comfort NoFrost GNP 3013-2	Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany
Gas burner	1230/1 natural gas	Carl Friedrich Usbeck KG, Radevormwald, Germany
Gel electrophoresis chamber		Peqlab Biotechnologie GmbH, Erlangen, Germany
Gel electrophoresis system	Mini PROTEAN® Tetra Cell	Bio-Rad Laboratories, Inc., Hercules, CA, USA

Table 1 (continued)

Equipment	Model	Manufacturer
Heating block	Dri-Block® 3	Cole-Parmer, Staffordshire, Stone, UK
Heating cabinet	TC 135 S; Heraeus B5042E	Tintometer GmbH, Lovibond Water Testing, Dortmund, Germany; Heraeus Instruments GmbH, Hanau, Germany
Homogenizer	Fastprep®-24; Bag Mixer	MP Biomedicals Germany GmbH, Eschwege, Germany; Interscience, St Nom la Bretèche, France
Magnetic stirrer	RCT basic; WiseStir MSH-20A; ARE	IKA®-Werke GmbH & Co. KG, Staufen, Germany; Witeg Labortechnik GmbH, Wertheim, Germany; VELP Scientifica, Usmate, Italy
Micro scale	SI-234	Denver Instruments, Bohemia, NY, USA
Microscope	Axiolab E	Carl Zeiss AG, Oberkochen, Germany
Multichannel pipette	RAININ 20-300 µL LTS	Mettler-Toledo GmbH, Gießen, Germany
Multipette®	Multipette® stream E3/E3x, 1000 µL	Eppendorf AG, Hamburg, Germany
Owl™ semi-dry electroblotting 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; Emax precision microplate reader; FLUOstar Omega	Biochrom Ltd., Cambourne, UK; Molecular Devices, San Jose, CA, USA; BMG LABTECH GmbH, Ortenberg, Germany
Pipette	Pipetman (5000, 1000, 200, 100, 20, 10 µL)	Gilson, Inc., Middleton, WI, USA
Power supply	Power Pac™ Basic; Power Pack P25	Bio-Rad Laboratories, Inc., Hercules, CA, USA; Biometra GmbH, Göttingen, Germany
Pulse control	Pulse Controller Plus, Model no. 165-2110	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Refrigerator	Profiline	Robert Bosch Hausgeräte GmbH, Munich, Germany

Table 1 (continued)

Equipment	Model	Manufacturer
Rocking shaker	Unitwist RT	UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg, Germany
Rotary shaker	Unimax 2010; Unitwist 300	Heidolph Instruments GmbH & Co. KG. Schwabach, Germany; UniEquip Laborgerätebau- und Vertriebs GmbH, Planegg, Germany
Scale	Scaltec; Kern 572	Denver Instrument, Bohemia, NY, USA; Kern & Sohn GmbH, Balingen-Frommern, Germany
Scanner	Bio-5000 Microtek	SERVA Electrophoresis GmbH, Heidelberg, Germany
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
Thermostat	Lauda Alpha	LAUDA Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany
Tube shaker	Reax 2	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Ultra-low temperature freezer	MDF-U700VX	Panasonic Healthcare Co., Ltd., Gunma, Japan
Ultrasonic homogenizer	SONOPULS HD 2070	BANDELIN electronic GmbH & Co. KG, Berlin, Germany
UV table	UVT-28 M	Herolab GmbH Laborgeräte, Wiesloch, Germany
Vacuum pump	PC 3003 VARIO	VACUUBRAND GmbH & Co. KG, Wertheim, Germany
Vortex mixer	Vortex Genie 2	Scientific Industries Inc., Bohemia, NY, USA

2.1.2 Consumables

The consumables that were used in the current study are listed in Table 2.

Table 2: Consumables
The used consumables and their type and manufacturer are listed.

Consumable	Type	Manufacturer
Apples		Local mart, Freising, Germany
Blender bags		VWR International, Radnor, PA, USA
Blotting paper sheets	Grade BF3, 330 g/m^2, 200 x 200 mm	Ahlstrom-Munksjö, Stockholm, Sweden
Cannula	Sterican®, 0.6 x 30 mm	B. Braun Biotech International, Melsungen, Germany
Combitips® advanced	10, 5, 1.5, 0.1 mL	Eppendorf AG, Hamburg, Germany
Cover glass	20 x 20 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Crown caps	FER E4, Ø 29 mm, seal 802	SOLOCAP-MAB S.A., Contrexéville, France
Cryogenic vial	Nunc® CryoTubes®	Sigma-Aldrich, St. Louis, MO, USA
Dialysis tube	Membra-Cel®, MWCO 3500, Ø 16 mm	SERVA Electrophoresis GmbH, Heidelberg, Germany
Electroporation cuvettes	Gene Pulser®, 2 mm gap width	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Filter discs	Grade 3 hw, Ø 70 mm	Ahlstrom-Munksjö, Stockholm, Sweden
Filter pipette tips	TipOne (1000, 200, 100, 20, 10 µL)	STARLAB GmbH, Hamburg, Germany
Glass beads	Ø 2.85-3.45 mm and Ø 1.25-1.65 mm; Ø 0.5 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany; Scientific Industries, Bohemia, NY, USA
Grapes		Local mart, Freising, Germany
HisPur Ni-NTA Chromatography Cartridge	5 mL	Thermo Fisher Scientific Inc., Waltham, MA, USA
Immun-Blot™ PVDF Membrane	For protein blotting, 10 x 15 cm, Ø 0.2 µm	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Inoculating loop	1 µL, 10 µL	VWR International, Radnor, PA, USA
Microscope slides	76 x 26 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Microtest plate 96 well		Sarstedt AG & Co., Nürnbrecht, Germany
Microtest plate 96 well NUNC-IMMUNO Module	F8, Maxisorp	Thermo Fisher Scientific Inc., Waltham, MA, USA

Table 2 (continued)

Consumable	Type	Manufacturer
Muslin bandage	100 % polyester, elastic	Altapharma Naturprodukte, Hamburg, Germany
Parafilm®	4" x 125'	Bemis Company, Inc., Oshkosh, WI, USA
Pasteur pipette	Pastette®, 3 mL graduated	Alpha Laboratories, Hampshire, UK
PCR tubes	0.2 mL 8-Strip	STARLAB GmbH, Hamburg, Germany
Petri dishes	92 x 16 mm, with cams (bacteria, yeasts) and without cams (filamentous fungi)	Sarstedt AG & Co., Nürnbrecht, Germany
Photometer cuvette	10 x 4 x 45 polystyrene	Sarstedt AG & Co., Nürnbrecht, Germany
Pipette tips	TipOne (1000, 200, 100, 20, 10 µL), 5 mL; RAININ 300 µL	STARLAB GmbH, Hamburg, Germany; Mettler-Toledo GmbH, Gießen, Germany
Reaction tube	15 mL, 50 mL	Sarstedt AG & Co., Nürnbrecht, Germany
Reaction vessel	1.5 mL, 2 mL	Sarstedt AG & Co., Nürnbrecht, Germany
Sea sand		Merck KGaA, Darmstadt, Germany
Spreaders	L-shaped	VWR International, Radnor, PA, USA
Sterile filter	Filtropur S 0.2 µm; Nalgene™ Rapid-Flow™ 0.2 µm, 0.45 µm	Sarstedt AG & Co., Nürnbrecht, Germany; Thermo Fisher Scientific, Waltham, MA, USA
Syringe	Injekt 20 mL/ 2 mL; HSW NORM-JECT 50 mL	B. Braun Melsungen AG, Melsungen, Germany; Henke Sass Wolf, Tuttlingen, Germany
Toothpick	NatureStar	Franz Mensch GmbH, Buchloe, Germany
Whatman™ 3MM Chr Chromatography Paper	46 x 57 cm	Thermo Fisher Scientific Inc., Waltham, MA, USA
White grape juice	100 % NFC juice	Eckes-Granini Deutschland GmbH, Nieder-Olm, Germany

2.1.3 Chemicals

The chemicals that were used in the current study are listed in Table 3.

Table 3: Chemicals
The used chemicals and their purity grade and manufacturer are listed.

Chemical	Purity grade	Manufacturer
10x Incubation mix *Taq* polymerase with $MgCl_2$		MP Biomedicals GmbH, Eschwege, Germany
2-mercaptoethanol	BioReagent, 99 %	Sigma-Aldrich, St. Louis, MO, USA
3-(N-morpholino)-propanesulfonic acid (MOPS)		Gerbu Biotechnik GmbH, Heidelberg, Germany
5-bromo-4-chloro-3-indolyl phosphate (BCIP) toluidine salt		Gerbu Biotechnik GmbH, Heidelberg, Germany
5x Phusion® GC reaction buffer		New England BioLabs GmbH, Ipswich, MA, USA
5x Phusion® HF reaction 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, Deutschland
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
Albumin fraction V (bovine serum albumin (BSA))	≥ 98 %, powdered, for molecular biology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ammonium acetate	For analysis	Merck KGaA, Darmstadt, Deutschland
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, Deutschland
BD Difco™ Yeast Carbon Base (YCB)	Laboratory use	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Bromophenol blue	For electrophoresis	PanReac AppliChem GmbH, Darmstadt, Germany
Calcium chloride dihydrate	For analysis	Merck KGaA, Darmstadt, Germany

Table 3 (continued)

Chemical	Purity grade	Manufacturer
Citric acid	≥ 99.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Coomassie Protein Assay Reagent		Thermo Fisher Scientific Inc., Waltham, MA, USA
Coomassie R-250		Bio-Rad Laboratories, Inc., Hercules, CA, USA
D(+)-Biotin	≥ 98.8%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
D(+)-Glucose monohydrate	For microbiology	Merck KGaA, Darmstadt, Germany
Diethanolamine		Merck KGaA, Darmstadt, 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
Di-sodium hydrogen phosphate monohydrate	≥ 98 %, p. a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	For microbiology	Gerbu Biotechnik GmbH, Heidelberg, Deutschland
dNTPs 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 disodium salt dihydrate (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
Formamide		Gerbu Biotechnik GmbH, Heidelberg, Germany
GeneRuler 1 kb DNA ladder		Thermo Fisher Scientific Inc., Waltham, MA, USA
GeneRuler 100 bp Plus DNA ladder	0.5 µg/µL	Thermo Fisher Scientific Inc., Waltham, MA, USA
Glycerol	High purity 87 %	Gerbu Biotechnik GmbH, Heidelberg, Germany
Glycine	99.56 %	Gerbu Biotechnik GmbH, Heidelberg, Germany

Table 3 (continued)

Chemical	Purity grade	Manufacturer
HPLC grade water		J.T. Baker, Center Valley, PA, USA
Hydrochloric acid	37 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Imidazole	Puriss. p.a., ≥ 99.5 % (GC)	Sigma-Aldrich, St. Louis, MO, USA
Isopropyl alcohol	≥ 99.5 %, for synthesis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
L(+)-Ascorbic acid	≥ 99 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lithium acetate dihydrate		Sigma-Aldrich, St. Louis, MO, USA
L-Leucine	Research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Lysozyme	From chicken egg, 100,000 units/mg	SERVA Electrophoresis GmbH, Heidelberg, Germany
Magnesium chloride	≥ 98.5 %, anhydrous	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Malt extract	For microbiology	PanReac AppliChem GmbH, Darmstadt, Germany
Methanol	≥ 98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Monosodium glutamate		Sigma-Aldrich, St. Louis, MO, USA
N,N-Dimethylformamide (DMF)	99.8 %, anhydrous	Sigma-Aldrich, St. Louis, MO, USA
Neutral red	Research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Ni-NTA Agarose Resin		SERVA Electrophoresis GmbH, Heidelberg, Germany
Nitro blue tetrazolium chloride (NBT)	Analytical grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
PageRuler™ Plus Prestained Protein Ladder	10 to 250 kDa	Thermo Fisher Scientific Inc., Waltham, MA, USA
para-Nitrophenylphosphate (pNPP)		Gerbu Biotechnik GmbH, Heidelberg, Germany
Peptone ex soya	Papainic digest, for microbiology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Phosphoric acid	85 %	J.T. Baker, Center Valley, PA, USA
Potassium chloride	≥ 99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Table 3 (continued)

Chemical	Purity grade	Manufacturer
Potassium dihydrogen phosphate	For analysis	Merck KGaA, Darmstadt, Germany
Roti®-Blue	5x concentrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
SERVA Triple Color Protein Standard III	5 to 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, Germany
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)	In pellets, research grade	SERVA Electrophoresis GmbH, Heidelberg, Germany
Sodium hydroxide	≥ 99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hypochlorite solution	12 % Cl	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium phosphate monobasic	≥ 98 %, p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium thiosulfate pentahydrate	For analysis	Merck KGaA, Darmstadt, Germany
T4 DNA ligase buffer	10x	Thermo Fisher Scientific Inc., Waltham, MA, USA
Tetramethylethylenediamine (TEMED)	~99 %	Sigma-Aldrich, St. Louis, MO, USA
Tricine	Pufferan®, ≥ 99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trifluoroacetic acid (TFA)	≥ 99 %	Sigma-Aldrich, St. Louis, MO, USA
Tris hydrochloride (Tris-HCl)	Molecular biology	Gerbu Biotechnik GmbH, Heidelberg, Germany
Tris-(hydroxymethyl)-aminomethane (Tris)	Ultrapure, analytical grade	Gerbu Biotechnik GmbH, Heidelberg, Germany
tri-Sodium citrate dihydrate	≥ 99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tryptone/Peptone ex casein	Granulated	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tween 20	For bacteriology	Gerbu Biotechnik GmbH, Heidelberg, Germany

Table 3 (continued)

Chemical	Purity grade	Manufacturer
Urea	Ultrapure reagent	Gerbu Biotechnik GmbH, Heidelberg, Deutschland
Yeast extract	Micro-granulated, for bacteriology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Yeast invertase (YI)		Sigma-Aldrich, St. Louis, MO, USA
Yeast nitrogen base	w/o amino acids	Becton, Dickinson and Company, Franklin Lakes, NJ, USA
Zeocin™		InvivoGen, San Diego, CA, USA
α-Lactose monohydrate	≥ 99 %	Sigma-Aldrich, St. Louis, MO, USA

2.1.4 Media and buffers

The composition of the media and buffers that were used in the current study are listed in the following tables.

All components of the respective media were weighted and filled up with deionized water to the desired volumes. Where necessary, the pH was adjusted with either sodium hydroxide or hydrochloric acid. After autoclaving or sterile filtration, liquid media were stored at 4 °C or ambient temperature (~23 °C, AT). Solid media were poured into Petri dishes with cams for yeasts and bacteria and without cams for filamentous fungi and were stored at 4 °C. Media containing antibiotics were freshly prepared and stored for max. two weeks at 4 °C. Media containing Zeocin™ were handled in the dark. Sugar components were sterile filtered separately and added to the media after autoclaving.

Table 4: Media
Components of the different media are listed.

Malt extract (ME) medium/agar	
Malt extract	2.00 % (w/v)
Peptone ex soya	0.20 % (w/v)
Agar agar (optionally)	1.50 % (w/v)
pH adjusted to 5.6	
Yeast carbon base (YCB) medium (component I: component II) (9:1)	
Component I	autoclaved
Ammonium sulfate	0.55 % (w/v)
Component II	sterile filtered 0.2 µm
Difco™ Yeast Carbon Base	11.70 % (w/v)
D-Glucose monohydrate	5.50 % (w/v)

Table 4 (continued)

Yeast extract-Peptone-Dextrose (YPD) medium/agar	
Yeast extract	1.00 % (w/v)
Tryptone/Peptone	2.00 % (w/v)
Agar agar (optionally)	1.80 % (w/v)
D-Glucose monohydrate	2.00 % (w/v)
Zeocin™ (optionally)	300 µg/mL
Yeast extract-Peptone-Dextrose-Sorbitol (YPDS) medium/agar	
Yeast extract	1.00 % (w/v)
Tryptone/Peptone	2.00 % (w/v)
Agar agar (optionally)	1.80 % (w/v)
D-Glucose monohydrate	2.00 % (w/v)
Sorbitol	1 M
Zeocin™ (optionally)	300 µg/mL
Buffered Minimal Glycerol Medium (BMG)	
Difco™ Yeast Nitrogen Base	1.34 % (w/v)
Biotin	4×10^{-5} % (w/v)
Tripotassium phosphate buffer (see Table 5)	100 mM
Glycerol	1.00 % (v/v)
Buffered Minimal Methanol Medium (BMM)	
Difco™ Yeast Nitrogen Base	1.34 % (w/v)
Biotin	4×10^{-5} % (w/v)
Tripotassium phosphate buffer (see Table 5)	100 mM
Methanol	0.50 % (v/v)
Buffered Complex Glycerol Medium (BMGY)	
Difco™ Yeast Nitrogen Base	1.34 % (w/v)
Biotin	4×10^{-5} % (w/v)
Tripotassium phosphate buffer (see Table 5)	100 mM
Yeast extract	1.00 % (w/v)
Tryptone/Peptone	2.00 % (w/v)
Glycerol	1.00 % (v/v)
Buffered Complex Methanol Medium (BMMY)	
Difco™ Yeast Nitrogen Base	1.34 % (w/v)
Biotin	4×10^{-5} % (w/v)
Tripotassium phosphate buffer (see Table 5)	100 mM
Yeast extract	1.00 % (w/v)
Tryptone/Peptone	2.00 % (w/v)
Methanol	0.50 % (v/v)
Low-salt lysogeny broth (LB) medium/agar	
Tryptone/Peptone	1.00 % (w/v)
Yeast extract	0.50 % (w/v)
Sodium chloride	0.50 (w/v)
Agar agar (optionally)	1.50 % (w/v)
Zeocin™ (optionally)	30 µg/mL
pH adjusted to 7.5	

Table 4 (continued)

Glycerol stock medium	
Monosodium glutamate	1.00 % (w/v)
α-Lactose monohydrate	1.60 % (w/v)
Agar agar	0.10 % (w/v)
Ascorbic acid	0.01 % (w/v)
Glycerol	12.00 % (v/v)

Table 5: Tripotassium phosphate buffer for BMG, BMM, BMGY, and BMMY medium
Components of the tripotassium phosphate buffer for BMG, BMM, BMGY, and BMMY medium are listed.

Tripotassium phosphate buffer	
Dipotassium phosphate	1 M
Monopotassium phosphate	1 M
pH adjusted to 6.0	

Table 6: Breaking buffer for preparation of cell lysates
Components of the breaking buffer for preparation of cell lysates of expression cultures are listed.

Breaking buffer	
Monosodium phosphate	50 mM
pH adjusted to 7.4	
Phenylmethylsulfonyl fluoride (freshly prepared, added right before use)	1 mM
Ethylenediaminetetraacetic acid	1 mM
Glycerol	5.00 % (v/v)

Table 7: Buffers for SDS-PAGE
Components of the different buffers and solutions for SDS-PAGE are listed.

5 x Anode buffer	
Tris	1 M
pH adjusted to 8.9	
5 x Cathode buffer	
Tris	0.5 M
Tricine	0.5 M
Sodium dodecyl sulfate	0.50 % (w/v)
pH adjusted to 8.25	
Gel buffer	
Tris	3 M
pH adjusted to 8.45	
Sodium dodecyl sulfate (SDS) solution	
Sodium dodecyl sulfate	25.00 % (w/v)

Table 7 (continued)

Application buffer		
Tris hydrochloride, pH 8.45		250 mM
Sodium dodecyl sulfate		7.50 % (w/v)
Glycerol		25.00 % (v/v)
Bromophenol blue		0.25 mg/mL
2-Mercaptoethanol		12.50 % (v/v)
	Separating gel (12 % acrylamide, 1 M Tris, pH 8.45)	**Stacking gel** (4 % acrylamide, 0.74 M Tris, pH 8.45)
Acrylamide/Bis, 30 %, 37.5:1	4.00 mL	0.68 mL
Gel buffer	3.33 mL	1.29 mL
Sodium dodecyl sulfate solution	0.04 mL	0.016 mL
$_dH_2O$	2.56 mL	3.21 mL
Tetramethylethylenediamine	0.007 mL	0.007 mL
Ammonium persulfate (10 % (w/v))	0.05 mL	0.033 mL

Table 8: Solutions for silver staining

Components of the different solutions for silver staining of SDS-PAGE gels are listed.

Fixation solution	
Ethanol	40.00 % (v/v)
Acetic acid	10.00 % (v/v)
Washing solution	
Ethanol	30.00 % (v/v)
Thiosulfate solution	
Sodium thiosulfate pentahydrate	0.02 % (w/v)
Silver nitrate solution	
Silver nitrate	0.20 % (w/v)
Developing solution	
Sodium carbonate	3.00 % (w/v)
Sodium thiosulfate pentahydrate	0.0005 % (w/v)
Formaldehyde (37 % (v/v))	0.10 % (v/v)
Stop solution	
Glycine	0.50 % (w/v)

Table 9: Solutions for Coomassie staining

Components of the different solutions for Coomassie staining of SDS-PAGE gels are listed.

Fixation solution	
Phosphoric acid	1.00 % (v/v)
Methanol	20.00 % (v/v)
Staining solution	
Methanol	20.00 % (v/v)
Roti®-Blue (5x)	20.00 % (v/v)
Washing solution	
Methanol	25.00 % (v/v)

Table 10: Buffers for Western blot and dot blot analysis
Components of the different buffers and solutions for Western blot and dot blot analysis are listed.

Transfer buffer	
Tris	50 mM
Glycine	190 mM
Sodium dodecyl sulfate	0.10 % (w/v)
Methanol	20.00 % (v/v)
Blocking solution	
Tris	0.02 M
Sodium chloride	0.2 M
Bovine serum albumin	3.00 % (w/v)
pH adjusted to 7.4	
Phosphate-buffered saline (PBS) buffer	
Monopotassium phosphate	4 mM
Disodium phosphate	16 mM
Sodium chloride	115 mM
pH adjusted to 7.4	
Phosphate-buffered saline-Tween (PBS-T) buffer	
Monopotassium phosphate	4 mM
Disodium phosphate	16 mM
Sodium chloride	115 mM
Tween 20	0.10 % (v/v)
pH adjusted to 7.4	
Alkaline Phosphatase (AP) buffer	
Tris hydrochloride	100 mM
Sodium chloride	100 mM
Magnesium chloride	5 mM
pH adjusted to 8.8	
Nitro blue tetrazolium chloride (NBT) solution	
Nitro blue tetrazolium chloride	75 mg/mL
Dimethylformamide	70.00 % (v/v)
5-Bromo-4-chloro-3-indolyl phosphate (BCIP) solution	
5-Bromo-4-chloro-3-indolyl phosphate	60 mg/mL
Dimethylformamide	100.00 % (v/v)

Table 11: Buffers for ELISA
Components of the different buffers for ELISA are listed.

Bicarbonate buffer	
Sodium carbonate	50 mM
Sodium hydrogen carbonate	50 mM
pH adjusted to 9.6	
Phosphate-buffered saline (PBS) buffer	
Monopotassium phosphate	1.5 mM
Disodium phosphate	8 mM
Sodium chloride	136 mM
Potassium chloride	2.7 mM
pH adjusted to 7.5	
Phosphate-buffered saline-Tween (PBS-T) buffer	
Monopotassium phosphate	1.5 mM
Disodium phosphate	8 mM
Sodium chloride	136 mM
Potassium chloride	2.7 mM
Tween 20	0.05 % (v/v)
pH adjusted to 7.5	
Blocking buffer	
Bovine serum albumin	2.00 % (w/v) in
pH adjusted to 7.5	PBS-T buffer
Diethanolamine buffer	
Diethanolamine	9.60 % (v/v)
Magnesium chloride	1 mM
pH adjusted to 9.8	

Table 12: Buffer for agarose gel electrophoresis
Components of the TAE buffer for agarose gel electrophoresis are listed.

50 x Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) buffer	
Tris hydrochloride	2 M
Ethylenediaminetetraacetic acid	0.5 M
Acetic acid	1 M
pH adjusted to 8.2	

Table 13: Buffer for gDNA isolation of filamentous fungi
Components of the extraction buffer for gDNA isolation of filamentous fungi are listed.

Extraction buffer	
Tris hydrochloride, pH 8.5	200 mM
Sodium chloride	250 mM
Ethylenediaminetetraacetic acid	25 mM
Sodium dodecyl sulfate	0.50 % (w/v)

Table 14: Solutions for cloning procedures
Components of calcium chloride solution and lithium acetate/ dithiothreitol solution are listed.

Calcium chloride solution	
Calcium chloride	60 mM
Glycerol	15.00 % (v/v)
3-(N-morpholino) propanesulfonic acid	10 mM
pH adjusted to 7.0	
Lithium acetate/ dithiothreitol solution	
Lithium acetate	100 mM
Sorbitol	0.6 M
Tris hydrochloride	10 mM
Dithiothreitol (freshly added)	10 mM
pH adjusted to 7.5	

Table 15: Buffer for LAMP
Components of the ammonium sulfate buffer for LAMP are listed.

10 x Ammonium sulfate buffer	
Ammonium sulfate	100 mM
Potassium chloride	100 mM
pH adjusted to 8.7	

Table 16: Buffers for protein purification
Components of the different buffers for protein purification are listed.

Buffer B1	
Tris	0.1 M
Ethylenediaminetetraacetic acid	10 mM
2-Mercaptoethanol	0.40 % (v/v)
Potassium chloride	100 mM
Dithiothreitol (freshly prepared)	10.00 % (w/v)
pH adjusted to 8.9	
Buffer B2	
Ammonium acetate	0.1 M
Methanol	100.00 % (v/v)
Buffer B3	
Ammonium acetate	0.1 M
Dithiothreitol	10 mM
Methanol	100.00 % (v/v)
Buffer B4	
Dithiothreitol	10 mM
Acetone	80.00 % (v/v)

Table 17: Buffers for protein purification by FPLC
Components of the different buffers for protein purification by FPLC are listed.

Binding buffer	
Sodium phosphate	50 mM
Sodium chloride	300 mM
Imidazole	10 mM
pH adjusted to 8.0	
Wash buffer	
Sodium phosphate	50 mM
Sodium chloride	300 mM
Imidazole	20 mM
pH adjusted to 8.0	
Elution buffer	
Sodium phosphate	50 mM
Sodium chloride	300 mM
Imidazole	400 mM
pH adjusted to 8.0	

2.1.5 Primers

Table 18 shows the sequences of the primers that were used in the LAMP assay for the detection of *P. oxalicum* (Vogt et al., 2017a). They are specific for the gene coding for the protein PDE_07106 from *P. oxalicum*.

Table 18: LAMP primers for the detection of *P. oxalicum*
The sequences and melting temperatures (T_m) of the LAMP primers for the detection of *P. oxalicum* are listed (Vogt et al., 2017a). A hyphen indicates the junction between the parts F1c/B1c and F2/B2 of FIP/BIP primers.

Primer	Sequence 5'-3'	T_m [°C]
FIP-RET21-ID1	TCACCGCAGTTGACGGGTCC-CCTTGCACACTCGTCGTGAC	>75.0
BIP-RET21-ID1	CCTCAGGCTGGAGCGGTCAAT-CTGGCGGCTCTTGTTGTTGA	>75.0
F3-RET21-ID1	CTGGACCTTTGGCATCTACC	59.4
B3-RET21-ID1	TGTCGGTGTAAGCAGGGTAG	59.4
LF-RET21-ID4	TGGACTGGGAGGCCTTTTGG	61.4
LB-RET21-ID4	GTCCCGGCAATGGCTTCACC	63.5

Table 19 shows the sequences of the primers that were used in the LAMP assay for the detection of *P. expansum*. They are specific for the gene coding for the protein PEX2_044840 from *P. expansum*. Primers F2_PEX2 and B2_PEX2 were used for PCR-based amplification of the smallest LAMP amplification product (see 2.2.4.6.2) and for sequencing of that product (see 2.2.4.5).

Table 19: LAMP primers for the detection of *P. expansum*
The sequences and melting temperatures (T_m) of the LAMP primers for the detection of *P. expansum* are listed. A hyphen indicates the junction between the parts F1c/B1c and F2/B2 of FIP/BIP primers.

Primer	Sequence 5'-3'	T_m [°C]
F3_PEX2	TGCAGACCGAATTCACTTGG	57.3
B3_PEX2	CGCGGGATTTCTTACCGAG	58.8
FIP_PEX2	TAGTCCAGCCCGGAAATGTGTC-CACCAACTATGGCTGTGAGT	>75.0
BIP_PEX2	TATCGTTATCAACGCCGGCA-GCGTTCCGACCATTGGAAGGT	>75.0
LF_PEX2	GAAGAATTTTCCAAAGTATGTGGCG	59.7
LB_PEX2	AGCGCAGGATACTCCATCAA	57.3
F2_PEX2	CACCAACTATGGCTGTGAGT	57.3
B2_PEX2	GCGTTCCGACCATTGGAAGGT	61.8

Table 20 shows the sequences of the primers that were used in the LAMP assay for the detection of *B. cinerea* (Tomlinson et al., 2010). They are specific for the intergenic spacer of the *B. cinerea* nuclear ribosomal DNA sequence.

Table 20: LAMP primers for the detection of *B. cinerea*
The sequences and melting temperatures (T_m) of the LAMP primers for the detection of *B. cinerea* are listed (Tomlinson et al., 2010). A hyphen indicates the junction between the parts F1c/B1c and F2/B2 of FIP/BIP primers.

Primer	Sequence 5'-3'	T_m [°C]
F3-Bcin	TCGGAGTGTCCTAGGAATGC	59.4
B3-Bcin	TGAGATGGCCAACTCTCAGA	57.3
FIP-Bcin	GCCTGCTCACCGGTAGTAGTGT-GTGAGCCCTTGGTCTAAAGC	>75.0
BIP-Bcin	GCAGAATCTGTCCCCGGTGAG-CGGGAGCAACAATTAATCGC	>75.0
Lf-Bcin	TGGGGTTAACTAGTCACCTATACG	61.0
Lb-Bcin	AGGTCACCTTGCAATGAGTGGA	60.3

Table 21 shows the sequences of the primers that were used in the LAMP assay for the detection of patulin-producing *Penicillium* species (Frisch and Niessen, 2019). They are specific for the gene coding for isoepoxydon dehydrogenase from the patulin biosynthetic pathway.

Table 21: LAMP primers for the detection of patulin-producing *Penicillium* species

The sequences and melting temperatures (T_m) of the LAMP primers for the detection of patulin-producing *Penicillium* species are listed (Frisch and Niessen, 2019). A hyphen indicates the junction between the parts F1c/B1c and F2/B2 of FIP/BIP primers. Wobble bases are defined as Y (C/T), N (A/C/G/T), R (G/A), and V (G/A/C).

Primer	Sequence 5'-3'	T_m [°C]
F3-IDH-ID5	AGTTTYGCGATCGATGTCAT	54.2
B3-IDH-ID5	CTTNGGCCCYAAGAAGTGG	58.8
FIP-IDH-ID5	TGGATGCCTGGGGRGACTTT-GGGYTTCGTYGAGCTGGT	>75.0
BIP-IDH-ID5	CGGGAATTCTACCGGTCCCCT-CAATTCCTGVACATGCTGC	>75.0
LF-IDH-ID5	AGTAGGGAGTAGCCGCCTT	58.8
LB2-IDH	GCAGCCTACGGGCCCTGC	65.1

Table 22 shows the sequences of the PCR-primers bt2a and bt2b that were used for sequencing-based identification of *Penicillium* species (Glass and Donaldson, 1995). They are specific for the *beta tubulin* gene in fungi.

Table 22: Primers for identification and sequencing of *Penicillium* species

The sequences and melting temperatures (T_m) of the primers for sequencing-based identification of *Penicillium* species are listed (Glass and Donaldson, 1995).

Primer	Sequence 5'-3'	T_m [°C]
bt2a (forward)	GGTAACCAAATCGGTGCTGCTTTC	62.7
bt2b (reverse)	ACCCTCAGTGTAGTGACCCTTGGC	66.1

Table 23 shows the sequences of the primers that were used for cloning procedures with the proteins PDE_04519 and PDE_07106 from *P. oxalicum* and PEX2_044840 from *P. expansum*.

Table 23: Primers for cloning procedures with PDE_04519 and PDE_07106 from *P. oxalicum* and PEX2_044840 from *P. expansum*

The sequences, melting temperatures (T_m), and annealing temperatures (T_a) of the primers for cloning procedures with PDE_04519 and PDE_07106 from *P. oxalicum* and PEX2_044840 from *P. expansum* are listed. Sequences that were added by the primers are written in lower case letters. Two annealing temperatures (T_a) (separated by a slash) were used for two-step PCR, the annealing temperature that is written behind the comma was used for colony PCR.

Primer	Sequence 5'-3'	T_m [°C]	T_a [°C]
Seq_Plasmid_f	CCAACAGCACAAATAACGGG	57.3	61.0
Seq _Plasmid_r	ATGGTCGACGGCGCTATTC	58.8	61.0
pPICZseq_f	GACTGGTTCCAATTGACAAGC	64.0	65.0
pPICZseqa_f	TACTATTGCCAGCATTGCTGC	66.0	65.0
pPICZseq_r	GCAAATGGCATTCTGACATCC	64.0	65.0
Acc65I_PDE_04519_f	tataggtaccGCGCCCACCAGCC	67.8	48.0/67.0, 69.0
XbaI_PDE_04519_r	gtgttctagagcGATGTACTGCCAGGC	68.0	48.0/67.0, 69.0

Table 23 (continued)

Primer	Sequence 5'-3'	T_m [°C]	T_a [°C]
MC_PDE_04519_f	TGAGCTTCACCCAGGCCCACTC	65.8	62.0
MC_PDE_04519_r	GGCACCAGTGGGATCGACGTAG	65.8	62.0
Acc65I_PDE_07106_f	tataggtaccGCTCCTGCCAGC	64.0	42.0/64.0, 63.0
XbaI_PDE_07106_r	ctcttctagAGGGAGAGCGTAGG	64.2	42.0/64.0, 63.0
MC_PDE_07106_f	GTCAACAACAAGAGCCGCCAG	61.8	58.0
MC_PDE_07106_r	CACGGCCAAGGTGGTGAAG	61.0	58.0
Acc65I_PEX2_f	gagaggtaccGCTCCAGTTGCTATTAC	66.5	50.0/66.0, 55.0
XbaI_PEX2_r	gagatctagagcGATGTACTCGGTAGTCAAC	68.2	50.0/66.0, 55.0
MC_PEX2_f	GAACGGAACTAACGCTTTGG	63.4	59.0
MC_PEX2_r	CAGGTAGATGGAGGAAGCAATG	64.6	59.0

2.1.6 Antibodies

The antibodies that were used in the current study are listed in Table 24.

Table 24: Antibodies
The used antibodies and their function and manufacturer are listed.

Antibodies	Function	Manufacturer
Anti-VOG-APA-IgG (polyclonal, produced in chicken)	Primary antibody for detection of PDE_07106 from *P. oxalicum* (Vogt-Hrabak, 2017)	Davids Biotechnologie GmbH, Regensburg, Germany
Anti-VOG-EFA-IgG (polyclonal, produced in chicken)	Primary antibody for detection of PDE_04519 from *P. oxalicum* (Vogt-Hrabak, 2017)	Davids Biotechnologie GmbH, Regensburg, Germany
Anti-Chicken-IgY-AP (produced in rabbit)	Secondary antibody for detection of PDE_07106 (Anti-VOG) and PDE_04519 from *P. oxalicum*	Sigma-Aldrich GmbH, Schnelldorf, Germany
Anti-6xHis-tag (monoclonal, produced in mouse)	Primary antibody for expression samples	Thermo Fisher Scientific Inc., Waltham, MA, USA
Pierce® Anti-Mouse IgG-AP (produced in goat)	Secondary antibody for expression samples	Thermo Fisher Scientific Inc., Waltham, MA, USA
Anti-PEX2_044840 (polyclonal, produced in rabbit)	Primary antibody for detection of PEX2_044840 from *P. expansum*	Davids Biotechnologie GmbH, Regensburg, Germany
Anti-PDE_07106 (polyclonal, produced in rabbit)	Primary antibody for detection of PDE_07106 from *P. oxalicum*	Davids Biotechnologie GmbH, Regensburg, Germany

Table 24 (continued)

Antibodies	Function	Manufacturer
Anti-ns-LTP1-*Vv* (polyclonal, produced in rabbit)	Primary antibody for detection of ns-LTP1 from *Vitis vinifera*	Davids Biotechnologie GmbH, Regensburg, Germany
Anti-nsLtp1-P2-IgG (polyclonal, produced in rabbit)	Primary antibody for detection of ns-LTP1 from *Hordeum vulgare* (Specker, 2014)	ImmnuoK, Amsbio, AMS Biotechnology, Oxfordshire, UK
Anti-Rabbit-IgG-AP (produced in goat)	Secondary antibody for detection of PEX2_044840 from *P. expansum*, PDE_07106 from *P. oxalicum* (primary antibody Anti-PDE_07106), ns-LTP1 from *Vitis vinifera* and ns-LTP1 from *Hordeum vulgare*	Sigma-Aldrich GmbH, Schnelldorf, Germany

2.1.7 Kits

Kits that were used in the current study are listed in Table 25.

Table 25: Kits
The used kits and their manufacturer are listed.

Kit	Manufacturer
FastDNA™ SPIN Kit for Soil	MP Biomedicals GmbH, Eschwege, Germany
GeneJet Gel Extraction Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
Pierce™ Coomassie (Bradford) Protein Assay Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA
QIAprep Spin MiniPrep Kit	Qiagen N. V., Venlo, the Netherlands
QIAquick PCR Purification Kit	Qiagen N. V., Venlo, the Netherlands

2.1.8 Enzymes

Enzymes that were used in the current study are listed in Table 26.

Table 26: Enzymes
The used enzymes and their manufacturer are listed.

Enzyme	Manufacturer
Acc65I FastDigest	Thermo Fisher Scientific Inc., Waltham, MA, USA
Bst Polymerase	New England BioLabs GmbH, Ipswich, MA, USA
DraI FastDigest	Fermentas, Waltham, MA, USA
Phusion HF Polymerase	New England BioLabs GmbH, Ipswich, MA, USA
T4 DNA Ligase	Fermentas, Waltham, MA, USA
Taq Polymerase	MP Biomedicals, Santa Ana, CA, USA
XbaI FastDigest	Fermentas, Waltham, MA, USA

2.1.9 Plasmids

Plasmids that were used in the current study are listed in Table 27.

Table 27: Plasmids
The used plasmids, their features, and their manufacturer are listed.

Plasmid	Feature	Manufacturer
pPICZαA	*AOX1* promoter for methanol-induced expression of gene of interest, α-factor secretion signal for secreted expression of recombinant protein, Zeocin™ resistance gene for selection in *E. coli* and *Pichia pastoris*, 6xHis-tag and *c-myc* epitope for purification and detection of recombinant protein	Thermo Fisher Scientific Inc., Waltham, MA, USA
pMA-RQ	Ampicillin resistance gene for selection in *E. coli* Codon-optimized *PEX2_044840* gene	Thermo Fisher Scientific Inc., Waltham, MA, USA

2.1.10 Software and databases

The software programs and databases that were used in the current study are listed in Table 28.

Table 28: Software and databases
The used software and databases are listed with their function and web address.

Company/Program	Function	Web address
Adobe Inc., Photoshop	Editing of pictures	Installed on computer
Bioinformatics resource portal of the Swiss Institute of Bioinformatics, ExPASy ProtParam	Calculation of protein properties	https://web.expasy.org/protparam/
BMG LABTECH, SPECTROstar Nano	Operation and analysis of microplate reader	Installed on a computer at TMW
Center for Biological Sequence Analysis at Technical University of Denmark, SignalP-5.0 Server	Prediction of signal sequence	http://www.cbs.dtu.dk/services/SignalP/
Cytiva, Unicorn 6.3	Data analysis of FPLC results	Installed on a computer at Chair of Microbiology (TUM)
Eiken Genome, PrimerExplorer V.5	Design of LAMP primers	http://primerexplorer.jp/e/
Eurofins, Oligo Analysis Tool	Calculation of primer parameters	https://www.eurofinsgenomics.eu/de/ecom/tools/oligo-analysis/
GATC Biotech/ Eurofins	Sequencing	https://www.eurofinsgenomics.eu
Intas, GDS Application	Documentation of agarose gels	Installed on a computer at TMW

Table 28 (continued)

Company/Program	Function	Web address
Microsoft, Office	Analysis of data and writing	Installed on computer
Microtec, Scan Wizard Bio	Scanning of acrylamide gels	Installed on a computer at TMW
Molecular Devices, Soft Max®	Operation and analysis of microplate reader	Installed on a computer at TMW
National Center for Biotechnology Information, BLAST	Sequence-based research about proteins/genes	https://www.ncbi.nlm.nih.gov
Sigma Aldrich, DNA Oligos in Tubes	Calculation of primer parameters	https://www.sigmaaldrich.com/pc/ui/tube-home/standard
SnapGene, SnapGene® Viewer 4.2.9	Graphical depiction of vector maps	http://www.snapgene.com/products/snapgene/release_notes/?referrer=SnapGene%2520Viewer
Thermo Fisher, NanoDrop ND-1000	Documentation and analysis of Nanodrop results	Installed on a computer at TMW
Tom Hall for Ibis Therapeutics, BioEdit	Multiple sequence alignment	http://www.softpedia.com/get/Science-CAD/BioEdit.shtmL
Westerdijk Fungal Biodiversity Institute, CBS-KNAW	Sequence alignment and identification tool	http://www.westerdijkinstitute.nl
Zeiss, Zen 2	Documentation by microscope camera	Installed on a computer at TMW

2.1.11 Sparkling wines, base wines, and musts

The sparkling wines and base wines that were used in the current study are listed in Table 29.

Table 29: Sparkling wines and base wines

The used sparkling wines and base wines are listed with information about white or red variety and gushing potential.

Sample number	Type	Gushing-potential according to manufacturer	Gushing-potential after opening of the bottle
1	White		Gushing-negative
2	White		Gushing-negative
3	Red		Gushing-negative
4	Red		Gushing-negative
5	White		Gushing-negative
6	White		Gushing-negative

Table 29 (continued)

Sample number	Type	Gushing-potential according to manufacturer	Gushing-potential after opening of the bottle
7	Red	Gushing-positive	
8	White	Gushing-positive	
9	White	Gushing-negative	
10	Red	Gushing-negative	
11	Red	Gushing-negative	
12	White	Gushing-positive	
13	Red	Gushing-positive	
14	White	Gushing-negative	
15	White	Gushing-negative	
16	Red	Gushing-positive	Gushing-negative
17	White	Gushing-positive	
18	Red	Gushing-positive	
19	White	Gushing-positive	
20	Red	Gushing-positive	Gushing-negative
21	White	Gushing-positive	
22	Base wine, Red	Gushing-negative	
23	Red	Gushing-positive	Gushing-negative
24	Base wine, Red	Gushing-negative	
25	Base wine, Red	Gushing-negative	
26	White	Gushing-negative	
27	White	Gushing-negative	
28	Red	Gushing-negative	
29	White	Gushing-negative	
30	White	Gushing-positive	
31	White	Gushing-positive	Gushing-negative
32	White	Gushing-positive	Gushing-negative
33	White	Gushing-positive	Gushing-negative
34	White	Gushing-positive	Gushing-negative
35	Red	Gushing-positive	Gushing-negative
36	White	Gushing-positive	Gushing-negative
37	White	Gushing-positive	
38	White	Gushing-positive	
39	White	Gushing-negative	
40	Base wine, White	Gushing-negative	
41	Base wine, White	Gushing-negative	
42	White	Gushing-negative	
43	Base wine, White	Gushing-negative	
44	Base wine, White	Gushing-negative	
45	Base wine, White	Gushing-negative	
46	Base wine, White	Gushing-negative	

The musts that were used in the current study and were provided by project partners from Hochschule Geisenheim University (HGU) are listed in Table 30.

Table 30: Musts
The used musts are listed with information about variety of the grapes and nutrient addition.

Must name	Variety	Nutrient addition
Must A	Riesling	none
Must B	Riesling	0.5 g/L diammonium hydrogen phosphate (DAP)

2.1.12 Organisms

Organisms that were mainly used in the current study are listed in Table 31. Additional organisms are listed in Table 47, Table 48, and in section 3.1.

Table 31: Organisms
Organisms that were mainly used in the current study are listed with their denotation, source, and number in the non-public strain collection at the Chair of Technical Microbiology, Weihenstephan, Germany (TMW).

Species	Strain denotation	Source	TMW number
E. coli	TOP10	TMW strain collection	2.580
E. coli	TOP10 pPICZαA	TMW strain collection	2.651
E. coli	Transformant PDE_04519	This study	2.2196
E. coli	Transformant PDE_07106	This study	2.2197
E. coli	Transformant PEX2_044840	This study	2.2198
Pichia pastoris	X33	TMW strain collection	3.0177
Pichia pastoris	Transformant with empty pPICZαA vector	This study	3.1068
Pichia pastoris	Transformant PDE_07106	This study	3.1069
Pichia pastoris	Transformant PDE_04519	This study	3.1079
Pichia pastoris	Transformant PEX2_044840	This study	3.1092
Pichia pastoris	Transformant PEX2_044840	This study	3.1089
P. expansum		TMW strain collection	4.2808
P. expansum		TMW strain collection	4.2806
P. expansum		TMW strain collection	4.2805
P. oxalicum		TMW strain collection	4.2539
P. oxalicum		TMW strain collection	4.2553
B. cinerea		TMW strain collection	4.2527
B. cinerea		TMW strain collection	4.2743

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Cultivation of organisms

Filamentous fungi were cultivated on ME agar plates (see Table 4) at AT for 7 d.
For production of fungal spumate, 600 mL of YCB medium (see Table 4) were inoculated with a conidial suspension (1 x 10^6 spores) and incubated in a Fernbach flask at AT shaken at 80 rpm for 7 d. Following filtration through a sterile muslin bandage and subsequently through a sterile filter cartridge (0.45 µm), the cell-free supernatant was used for foam fractionation (see 2.2.1.4).

Yeasts were cultivated on YPD agar plates (see Table 4) at 30 °C for 2-3 d. Liquid cultures of yeasts were shaken under the same conditions at 240 rpm.
For protein expression in yeasts, 25 mL BMGY/BMG medium (see Table 4) were inoculated with a single yeast colony in a 250 mL baffled flask closed with sterile gauze and incubated at 30 °C shaken at 240 rpm overnight to an OD_{600} = 2-4. The cells were harvested at 2,500 x g for 5 min at AT and resuspended in BMMY/BMM medium (see Table 4) to an OD_{600} = 1 (~250 mL). The culture was incubated in 1 L baffled flasks closed with sterile gauze at 30 °C shaken at 240 rpm for 24 h (PEX2_044840 transformants) to 48 h (PDE_04519 and PDE_07106 transformants). Methanol (100 % (v/v)) was added to the culture every 24 h to maintain a methanol concentration of 0.5 % in the culture.

Bacteria were cultivated on LB agar plates (see Table 4) at 37 °C overnight. Liquid cultures of bacteria were shaken under the same conditions at 180 rpm.

2.2.1.2 Cryoconservation of organisms

For cryoconservation of filamentous fungi, cultures were grown in 50 mL ME broth (see Table 4) mixed with porous clay granules without shaking at AT. After 4-7 d, clay granules were transferred to 80 % (v/v) glycerol in cryogenic vials and stored at -80 °C.

For cryoconservation of yeasts, cultures grown for 3 d on YPD agar plates were transferred to ME agar plates (see Table 4) with a sterile inoculating loop. After 2 d of incubation at AT, the cells were suspended in 5 mL glycerol stock medium (see Table 4) and harvested after homogenization of the culture surface with a Drigalski spatula. The suspension was vortexed and stored at 4 °C overnight. The next day, 1.8 mL of the suspension were transferred into a cryogenic vial, vortexed, and stored at -80 °C.

For cryoconservation of bacteria, 10 mL overnight culture in LB medium (see Table 4) were centrifuged at 4,000 x g for 5 min. The pellet was resuspended in 800 µL freshly prepared LB medium with respective antibiotic. The suspension was added to 1 mL sterile glycerol (80 % (v/v)) in a cryogenic vial, vortexed, and stored at -80 °C.

2.2.1.3 Preparation of conidial suspensions

Fungal cultures grown on ME agar plates (see Table 4) were suspended in 5 mL sterile deionized water with 300 µL Tween 20 and harvested after homogenization of the culture surface with a Drigalski spatula. After centrifugation at 7,379 x g for 5 min at AT, the pellet was washed three times with 5 mL sterile deionized water, respectively. The resulting pellet was stored in 50 % (v/v) glycerol at 4 °C. When immediate use was required instead of storage in glycerol, the resulting pellet was suspended in 1 mL sterile deionized water and glass beads (0.1 g Ø 0.5 mm, 0.3 g Ø 1.25-1.65 mm) were added, followed by vortexing for 10 min. Total conidial numbers were counted using a Thoma hemocytometer.

2.2.1.4 Foam fractionation

The cell-free supernatant of fungal liquid cultures (see 2.2.1.1) was transferred into a conical flask with lateral outlet and nitrogen was advected (20 NL/h to 80 NL/h) through a porous glass frit to generate foam in the upper part of the flask. The foam ("spumate") was collected at the lateral outlet of the flask.

2.2.1.5 Gushing tests

Commercial sparkling wine bottles were precooled to 4 °C before opening and inoculated with the desired amount of protein purified from culture supernatant or cell lysate. After re-sealing with sterile crown caps, the bottles were incubated for 20 h with horizontal shaking at 40 rpm at AT. Before opening, the bottles were placed upright for 2 h at AT. To determine the weight loss due to gushing the bottles were weighted before and after opening. The loss of CO_2 during the initial opening of bottles was subtracted from all obtained gushing results. Untreated bottles and bottles treated with proteins purified from *Pichia pastoris* wild type strain X33 culture supernatant or cell lysate as negative controls were used in each experiment. The experiments were conducted in duplicates. The sparkling wines for the gushing tests with protein PEX2_044840 and respective negative controls were all from the same production lot. A different production lot was used for the tests with protein PDE_07106 and respective negative controls. Statistical analysis of the gushing test results was conducted with a paired t-test ($\alpha = 0.05$).

2.2.1.6 Artificial contamination of sample material

Grapes and apples from a local mart were surface-sterilized in 70 % (v/v) ethanol for 5 min, followed by immersion in 1 % (v/v) sodium hypochlorite for 30 sec. Each step was followed by two washing steps in sterile deionized water. Prick-infection of grapes and apples was conducted after dipping a sterile toothpick into a conidial suspension of *P. expansum* TMW 4.2805 (6 x 10^8 spores/mL). As negative control *P. brevicompactum* TMW 4.2921 (8 x 10^8 spores/mL) and as non-inoculated control sterile deionized water were used. For prick-infection of apples, the fruit was cut into three pieces excluding the core. Infected grapes were

incubated in sterile 50 mL reaction tubes at AT over a period of 5 d, each tube filled with three grapes. Infected apples were incubated in sterile blender bags at AT over a period of 5 d, each bag filled with one apple piece.

Ten milliliters of commercial apple juice, grape juice, and apple puree (diluted 2:1 with sterile deionized water) were inoculated with 1 x 10^7 spores of *P. expansum* TMW 4.2808. As negative control *P. brevicompactum* TMW 4.2921 (1 x 10^8 spores) and as non-inoculated control 500 µL sterile deionized water were used. Infected matrices were incubated at AT over a period of 5 d.

2.2.1.7 Must inoculation

For the testing of antibodies, sterile must (see Table 30, must A and B) was inoculated with *P. oxalicum* (TMW 4.2553) by project partners at HGU (Geisenheim, Germany). Therefore, the fungi were grown on grape juice agar (grape juice with 70 °Oe, pH adjusted to 6.0, 2.00 % (w/v) agar agar) plates for 5 d at 25 °C at HGU. From the overgrown plates, three small plates (Ø 1.5 cm) were cut and transferred to 750 mL sterile must, respectively. After incubation for 3 weeks at AT, the cultures were decanted to remove the mycelium and sent to TMW. The cell-free must was dialyzed (see 2.2.2.1) and lyophilized (see 2.2.2.2) for further analysis.

2.2.2 Protein-chemical methods

2.2.2.1 Dialysis

Samples were dialyzed to remove ethanol, salts, and other small interfering substances. Therefore, the samples were transferred into a dialysis tube with a molecular weight cut-off of 3.5 kDa and dialyzed for 2 d against twenty times their volume of circulating deionized water at 4 °C. The water was changed three times a day.

2.2.2.2 Lyophilization

Lyophilization was performed in order to concentrate the proteins of the samples. After dialysis, the samples were frozen and freeze-dried for 1-2 d. Lyophilized samples were stored at 4 °C. Purified lyophilized protein was weighted before storage.

2.2.2.3 Protein purification

Protein purification of lyophilized samples for further analysis by SDS-PAGE was performed according to Vogt et al. (2016). The used buffers are listed in Table 16. Purified samples were stored at -20 °C.

2.2.2.4 Expression and purification of recombinant proteins

For determination of optimal expression conditions and screening for best transformants, 1 mL samples of BMMY yeast expression cultures were withdrawn after different time points of incubation. After centrifugation at 15,000 x g for 3 min at 4 °C, the supernatant was transferred

to a new reaction vessel and both supernatant and pellet were stored at -80 °C for further analysis.

For optimized expression experiments, the expression cultures were centrifuged at 15,000 x g for 30 min at 10 °C after incubation for 24-48 h. The supernatant was directly used for protein analysis or purification, whereas a cell lysate was needed from the cell pellet. Therefore, for each culture, one-tenth freshly prepared breaking buffer (see Table 6) was added to the cell pellet. For treatment in a FastPrep homogenizer, 0.2 g sterile glass beads (Ø 0.5 mm, 1.25–1.55 mm, 2.85-3.45 mm) were added. Treatment in FastPrep (45 sec, 24*2, 5 m/sec) or with ultrasonication (45 sec, 90 % power, 50 % cycle, on ice) followed four times, respectively, with short intermediate storage periods of samples on ice to prevent overheating. After centrifugation at 17,000 x g for 30 min at 4 °C, the supernatants (= cell lysates) were transferred to new reaction vessels.

For protein analyses, the culture supernatants or cell lysates were used for affinity purification of expressed proteins using Ni-NTA agarose resins according to the manufacturer's instructions.

For gushing tests and to obtain preparative quantities, protein purification was performed by immobilized metal affinity chromatography (IMAC) using an ÄKTApure 25L1 FPLC system (see 2.2.2.6).

2.2.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with staining methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Schägger and Jagow (1987) in order to analyze proteins and their molecular weight. The used buffers and solutions are listed in Table 7. Stacking and separating gels were freshly prepared and successively poured. Lyophilized samples were resolved in deionized water before mixing with application buffer 5:1, followed by heating to 95 °C for 10 min before application to the gel. SERVA Triple Color Protein Standard 3 was used as molecular weight marker. The electrophoresis was conducted at 80 V for 10 min for accumulation of the proteins in the stacking gel and subsequently at 110 V for 1 h for separation of proteins.

Silver staining of SDS-PAGE gels was performed comparable to Blum et al. (1987). The different steps are shown in Table 32 and the used solutions in Table 8.

Table 32: Silver staining of SDS-PAGE gels
The different steps of the silver staining of SDS-PAGE gels are listed (comparable to Blum et al. (1987)).

Step	Reagent	Duration
Fixation	Fixation solution	> 3 h or overnight
Washing	Washing solution	2 x 20 min
Washing	$_dH_2O$	20 min
Sensitization	Thiosulfate solution	max. 1 min
Washing	$_dH_2O$	3 x 20 sec
Silver staining	Silver nitrate solution	20 min
Washing	$_dH_2O$	3 x 20 sec
Development	Developing solution	1-3 min
Washing	$_dH_2O$	3 x 20 sec
Stop	Stop solution	5 min
Storage	$_dH_2O$	3 x 20 sec

Coomassie staining of SDS-PAGE gels was performed according to the manufacturer's recommendations. The different steps are shown in Table 33 and the used solutions in Table 9.

Table 33: Coomassie staining of SDS-PAGE gels
The different steps of the Coomassie staining of SDS-PAGE gels are listed.

Step	Reagent	Duration
Fixation	Fixation solution	60 min
Staining	Staining solution	2-15 h
Washing	Washing solution	5 min

The staining procedures were stopped after the occurrence of visible protein bands. Stained gels were photographed for documentation. To analyze specific proteins bands on the gel, the bands were excised.

2.2.2.6 Fast protein liquid chromatography (FPLC)

Protein purification was performed by immobilized metal affinity chromatography (IMAC) using an ÄKTApure 25L1 FPLC system equipped with a 5 mL HisPur™ Ni-NTA Chromatography Cartridge following the manufacturer's recommendations. The buffers are listed in Table 17 and were filtered and degassed before use. Before sample application (1 L culture supernatant), the column was washed (5 column volumes (CV) water) and equilibrated (5 CV binding buffer) as well as washed (10 CV wash buffer) to flush unbound proteins out. The elution of the target protein was performed by using a two-step elution (1. 20 % (v/v) elution buffer (2 CV); 2. 100 % (v/v) elution buffer (8 CV)), followed by washing (5 CV wash buffer, 5 CV water) and re-equilibration (5 CV binding buffer) or storage of the column in 20 % (v/v) ethanol (5 CV ethanol). Eluted proteins were detected with a UV detector at 280 nm and 1.5 mL

fractions were collected. Fractions were dialyzed and if necessary lyophilized for further analyses.

2.2.2.7 Protein quantification

Proteins were quantified using the Pierce™ BCA Protein Assay Kit and Pierce™ Coomassie (Bradford) Protein Assay Kit (Table 25) according to the manufacturers' instructions. Triplicates were measured with a microplate reader from all samples. As standards, a 1:2 serial dilution starting at 2 mg/mL BSA or YI was used. PBS buffer was used as BLANK. The BLANK extinction value was subtracted from the measured values of the samples and the standards (both diluted in PBS buffer). The concentration of samples was determined with the standard curve. Potential dilutions of samples were included in the calculation.

2.2.2.8 Protein identification

For verification of amino acid sequence identity of proteins, the proteins to be analyzed were separated by SDS-PAGE (see 2.2.2.5) and excised.

Analysis of the excised protein bands was performed with nano-ESI-LC-MS/MS, followed by Mascot analysis in the UniProt database. This proteomic analysis was performed at the Protein Analysis Unit (ZfP) of the Ludwig Maximilians University of Munich, Germany, a registered research infrastructure of the Deutsche Forschungsgemeinschaft (DFG, RI-00089).

Analysis of the excised protein bands was also performed by BayBioMS (Bavarian Center for Biomolecular Mass Spectrometry, Freising, Germany). Respective bands were digested with trypsin according to standard procedures (Shevchenko et al., 2006). Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was performed, followed by label-free quantification via MaxQuant (Cox and Mann, 2008) and data analysis via Perseus (Tyanova et al., 2016).

For protein sequencing by Edman degradation, the proteins were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane (see 2.2.3.1). The membrane was stained after blotting of the proteins from the gel onto the membrane (Goldman et al., 2016). Therefore, the membrane was washed three times in HPLC grade water for 5 min, respectively. Incubation for 5 min in solution 1 (0.025 % (w/v) Coomassie R-250 in 40 % (v/v) methanol) was followed by incubation for 5-10 min in 50 % (v/v) methanol. The stained membrane was washed with HPLC grade water and air-dried. Interesting protein bands were excised and sent to TOPLAB GmbH (Martinsried, Germany) for N-terminal protein sequencing by Edman degradation.

2.2.3 Immunochemical methods

2.2.3.1 Western blot analysis

Western blot analysis was conducted for immunochemical detection of proteins by specific antibodies. The used buffers are listed in Table 10. As positive controls, GFP with His-tag or the corresponding peptides were used. After separation of proteins by SDS-PAGE (see 2.2.2.5), the proteins were blotted onto a PVDF membrane by semi-dry electroblotting. Thus, the membrane was saturated in 100 % (v/v) methanol for 30 sec, followed by washing in deionized water for 2 min. The membrane, the acrylamide gel, and six blotting papers were equilibrated in transfer buffer for 20 min on a rocking shaker. The blotting was conducted at 50 mA for 1 h with the following assembly: Cathode – three blotting papers – gel – membrane – three blotting papers – anode. Afterwards, the membrane was incubated in blocking solution overnight at 4 °C or for at least 2.5 h at AT. After blocking, the membrane was washed three times with PBS-T buffer for 10 min, respectively. The respective primary antibody (see Table 24, diluted 1:2,000 in 25 mL PBS-T buffer) was applied for 1.5 h at AT. Following three washing steps with PBS-T buffer, the secondary antibody (see Table 24, diluted 1:5,000 in 25 mL PBS-T buffer) was applied for 1.5 h at AT. After two washing steps with PBS-T buffer and PBS buffer for 5 min, respectively, and one washing step with AP buffer for 5 min, the membrane was stained with 7.5 µL NBT solution and 30 µL BCIP solution in 15 mL AP buffer. The staining procedure was stopped with deionized water after the occurrence of a blue coloration of specifically detected protein bands. Stained membranes were photographed for documentation.

To increase the stringency of binding, the sodium chloride content in PBS-T and PBS buffer was increased in some experiments from 115 mM to 150 mM.

2.2.3.2 Dot blot analysis

The analysis of proteins with a dot blot facilitates a rapid immunochemical detection with specific antibodies. It is comparable to Western blot analysis, but dot blot analysis detects no molecular weights. Therefore, no separating SDS-PAGE prior to the analysis is needed and up to 96 samples can be tested simultaneously. The used buffers are listed in Table 10. A PVDF membrane was saturated in 100 % (v/v) methanol for 30 sec and washed in deionized water for 2 min, while a blotting paper was saturated in deionized water. Blotting paper and membrane were fixed in the dot blot apparatus and 50 µL of sample (400 µL for experiments shown in section 3.3.3 and Figure 30) were applied per cavity. As positive controls, GFP with His-tag or the corresponding peptides were used. Vacuum was applied until all samples were soaked in the membrane. Subsequently, the membrane was incubated in blocking solution overnight at 4 °C or for at least 2.5 h at AT. Washing and staining steps were performed according to the procedure in the Western blot analysis (see 2.2.3.1).

2.2.3.3 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) enables a quantitative immunochemical detection of proteins. Protocols for the ELISAs applied in this study were optimized and these optimized protocols are described in the following sections. The used buffers and antibodies are listed in Table 11 and Table 24. Triplicates were measured per samples. Washing steps were performed for 5 min, respectively.

2.2.3.3.1 ELISA for the detection of PEX2_044840 from *P. expansum*

All solutions containing purified PEX2_044840 protein or antibodies were freshly prepared in glass tubes. The microtiter plate was coated with 100 µL lyophilized purified PEX2_044840 protein per cavity (diluted in bicarbonate buffer to 1,000 ng/mL and heated for 10 min at 60 °C), whereas the BLANK cavities remained uncoated. After incubation at 4 °C overnight, the wells were washed three-times with 200 µL PBS-T-buffer, respectively. Afterwards, 300 µL blocking buffer were applied to each well to prevent unspecific binding. The plate was incubated at AT for 2 h on a rotary shaker (40 rpm). In the meantime, the primary antibody (Anti-PEX2_044840, see Table 24) was diluted in PBS buffer to a concentration of 20 µg/mL and 50 µL (1 µg primary antibody per well) were mixed with 50 µL of sample. The sample for the standard curve was purified lyophilized recombinant PEX2_044840 protein (diluted in PBS buffer and heated for 10 min at 60 °C) in concentrations ranging from 25 µg/mL to 1,000 µg/mL. Wine samples were diluted in PBS buffer prior to analysis. PBS buffer (heated for 10 min at 60 °C) was used as negative control and as BLANK. The primary antibody-sample-mix was incubated for 30 min at AT. After addition of 100 µL of the antibody-sample mix to the cavities, the plate was incubated for 1.5 h at 40 rpm at AT. Following three washing steps with 200 µL PBS-T-buffer, respectively, the secondary antibody (Anti-Rabbit-IgG-AP, see Table 24, diluted 1:5,000 in blocking buffer) was added. After incubation for 1.5 h at 40 rpm at AT, the cavities were washed three-times with 200 µL PBS-T-buffer and twice with 200 µL PBS buffer, respectively. Subsequently, 100 µL freshly prepared pNPP solution (diluted in diethanolamine buffer to 1 mg/mL) were added to each cavity, followed by an incubation for 30 min at 40 rpm at AT in the dark. After stopping of the reaction by addition of 50 µL 3 M NaOH per well, the extinction was measured in a spectrophotometer at 405 nm. The BLANK extinction value was subtracted from the measured values of the samples, controls, and standards. Potential dilutions of samples were included in the calculation. Absolute quantification was not possible, therefore, resulting amounts were compared relatively. The resulting amount of protein was normalized to the total protein content in the sample that was determined with the bicinchoninic acid (BCA) assay (see 2.2.2.7).

2.2.3.3.2 ELISA for the detection of ns-LTP1 *Vv*

An already developed ELISA for the detection of ns-LTP1 from *H. vulgare* (Specker, 2014) was optimized for the detection of ns-LTP1 from *V. vinifera* (*Vv*) (compare Kupfer (2018)). Moreover, an ELISA was developed for the specific detection of ns-LTP1 *Vv*.

The microtiter plate was coated with 100 µL of the respective peptide per cavity (diluted in bicarbonate buffer to 15 ng/mL), whereas the BLANK cavities remained uncoated. After incubation at 4 °C overnight, the wells were washed three-times with 200 µL PBS-T-buffer, respectively. The primary antibody (Anti-ns-LTP1-*Vv*, Anti-nsLtp1-P2-IgG (barley), see Table 24) was diluted 1:10 in PBS buffer and 2 µL were mixed with the following samples: 100 µL of lyophilized wine sample (diluted in PBS buffer), 100 µL of PBS buffer as BLANK and negative control, and 100 µL of lyophilized *Pichia pastoris* culture supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* (Lutterschmid et al., 2011) (diluted in PBS buffer to 15 mg/mL (10 mg/mL for barley ns-LTP1 ELISA)) as positive control. The antibody-sample mix was incubated for 30 min at AT. After addition of the antibody-sample mix to the cavities, the plate was incubated for 1.5 h at 40 rpm at AT. Following three washing steps with 200 µL PBS-T-buffer, respectively, the secondary antibody (Anti-Rabbit-IgG-AP, see Table 24, diluted 1:5,000 in PBS buffer) was added. After incubation for 30 min at 40 rpm at AT, the cavities were washed three-times with 200 µL PBS-T-buffer and twice with 200 µL PBS buffer, respectively. Subsequently, 100 µL freshly prepared pNPP solution (diluted in diethanolamine buffer to 0.5 mg/mL) were added to each cavity, followed by an incubation for 30 min at 40 rpm at AT in the dark. After stopping of the reaction by addition of 50 µL 3 M NaOH per well, the extinction was measured in a spectrophotometer at 405 nm. The BLANK extinction value was subtracted from the measured value of the samples and controls. The relative intensity was calculated by using the following formula:

$$Relative\ intensity\ in\ [\%] = \frac{A - C}{A - B} \times 100$$

- A Mean extinction of the triplicates of the negative control
- B Mean extinction of the triplicates of the positive control
- C Mean extinction of the triplicates of the sample

Potential dilutions of samples were included in the calculation. The resulting relative intensity was normalized to the total protein content in the sample that was determined with Bradford or BCA assay (see 2.2.2.7).

2.2.4 Moleculobiological methods

2.2.4.1 Isolation of DNA

2.2.4.1.1 Isolation of genomic DNA from filamentous fungi

Isolation of genomic DNA (gDNA) from filamentous fungi was performed according to Cenis (1992) with slight modifications: Cultures grown in 500 µL ME broth (see Table 4) or in grape juice, apple juice, or apple puree in a reaction vessel for 48 h on a rotary shaker at 100 rpm at AT were centrifuged at 12,470 x g for 5 min at AT. Following two washing steps with 500 µL sterile tap water, 300 µL extraction buffer (see Table 13) and 0.5 g sterile sea sand as well as 0.1 g sterile glass beads (Ø 0.5 mm) were added before treatment in a FastPrep homogenizer (45 sec, 24*2, 5.5 m/sec). Afterwards, 150 µL 3 M sodium acetate (pH 5.2) were added, followed by vortexing and the vessel was placed at -20 °C for 10 min. After centrifugation (12,470 x g, 5 min, AT), the supernatant was transferred into a new vessel and an equal volume of cold isopropyl alcohol (100 % (v/v) was added. The vessel was placed at -20 °C for 5 min. After centrifugation (12,470 x g, 5 min, AT), the pellet was washed with 500 µL cold ethanol (70 % (v/v)) and centrifuged twice. Afterwards, the pellet was dissolved in 50 µL $_{d}H_2O$ for 10 min at 50 °C and stored at 4 °C. DNA concentration was measured in a NanoDrop 1000 spectrophotometer.

2.2.4.1.2 Preparation of fungal DNA from sample material

The preparation of fungal DNA from grapes was performed according to Vogt et al. (2017a): 1.5 mL sterile tap water containing 1 % (v/v) Tween 20 were added to contaminated grapes which were transferred into 50 mL reaction tubes and the tubes were vigorously shaken manually. After shaking, the supernatant was transferred to a sterile 1.5 mL reaction vessel and centrifuged at 10,000 x g for 5 min. After two washing steps with 1 mL sterile tap water, respectively, the resulting pellet was suspended in 300 µL sterile deionized water and sterile glass beads (0.1 g ø 0.5 mm; 0.3 g ø 1.25-1.65 mm) were added. Following vortexing for 10 min and centrifugation, the supernatant was diluted 1:5 and 5 µL were used as template in LAMP.

For contaminated apples, 3 mL of sterile tap water containing 1 % (v/v) Tween 20 were added to a third of a contaminated apple in a blender bag. After homogenization in a bag mixer, this mixture was transferred to a reaction vessel and the preparation of fungal DNA followed the steps as described for contaminated grapes. Five microliters of the supernatant were directly used as template in LAMP.

For grape juice, apple juice, and apple puree, the isolation of fungal DNA followed the steps as described in section 2.2.4.1.1.

DNA preparation from soil and must samples was performed by use of the FastDNA™ Spin Kit for Soil (see Table 25) according to the manufacturer's recommendations. DNA concentration was measured in a NanoDrop 1000 spectrophotometer. For LAMP, the DNA concentration of soil samples was adjusted to 20 ng/µL. For must, 200 mM ammonium sulfate and potassium chloride, respectively, were used in the ammonium sulfate buffer (see Table 15) for the LAMP master mix to achieve a stronger buffering.

2.2.4.1.3 Plasmid isolation from bacteria

Plasmids were isolated from bacteria by using the QIAprep Spin MiniPrep Kit or the GeneJET Plasmid Miniprep Kit (see Table 25) according to the manufacturers' recommendations.

2.2.4.2 Polymerase chain reaction (PCR)

All PCR master mixes were pipetted on ice in 200 µL PCR tubes and were incubated in thermal cyclers. As negative control, deionized water was used instead of DNA. After the PCR reaction, PCR products were stored at 4 °C until further use. Amplification of DNA was checked by analysis of PCR products via agarose gel electrophoresis (see 2.2.4.3). Purification of PCR products was performed using the QIAquick PCR Purification Kit (see Table 25) according to the manufacturer's recommendations. DNA concentration was measured in a NanoDrop 1000 spectrophotometer.

2.2.4.2.1 PCR for amplification of *Penicillium* DNA

For the amplification of *Penicillium* DNA sequences, PCR with *Taq* polymerase was used. The PCR reagents which are shown in Table 34 were used with the primers bt2a and bt2b (see Table 22).

Table 34: PCR reagents for amplification of *Penicillium* DNA
The reagents for the PCR master mix for amplification of *Penicillium* DNA are shown.

Reagent	Volume [µL] per reaction
HPLC grade water	22.25
10 x Incubation Mix *Taq* Pol with magnesium chloride	2.50
dNTPs mix (10 mM each)	0.50
Primer forward (50 pmol/µL)	0.25
Primer reverse (50 pmol/µL)	0.25
Taq polymerase (5 U/µL)	0.25
DNA template	1.00

The used PCR program is shown in Table 35.

Table 35: PCR program for amplification of *Penicillium* DNA
Steps, function, temperature, and time of the PCR program for the amplification of *Penicillium* DNA are given.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	95.0	240
2	Denaturation	95.0	30
3	Annealing	62.7	30
4	Extension	72.0	45
	30 cycles of step 2-4		
5	Final extension	72.0	300

2.2.4.2.2 PCR for amplification of LAMP product

To confirm the specificity of the *P. expansum* LAMP assay, purified DNA obtained from LAMP products (see 2.2.4.6.2) needed to be amplified via PCR. The PCR program which is shown in Table 36 was used with the primers F2_PEX2 and B2_PEX2 (see Table 19). The composition of the PCR master mix is shown in Table 34.

Table 36: PCR program for amplification of LAMP product
Steps, function, temperature, and time of the PCR program for the amplification of the LAMP product are given.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	95.0	240
2	Denaturation	95.0	10
3	Annealing	67.0	10
	35 cycles of step 2-3		
4	Cooling	4.0	until further use

2.2.4.2.3 PCR for cloning procedures

High Fidelity Phusion Polymerase was used for cloning experiments due to its lower error rate. The DNA concentrations varied between 1 ng and 100 ng per reaction. The composition of the PCR master mix for amplification of genes of interest and sequencing of *E. coli* and *Pichia pastoris* transformants is shown in Table 37. The primer pair Acc65I_PDE_04519_f and XbaI_PDE_04519_r was used for the amplification of the coding gene for the protein PDE_04519, Acc65I_PDE_07106_f and XbaI_PDE_07106_r were used for amplification of the coding gene for the protein PDE_07106, and Acc65I_PEX2_f and XbaI_PEX2_r for the amplification of the coding gene for the protein PEX2_044840. For the sequencing of the *E. coli* transformants, primer pair Seq_Plasmid_f and Seq _Plasmid_r was used. For the sequencing of the *Pichia pastoris* transformants, primer pair pPICZseq_f and pPICZseq_r was used (see Table 23).

Table 37: PCR reagents for amplification of gene of interest and sequencing of *E. coli* and *Pichia pastoris* transformants

The reagents for the PCR master mix for amplification of gene of interest and sequencing of *E. coli* and *Pichia pastoris* transformants are shown.

Reagent	Volume [µL] per reaction
Buffer HF (5x) or GC	4.00
dNTPs mix (10 mM each)	0.40
Primer forward (10 pmol/µL)	1.00
Primer reverse (10 pmol/µL)	1.00
DNA	variable
Phusion polymerase (2,000 U/mL)	0.20
HPLC grade water	Fill up to 20.00 µL

The PCR programs are listed in the following tables. Annealing temperatures (T_a) are listed in Table 23.

Table 38: Two-step PCR program for amplification of gene of interest

Steps, function, temperature, and time of the two-step PCR program for amplification of gene of interest are given. Annealing temperatures (T_a) are listed in Table 23.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	98.0	30
2	Denaturation	98.0	10
3	Annealing	T_a	30
4	Extension	72.0	15
	10 cycles of step 2-4		
5	Denaturation	98.0	10
6	Annealing	T_a	30
7	Extension	72.0	15
	20 cycles of step 5-7		
8	Final extension	72.0	600

Table 39: PCR program for sequencing of *E. coli* transformants

Steps, function, temperature, and time of the PCR program for sequencing of *E. coli* transformants are given.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	98.0	30
2	Denaturation	98.0	10
3	Annealing	61.0	30
4	Extension	72.0	15
	30 cycles of step 2-4		
5	Final extension	72.0	600

Table 40: PCR program for sequencing of *Pichia pastoris* transformants
Steps, function, temperature, and time of the PCR program for sequencing of *Pichia pastoris* transformants are given.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	98.0	60
2	Denaturation	98.0	10
3	Annealing	65.0	30
4	Extension	72.0	70
	30 cycles of step 2-4		
5	Final extension	72.0	600

2.2.4.2.3.1 Colony PCR with *E. coli* and *Pichia pastoris*, multi-copy PCR, and determination of Mut phenotype

For the colony PCR with *E. coli*, the master mix was prepared (see Table 41) and a single bacterial colony was picked with a sterile pipette tip from an agar grown culture. The pipette tip was pressed onto a fresh LB Zeocin™ agar plate for inoculation and, subsequently, the rest of the picked colony was stirred in a corresponding PCR tube containing the respective PCR master mix. The used primer pairs were Acc65I_PDE_04519_f and XbaI_PDE_04519_r, Acc65I_PDE_07106_f and XbaI_PDE_07106_r, and Acc65I_PEX2_f and XbaI_PEX2_r (see Table 23).

For the colony PCR with *Pichia pastoris*, DNA was extracted from colonies according to Looke et al. (2011) with slight changes: Colonies were picked from YPDS Zeocin™ plates (see 2.2.4.4.3) and pressed onto a fresh YPDS agar plate for inoculation. Subsequently, the rest of the picked colony was mixed with 100 µL of a 200 mM LiAc, 1 % (w/v) SDS solution and incubated in a heating block for 10 min at 75 °C. Following addition of 300 µL 100 % (v/v) ethanol, centrifugation was performed at 15,000 x g for 3 min at AT. The pellet was washed with 150 µL 70 % (v/v) ethanol and centrifuged again. After drying of the pellet at 37 °C, it was dissolved in 50 µL deionized water. Following centrifugation at 15,000 x g for 1.5 min at AT, the supernatant was transferred to a new reaction vessel and 2 µL were used for PCR (see Table 41). The used primer pairs were pPICZseq_f, pPICZseqa_f, and pPICZseq_r (see Table 23).

Multi-copy PCR was conducted to analyze positive transformants for multiple insertions of the expression cassette into the *Pichia pastoris* genome. Primers were used which have their 3' ends positioned in opposite directions so that only one band is visible in agarose gel electrophoresis when at least one additional gene copy is present in the *Pichia pastoris* genome. The used primer pairs were MC_PDE_04519_f and MC_PDE_04519_r, MC_PDE_07106_f and MC_PDE_07106_r, and MC_PEX2_f and MC_PEX2_r (see Table 23). The composition of the master mix is shown in Table 41.

For determination of the Mut phenotype of the transformants, the PCR shown in Table 43 was conducted with the use of the primers pPICZseq_f and pPICZseq_r (see Table 23). The forward primer binds in the *AOX1* promoter region. The composition of the master mix is shown in Table 41.

Table 41: PCR reagents for colony PCR, multi-copy PCR, and determination of Mut phenotype
The reagents for the PCR master mix for colony PCR, multi-copy PCR, and determination of Mut phenotype are shown.

Reagent	Volumen [µL]
10 x Incubation Mix *Taq* Pol with magnesium chloride	1.50
dNTPs mix (10 mM each)	0.30
Primer forward (10 pmol/µL)	1.00
Primer reverse (10 pmol/µL)	1.00
DNA	Bacterial colony/ 2.00 µL of yeast DNA
Taq polymerase (5 U/µL)	0.20
HPLC grade water	11.00

The PCR programs are listed in the following tables. Annealing temperatures (T_a) are listed in Table 23.

Table 42: PCR program for colony PCR
Steps, function, temperature, and time of the PCR program for colony PCR are given. Annealing temperatures (T_a) are listed in Table 23.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	95.0	300
2	Denaturation	95.0	30
3	Annealing	T_a	30
4	Extension	72.0	30
	30 cycles of step 2-4		
5	Final extension	72.0	420

Table 43: PCR program for multi-copy PCR and determination of Mut phenotype
Steps, function, temperature, and time of the PCR program for multi-copy PCR and determination of Mut phenotype are given. Annealing temperatures (T_a) are listed in Table 23.

Step	Function	Temperature [°C]	Time [sec]
1	Initial denaturation	95.0	240
2	Denaturation	95.0	60
3	Annealing	T_a	45
4	Extension	72.0	140
	30 cycles of step 2-4		
5	Final extension	72.0	420

2.2.4.3 Agarose gel electrophoresis

For separation of DNA according to their molecular size, agarose gel electrophoresis was conducted. Therefore, 1-1.3 % (w/v) agarose gels were freshly prepared with 1 x TAE buffer (see Table 12). Five microliters of DNA sample mixed with 1 µL of 6x loading dye, and 4 µL of marker (Thermo Fisher, GeneRuler 100 bp Plus DNA Ladder or GeneRuler 1 kb DNA Ladder) were applied onto the gel which was run at 110 V for 1 h at AT. Afterwards, the gel was stained in an aqueous solution of dimidium bromide for 20 min, followed by washing in deionized water for 15 min. Gels were photographed under UV light (365 nm) for documentation. For further analysis of specific bands from the gel, the bands were excised and purified using the GeneJET Gel Extraction Kit (see Table 25) according to the manufacturer's recommendations.

2.2.4.4 Cloning procedure

2.2.4.4.1 Generation of vectors pPICZαA_PDE_04519, pPICZαA_PDE_07106, and pPICZαA_PEX2_044840

The nucleotide sequences of the proteins PDE_04519 and PDE_07106 from *P. oxalicum* were available under GenBank accession numbers KB644412.1 and KB644414.1, respectively (see appendix section 11.1 for sequences). The nucleotide sequence of the protein PEX2_044840 from *P. expansum* was available under GenBank accession number XM_016741759.1. An intron-free *PEX2_044840* gene sequence was synthesized with codon optimization for *Pichia pastoris* by GENEART GmbH (Regensburg, Germany) (see appendix section 11.1 for sequences). The vector backbone was pMA-RQ containing the optimized gene as insert and the ampicillin resistance gene *ampR* as selectable marker.

Amplification of the genes of interest was conducted by using primer pair Acc65I_PDE_04519_f and XbaI_PDE_04519_r for the coding gene of the protein PDE_04519, Acc65I_PDE_07106_f and XbaI_PDE_07106_r for the coding gene of the protein PDE_07106, and Acc65I_PEX2_f and XbaI_PEX2_r for the intron-free gene sequence of PEX2_044840 (see Table 23). The two-step PCR program shown in Table 38 was performed with the master mix composition listed in Table 37. For the reaction, 50 ng gDNA from *P. oxalicum* TMW 4.2539 and 100 ng pMA-RQ plasmid DNA were used, respectively. The used primer pairs added Acc65I and XbaI restriction sites to the resulting PCR products. The PCR products were analyzed by agarose gel electrophoresis (see 2.2.4.3) and purified by using the QIAquick PCR Purification Kit or excised from the gel and purified by using the GeneJet Gel Extraction Kit (see Table 25).

For the restriction digestion, 1 µg of the products was mixed with 1 µL Acc65I, 1 µL XbaI, 2 µL FastDigest buffer, and the volume was filled up with $_dH_2O$ to 20 µL. Incubation for 15 min at 37 °C was followed by incubation for 20 min at 65 °C to heat-inactivate the restriction enzymes. The same restriction digestion was conducted for the vector pPICZαA which was isolated from

an *E. coli* TOP10 strain (TMW 2.651) (see 2.2.4.1.3). After agarose gel electrophoresis (see 2.2.4.3) with the digested DNA, the resulting band was excised and purified.

The ligation of the constructs into the pPICZαA vector was conducted with the T4 DNA ligase. For the ligation mix, the insert DNA (28.6 ng PEX2_044840, 16 ng PDE_07106/04519) was mixed with 50 ng vector DNA, 2 µL T4 DNA ligase buffer, 1 µL T4 DNA ligase, and the volume was filled up with $_dH_2O$ to 20 µL. After incubation overnight (PEX2_044840) or for 1 h (PDE_07106/04519) at AT, the ligase was inactivated by incubation for 10 min at 65 °C. The resulting vectors were named pPICZαA_PDE_04519, pPICZαA_PDE_07106, and pPICZαA_PEX2_044840.

2.2.4.4.2 Preparation of chemically competent *E. coli* and transformation

The preparation of chemically competent *E. coli* TOP10 cells was performed with the calcium chloride method. Therefore, 500 µL of an *E. coli* TOP10 (TMW 2.580) overnight culture (see 2.2.1.1) were added to 50 mL fresh LB medium and incubated overnight to an OD_{600} = 0.375. For harvesting of the cells, 20 mL culture were transferred into two pre-cooled reaction tubes, respectively, and cooled for 10 min on ice. Following centrifugation at 1,600 x g for 7 min at 4 °C, the pellets were suspended in 4 mL sterile calcium chloride solution (see Table 14). After centrifugation at 1,100 x g for 5 min at 4 °C, the pellets were again suspended in 4 mL calcium chloride solution. Incubation on ice for 30 min was followed by centrifugation at 1,100 x g for 5 min at 4 °C. The resulting pellets were suspended in 800 µL calcium chloride solution and aliquots of 50 µL were shock-frosted in liquid nitrogen before storage at -80 °C until further use.

For the transformation of the plasmids pPICZαA_PEX2_044840, pPICZαA_PDE_04519, and pPICZαA_PDE_07106 into *E. coli*, these chemically competent *E. coli* cells were thawed on ice for 10 min. After addition of 20-25 ng plasmid DNA, the mixture was incubated for 30 min on ice. Following heat shock for 30 sec at 42 °C, the cells were cooled on ice for 5 min. Addition of 450 µL LB medium was followed by an incubation for 1 h at 37 °C at 150 rpm. After centrifugation at 2,000 x g for 1 min at AT, 450 µL of the supernatant were discarded. The cells were suspended in the remaining 50 µL and plated on LB Zeocin™ agar plates which were incubated overnight at 37 °C in the dark. The next day, grown colonies were analyzed by colony PCR (see 2.2.4.2.3.1) and agarose gel electrophoresis (see 2.2.4.3). The plasmid from successfully transformed colonies was isolated using the QIAprep Spin MiniPrep Kit (see Table 25) and sequenced using primer pair Seq_Plasmid_f and Seq_Plasmid_r (see Table 23) with the PCR program shown in Table 39. Transformants were cryo-conserved (see 2.2.1.2).

2.2.4.4.3 *Pichia pastoris* transformation by electroporation

For transformation into *Pichia pastoris*, the plasmids were isolated from *E. coli* transformants and linearized with the restriction enzyme DraI (see Table 26). Therefore, 2-5 µg plasmid DNA were mixed with 2 µL FastDigest buffer, 1 µL DraI, and the volume was filled up with $_dH_2O$ to 20 µL. An incubation of 5 min at 37 °C was followed by an incubation of 5 min at 65 °C. After analysis of the linearized plasmid by agarose gel electrophoresis (see 2.2.4.3), it was purified using the QIAquick PCR Purification Kit (see Table 25).

Pichia pastoris cells were transformed according to Wu and Letchworth (2004) with some changes according to De Schutter and Callewaert (2012): Here, 1 mL of a 5 mL overnight culture (see 2.2.1.1) of *Pichia pastoris* X33 wild type strain (TMW 3.0177) was transferred to 250 mL of YPD medium (see Table 4). After incubation at 30 °C at 250 rpm overnight until an OD_{600} = 1.5 was reached, cells were harvested by centrifugation at 1,500 x g for 5 min at 4 °C. The cells were resuspended in 200 mL freshly prepared sterile lithium acetate/ dithiothreitol solution (see Table 14) and incubated for 30 min at 100 rpm at AT. All further steps were performed on ice. After centrifugation at 1,500 x g for 5 min at 4 °C, the cells were resuspended in 35 mL ice-cold sterile 1 M sorbitol. After centrifugation at 1,800 x g for 5 min at 4 °C, the previous step was repeated twice, before cells were suspended in 2 mL ice-cold sterile 1 M sorbitol and stored on ice. For the transformation, 80 µL of prepared cells were mixed with linearized plasmid DNA in different concentrations (10-100 ng), respectively, and transferred to pre-cooled electroporation cuvettes. After incubation for 5 min on ice, the cuvette was put in the electroporator and cells were pulsed at 1.5 kV voltage, 400 Ω sample resistance, 25 µF capacity, and a time constant of 8 msec. Immediately afterwards, 1 mL ice-cold sterile 1 M sorbitol was added and the mixture was transferred to a reaction vessel. Following incubation for 2 h at 30 °C, 100 µL were plated on YPDS Zeocin™ (see Table 4) agar plates and incubated for 2-3 d at 30 °C in the dark. Grown colonies were analyzed by colony PCR (see 2.2.4.2.3.1) and agarose gel electrophoresis (see 2.2.4.3). Excised bands were sequenced using primer pair pPICZseq_f and pPICZseq_r (see Table 23) with the PCR program shown in Table 40 and cryo-conserved (see 2.2.1.2). The same primers were also used to determine the Mut phenotype (see 2.2.4.2.3.1). For determination of multi-copy clones, primer pairs MC_PDE_04519_f and MC_PDE_04519_r, MC_PDE_07106_f and MC_PDE_07106_r, and MC_PEX2_f and MC_PEX2_r, respectively, were used with the program shown in Table 43. The *Pichia pastoris* transformants contained a *S. cerevisiae* α-factor secretion signal for secretion of recombinant proteins into the culture supernatant and a C-terminal 6xHis-tag for purification and detection.

2.2.4.5 Sequencing of DNA

Purified PCR products were sent together with the respective primers to Eurofins Genomics Germany GmbH (Ebersberg, Germany) and sequencing (GATC service) was conducted according to Sanger. Sequences were aligned and analyzed (see Table 28).

2.2.4.6 Loop-mediated isothermal amplification (LAMP) assay

Loop-mediated isothermal amplification (LAMP) assays enable amplification of specific DNA sequences with high specificity, sensitivity, and rapidity under isothermal conditions. To avoid cross-contaminations, preparation of LAMP master mix (see Table 44) and addition of template DNA were done in separate rooms with different sets of pipettes. For all pipetting steps, sterile filter tips were used. Sterile deionized UV treated water was used as negative control. Positive controls were gDNA from *P. oxalicum*, *P. expansum*, and *B. cinerea*, respectively (see Table 31).

Table 44: LAMP master mix
The composition of the LAMP master mix is listed.

Reagent	Volume [µL] per reaction
10 x Ammonium sulfate buffer (see Table 15)	2.50
Magnesium chloride (200 mM)	1.00
dNTPs mix (10 mM each)	3.50
Primer mix (see Table 45)	2.60
Bst polymerase	1.00
Neutral red	1.00
$_{d}H_2O$ UV treated	8.40
DNA from sample	5.00

The composition of the LAMP primer mix is shown in Table 45. The primer sequences are listed in Table 18 to Table 21.

Table 45: LAMP primer mix
The composition of the LAMP primer mix is listed.

Primer (50 pmol/µL each)	Volume [µL]
Forward Inner Primer (FIP)	64.00
Backward Inner Primer (BIP)	64.00
Forward Outer Primer (F3)	8.00
Backward Outer Primer (B3)	8.00
Forward Loop Primer (LF)	32.00
Backward Loop Primer (LB)	32.00

The LAMP reactions were incubated in a thermal cycler for 60 min at 63 °C (assay for the detection of *B. cinerea*), 65 °C (assay for the detection of *P. oxalicum* and patulin-producing *Penicillium* species), and 68 °C (assay for the detection of *P. expansum*). A positive reaction

was indicated by a color change from orange to pink. Reactions were photographed for documentation.

2.2.4.6.1 Optimization and characterization of LAMP assays

Optimization and characterization of LAMP assays included determination of incubation temperature, sensitivity, and specificity.

Optimal incubation temperature was assessed by incubating reactions isothermally in a temperature gradient. Sensitivity of the assays was determined by testing of tenfold serially diluted gDNA or serially diluted conidial suspension. Specificity was investigated by testing purified gDNA from fungal strains.

2.2.4.6.2 Confirmation of LAMP product

The nucleotide sequence of the smallest amplified LAMP product obtained by a serial dilution of *P. expansum* TMW 4.2808 gDNA was analyzed to confirm the specificity of the *P. expansum* LAMP assay. Therefore, the LAMP products were applied onto an agarose gel and the smallest bands visible in all positive reactions were excised from the gel. Following extraction of DNA from the excised bands using the GeneJET Gel Extraction Kit (see Table 25), the purified DNA was amplified by PCR (see 2.2.4.2.2) with primers F2_PEX2 and B2_PEX (see Table 19). Agarose gel electrophoresis (see 2.2.4.3) with PCR product was conducted to confirm amplification and the PCR product was purified using the QIAquick PCR Purification Kit (see Table 25), followed by sequencing (see 2.2.4.5).

2.2.5 Bioinformatical methods

2.2.5.1 Primer design for cloning

Nucleotide and amino acid sequences were obtained from GenBank (PEX2_044840 XM_016741759.1, PDE_04519 KB644412.1, PDE_07106 KB644414.1). For amplification of the genes without the signal peptide, primers were designed which contained restriction sites for the enzymes Acc65I and XbaI, respectively. Moreover, additional non-binding nucleotides were added to the primers' 5'-ends for correct binding of restriction enzymes or correct reading frame alignment.

2.2.5.2 Primer design for LAMP

LAMP primers were designed using the PrimerExplorer V.5 software tool (http://primerexplorer.jp/e) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The primer design for the *P. expansum* specific LAMP assay was based on the coding gene of protein PEX2_044840 from *P. expansum* (GenBank accession number NW_015971172.1:c457334-456670). The nucleotide BLAST search tool on the NCBI database (Altschul et al., 1990) enabled *in silico* testing of the specificity of the designed primers.

3 Results

3.1 Cloning and heterologous expression of gushing-inducing proteins in *Pichia pastoris*

The gushing-inducing proteins PDE_07106 and PDE_04519 from *P. oxalicum* and PEX2_044840 from *P. expansum* were heterologously expressed in *Pichia pastoris* to produce high quantities of these proteins for gushing experiments and ELISA development.

3.1.1 Production of *Pichia pastoris* transformants

For amplification of the gene of interest, DNA from *P. oxalicum* TMW 4.2539 was used for work with proteins PDE_07106 and PDE_04519. By using the primer pairs Acc65I_PDE_07106_f and XbaI_PDE_07106_r, and Acc65I_PDE_04519_f and XbaI_PDE_04519_r, the respective signal peptides were excluded from the gene sequences by PCR and restriction sites for the enzymes Acc65I and XbaI were added (see appendix section 11.1 for sequences).

```
                    10        20        30        40        50        60
PEX2 native ATGCAATTCC TCACAACCCT TCTTCTGCTG GCTACCTCTG CCATCGCAGC TCCTGTCGCT
PEX2 mod.   *****G***T *G**C***T* GT*GT**T** *****T**C* *T**T**T** ***A**T***

                    70        80        90       100       110       120
PEX2 native ATCACTGCCA GGCAGTCCAA GCACTTCCAT CTTAAGTCTA CCGGTGCAAC CAACGAAAAT
PEX2 mod.   **T******* *A*****T** *********C T*G******* *T*****C** T*****G**C

                   130       140       150       160       170       180
PEX2 native CACAATAACC TATATGTCTA CTCCTACCAC ACGGGCGCAG GTCTCAGCGA TGCTGTGCTC
PEX2 mod.   *****C***T *G**C***** ********** **T**T**T* **T*GTCT** C*****TT*G

                   190       200       210       220       230       240
PEX2 native ACCAAGGATG CCAGCATAGC CAGTTCTATC TATCTGAATG GGACAAATGC ACTCGTCGAT
PEX2 mod.   **T*****C* *TTC***T** TTCC**C*** **C*****C* *A**T**C** TT*G**T**C

                   250       260       270       280       290       300
PEX2 native TTGCAGACCG AATTCACTTG GGGACTGATC GCCACTGGCA ACACCAACTA TGGCTCTTGG
PEX2 mod.   ********T* *G*****C** ***TT****T **T*****T* ********** C**T******

                   310       320       330       340       350       360
PEX2 native GAACCTATCG TTATCAACGC CGGCAGCGGC GGCAGCGCAG GATACTCCAT CAACAGCGAC
PEX2 mod.   **G**A**** ****T***** T**TTCT**T **TTC***T* *T*****T** T***TC****

                   370       380       390       400       410       420
PEX2 native AACACCTTCC AATGGTCGGA ACGCAGTGGC TTCGGTGGCT GGCTCGTCTG TGATTGGTAC
PEX2 mod.   *****T**** *G*****C** GA*ATC***T ********T* **T*G**T** ***C*****T

                   430       440       450       460       470       480
PEX2 native CACAATGCGC CCCAGCTCTT CTACTTGAAC CGATACTACA CCCCCACCAT CCCTTCCAGC
PEX2 mod.   **T**C**C* *A*****G** ********** A*G******* ****A**T** ***A***TC*

                   490       500       510
PEX2 native TGCTCCAAGG TCCAGTTGAC GACCGAATAT ATCTAA
PEX2 mod.   **T******** *T********* T*****G**C ******
```

Figure 7: Alignment of nucleotide sequences of native and modified *PEX2_044840* gene

The intron-free nucleotide sequence of the gene coding for the protein PEX2_044840 (GenBank accession number XM_016741759.1, PEX2 native) was aligned with the modified sequence (PEX2 mod.) which was codon-optimized for optimal expression in *Pichia pastoris*. Positions marked with an asterisk show identical nucleotides in both sequences.

For amplification of the coding gene for the protein PEX2_044840 from *P. expansum*, DNA from the ordered pMA-RQ plasmid containing the intron-free and *Pichia pastoris* codon-optimized *PEX2_044840* sequence ('modified *PEX2_044840*') was used as template. An alignment of the intron-free sequences of the native and modified gene is shown in Figure 7. Codon optimization led to changes in some of the nucleotide positions in the modified *PEX2_044840* gene sequence for optimal expression in *Pichia pastoris*, but the translated amino acid sequence was identical to the amino acid sequence of the native one. By using the primer pair Acc65I_PEX2_f and XbaI_PEX2_r, the signal peptide was excluded from the gene sequence by PCR and restriction sites were added to the product (see appendix section 11.1 for sequences).

The PCR products as well as the isolated vector pPICZαA were digested with the restriction enzymes Acc65I and XbaI and analyzed by agarose gel electrophoresis (see Figure 8). Even though the native nucleotide sequence of *PEX2_044840* contains a restriction site for Acc65I, the enzyme could still be used with the modified gene sequence because of nucleotide changes that resulted from codon optimization.

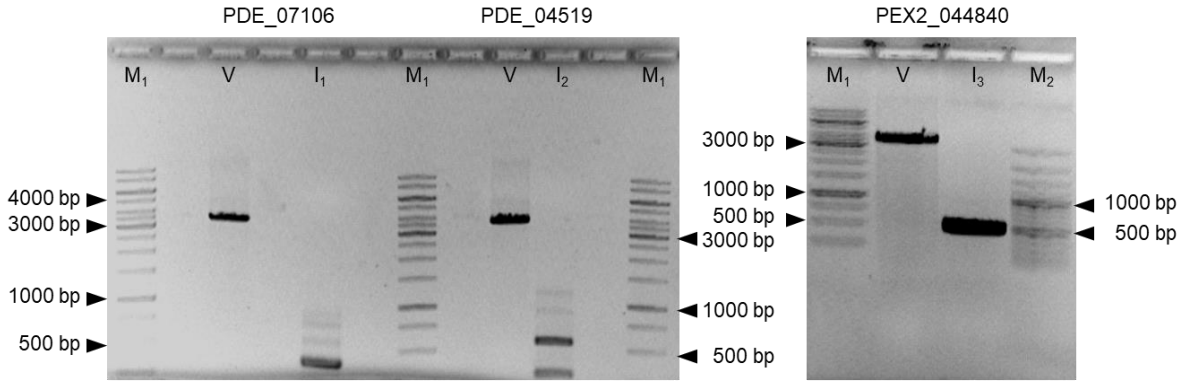


Figure 8: Digested vector and inserts
Reaction mixtures after restriction digestion of vector pPICZαA (V) and inserts (I_1 consisting of the *PDE_07106* gene sequence, I_2 consisting of the *PDE_04519* gene sequence, and I_3 consisting of the modified *PEX2_044840* gene sequence) were analyzed by agarose gel electrophoresis. M_1 = Marker GeneRuler 1 kb DNA ladder, M_2 = Marker GeneRuler 100 bp Plus DNA ladder.

As shown in Figure 8, the restriction digestion was successful. This was indicated by bands for the vector with a size of 3,563 bp as well as for the insert consisting of the *PDE_07106* gene sequence of 383 bp, for the insert consisting of the *PDE_04519* gene sequence of 527 bp, and for the insert consisting of the modified *PEX2_044840* gene sequence of 473 bp. The respective bands were excised from the gel, purified, and ligated into the pPICZαA vector. The resulting vectors were named pPICZαA_PDE_07106, pPICZαA_PDE_04519, and pPICZαA_PEX2_044840. In Figure 9, the vector map of pPICZαA_PEX2_044840 is shown exemplarily (see appendix Figure 62 for all vector maps).

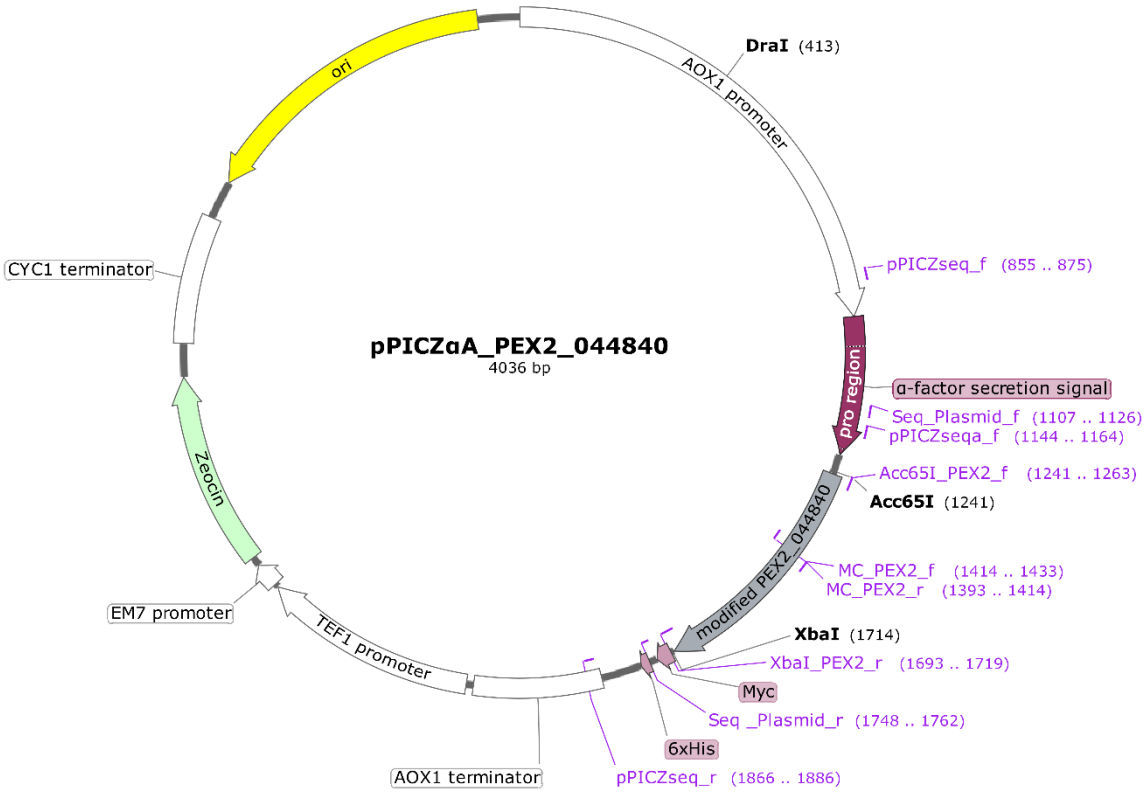


Figure 9: Vector map of pPICZαA_PEX2_044840
The vector map of pPICZαA_PEX2_044840 is shown. The modified *PEX2_044840* gene is under control of the methanol-inducible *AOX1* promoter. The α-factor secretion signal is responsible for the secretion of the expressed protein into the culture medium. The *myc* epitope and 6xHis-tag enable detection of the protein by specific antibodies and purification via IMAC. A Zeocin™ resistance gene and bacterial replication origin are encoded. Restriction sites for the enzymes DraI, Acc65I, and XbaI are marked in bold. Primer binding sites are marked in purple.

The constructs contained an α-factor secretion signal for secretion of recombinant protein into the culture supernatant and a C-terminal 6xHis-tag for purification and detection. For selection, a Zeocin™ resistance gene was used.

The constructs were transformed into *E. coli* TOP10 TMW 2.580. The resulting transformants were named *E. coli*_PDE_07106, *E. coli*_PDE_04519, and *E. coli*_PEX2_044840. Grown colonies were picked from LB Zeocin™ agar plates for colony PCR to check for positive transformation events. After colony PCR and agarose gel electrophoresis analysis, plasmids were isolated for selection of successful transformants. Results after PCR for sequencing with primers Seq_Plasmid_f and Seq_Plasmid_r, and subsequent agarose gel electrophoresis are shown in Figure 10.

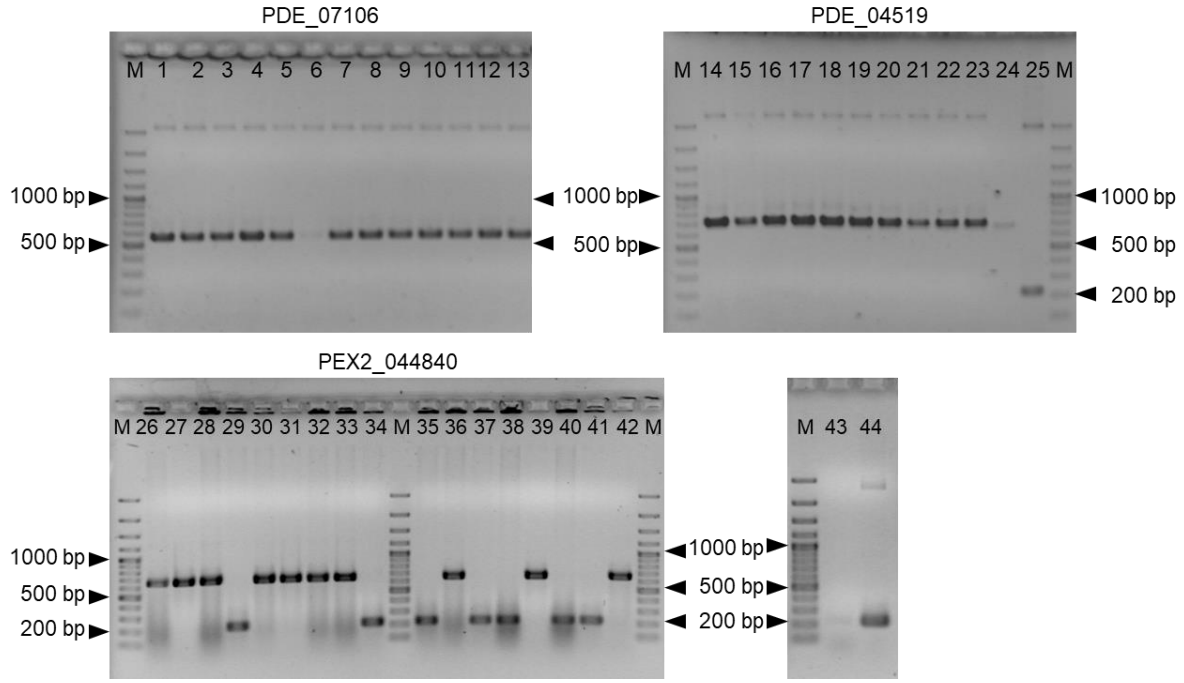


Figure 10: *E. coli* transformants

Plasmids were isolated from *E. coli* transformants and analyzed by agarose gel electrophoresis after PCR. 1-13 = plasmid DNA of *E. coli*_PDE_07106 transformants, 14-23 = plasmid DNA of *E. coli*_PDE_04519 transformants, 24 and 43 = negative control 1 ($_dH_2O$), 25 and 44 = negative control 2 (empty pPICZαA vector DNA), 26-42 = plasmid DNA of *E. coli*_PEX2_044840 transformants. M = Marker GeneRuler 100 bp Plus DNA ladder.

As shown in Figure 10, all *E. coli*_PDE_07106 transformants except the transformant from lane 6 carried the insert-containing vector indicated by a band with the size of 569 bp. All *E. coli*_ PDE_04519 transformants carried the insert-containing vector indicated by a band with the size of 713 bp. Ten *E. coli*_PEX2_044840 transformants carried the insert-containing vector indicated by a band with the size of 662 bp, while 7 transformants carried the empty pPICZαA vector. The empty pPICZαA vector was indicated by a band with the size of 216 bp that is also shown in the negative control 2 with DNA from the empty pPICZαA vector. Sequencing of plasmids revealed proper incorporation of the genes and confirmed the sequence identity of the respective genes.

As a negative control, the empty pPICZαA vector was isolated from *E. coli* TMW 2.651 and transformed into *Pichia pastoris* X33 TMW 3.0177.

For transformation into *Pichia pastoris* X33 TMW 3.0177, the plasmids were linearized by using enzyme DraI. The results of analysis by agarose gel electrophoresis are shown in Figure 11.

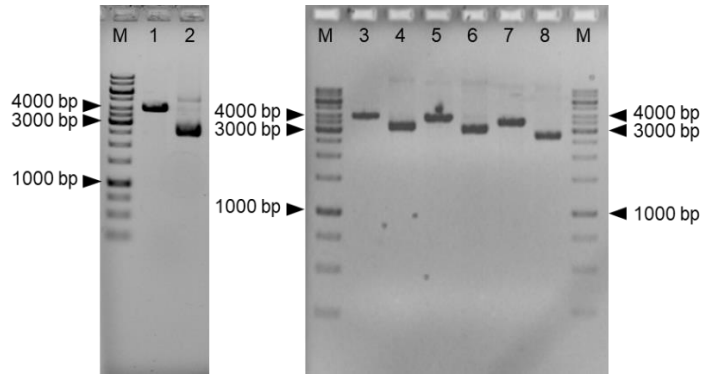


Figure 11: Linearization of plasmids

Plasmids were linearized by enzyme DraI and analyzed by agarose gel electrophoresis. 1 = linearized plasmid containing modified *PEX2_044840* gene sequence, 2 = circular plasmid containing modified *PEX2_044840* gene sequence, 3 = linearized plasmid containing *PDE_04519* gene sequence, 4 = circular plasmid containing *PDE_04519* gene sequence, 5 = linearized plasmid containing *PDE_07106* gene sequence, 6 = circular plasmid containing *PDE_07106* gene sequence, 7 = linearized empty pPICZαA vector, 8 = circular empty pPICZαA vector. M = Marker GeneRuler 1 kb DNA ladder.

As shown in Figure 11, the linearization was successful for all plasmids, indicated by a band size of 4,036 bp for plasmids containing the modified *PEX2_044840* gene sequence, 4,090 bp for plasmids containing the *PDE_04519* gene sequence, 3,946 bp for plasmids containing the *PDE_07106* gene sequence, and 3,593 bp for the empty pPICZαA vector. Circular DNA showed lower sizes.

The linearized plasmids were transformed into *Pichia pastoris*. The resulting transformants were named *Pichia pastoris*_PDE_07106, *Pichia pastoris*_PDE_04519, and *Pichia pastoris*_PEX2_044840. Grown colonies were picked from YPDS Zeocin™ agar plates for colony PCR. Results after PCR with primers pPICZseqa_f and pPICZseq_r, and subsequent agarose gel electrophoresis are shown in Figure 12.

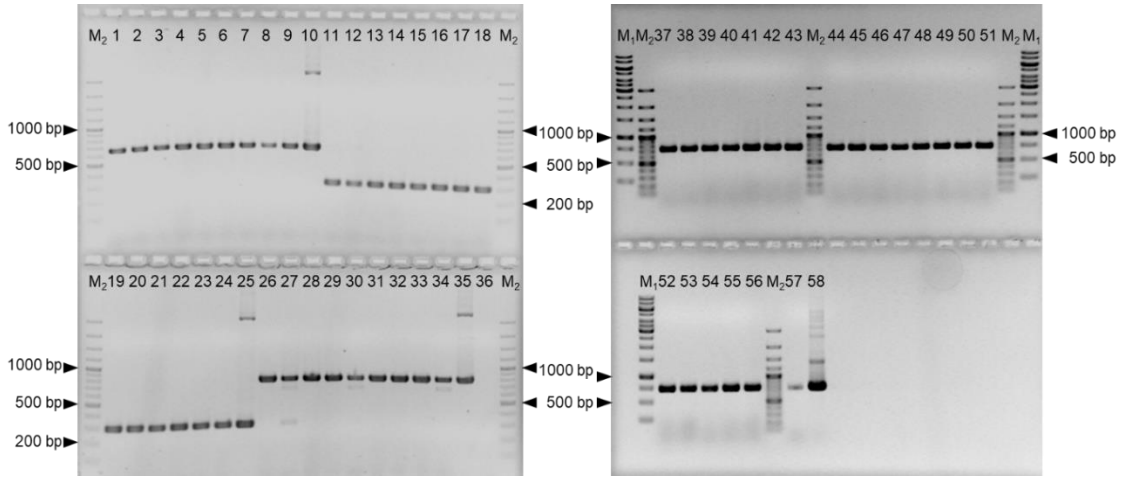


Figure 12: *Pichia pastoris* transformants

Colony PCR was conducted with *Pichia pastoris* transformants, followed by analysis via agarose gel electrophoresis. 1-9 = *Pichia pastoris*_PDE_07106 transformants, 10 = positive control (plasmid DNA containing *PDE_07106*), 11-24 = transformants containing empty pPICZαA vector, 25 = positive control (plasmid DNA empty pPICZαA vector), 26-34 = *Pichia pastoris*_PDE_04519 transformants, 35 = positive control (plasmid DNA containing *PDE_04519*), 36 and 57 = negative control ($_dH_2O$), 37-56 = *Pichia pastoris*_PEX2_044840 transformants, 58 = positive control (plasmid DNA containing modified *PEX2_044840*). M_1 = Marker GeneRuler 1 kb DNA ladder, M_2 = Marker GeneRuler 100 bp Plus DNA ladder.

As shown in Figure 12, all transformants showed the expected band sizes as did the respective positive controls with plasmid DNA. These were 653 bp for *Pichia pastoris*_PDE_07106 transformants, 300 bp for the empty pPICZαA vector, 797 bp for *Pichia pastoris*_PDE_04519 transformants, and 743 bp for *Pichia pastoris*_PEX2_044840 transformants, indicating insertion of the respective plasmids into *Pichia pastoris*.

For determination of Mut phenotype and sequencing of transformants, PCR with primers pPICZseq_f and pPICZseq_r was conducted. Results after analysis by agarose gel electrophoresis are shown in Figure 13.

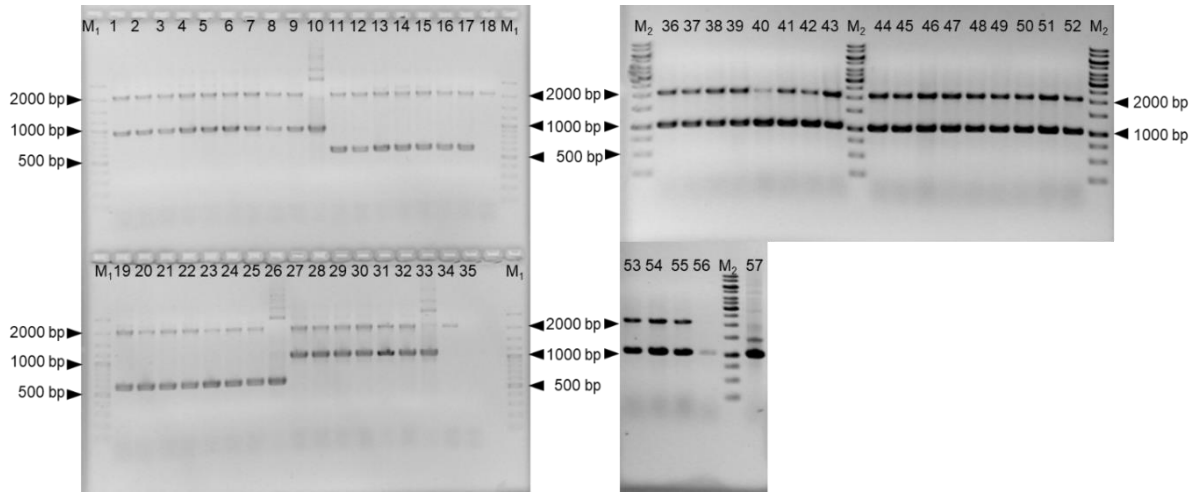


Figure 13: *Pichia pastoris* transformants screening for Mut phenotype
Colony PCR was conducted with *Pichia pastoris* transformants, followed by analysis via agarose gel electrophoresis to determine the Mut phenotype. 1-9 = *Pichia pastoris*_PDE_07106 transformants, 10 = positive control 1 (plasmid DNA containing *PDE_07106*), 11-17 and 19-25= transformants containing empty pPICZαA vector, 18 and 34 = positive control 2 (*Pichia pastoris* X33 WT TMW 3.0177 DNA), 26 = positive control 1 (plasmid DNA empty pPICZαA vector), 27-32 = *Pichia pastoris*_PDE_04519 transformants, 33 = positive control 1 (plasmid DNA containing *PDE_04519*), 35 and 56 = negative control ($_dH_2O$), 36-55 = *Pichia pastoris*_PEX2_044840 transformants, 57 = positive control 1 (plasmid DNA containing modified *PEX2_044840*). M_1 = Marker GeneRuler 100 bp Plus DNA ladder, M_2 = Marker GeneRuler 1 kb DNA ladder.

Using the primer pair pPICZseq_f and pPICZseq_r, a Mut^+ (Methanol utilization plus) phenotype for metabolization of methanol as carbon source is indicated by two bands, while a Mut^S (Methanol utilization slow) phenotype with a disrupted *AOX1* gene is indicated by one band. For Mut^+, the larger band at approximately 2.2 kb is deriving from the native undisrupted *AOX1* gene which is the case for the wild type X33 *Pichia pastoris* DNA (see Figure 13, positive control 2). The smaller band is deriving from insertion of the respective gene sequence into *Pichia pastoris* which is the case for the respective plasmid DNA (positive control 1). The sizes of the smaller bands were 942 bp for *Pichia pastoris*_PDE_07106 transformants, 589 bp for the empty pPICZαA vector, 1,086 bp for *Pichia pastoris*_PDE_04519 transformants, and 1,032 bp for *Pichia pastoris*_PEX2_044840 transformants. All transformants showed two bands indicating a Mut^+ phenotype. Only one *Pichia pastoris*_PEX2_044840 transformant in lane 40 showed a fainter second band, but Mut^+ phenotype was assumed.

Sequencing of PCR products confirmed complete integration of the respective gene inserts with correct orientation and positioning into the *Pichia pastoris* genome. Confirmed transformants were included in the TMW strain collection.

For screening of transformants that contain multiple copies of the expression cassette in the genome, multi-copy PCR was conducted. Therefore, primers were used that bind in the respective *PDE_07106*, *PDE_04519*, and *PEX2_044840* gene sequence. Their 3'-ends were directed away from each other so that a PCR product was only produced when at least two gene cassettes were integrated. The results analyzed by agarose gel electrophoresis are shown in Figure 14.

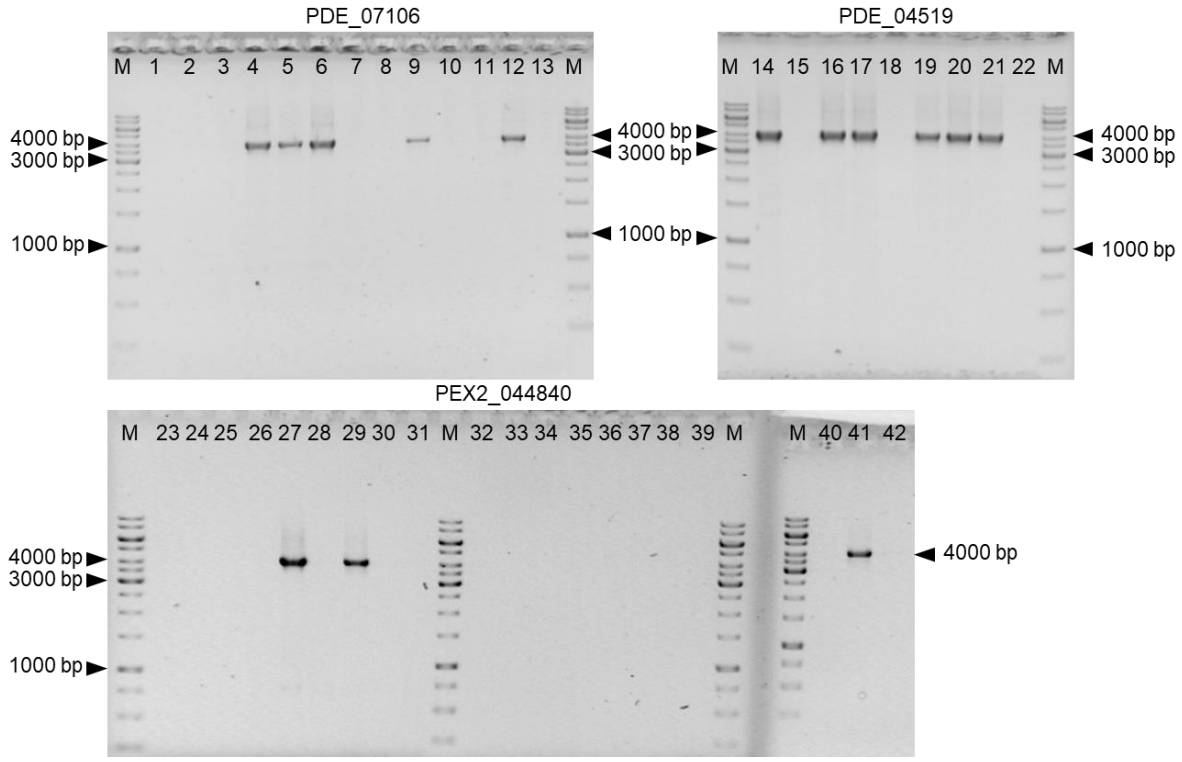


Figure 14: Screening for multi-copy *Pichia pastoris* transformants
Multi-copy PCR was conducted with *Pichia pastoris* transformants, followed by analysis via agarose gel electrophoresis to screen for multi-copy transformants. 1-9 and 11-12 = *Pichia pastoris*_PDE_07106 transformants, 10 and 13 = negative control ($_dH_2O$), 14-22 = *Pichia pastoris*_PDE_04519 transformants, 23-42= *Pichia pastoris*_PEX2_044840 transformants. M = Marker GeneRuler 1 kb DNA ladder.

As shown in Figure 14, 5 out of 11 *Pichia pastoris*_PDE_07106 transformants revealed a band with a size of approximately 4,000 bp indicating multi-copy transformants. Six out of 9 *Pichia pastoris*_PDE_04519 transformants were multi-copy transformants, and 3 out of 20 *Pichia pastoris*_PEX2_044840 transformants were of the multi-copy type. Multi-copy transformants were assumed to be the most promising clones for expression of the recombinant proteins with high yield due to the insertion of multiple copies of the expression cassette.

3.1.2 Production and purification of recombinant proteins

Transformants were tested for their ability to express recombinant proteins by dot blot analysis with an Anti-6xHis-tag antibody. Therefore, transformants were cultivated in BMMY medium and culture supernatants as well as cell lysates were tested after different incubation times.

The results for *Pichia pastoris*_PEX2_044840 transformants are shown in the following figure:

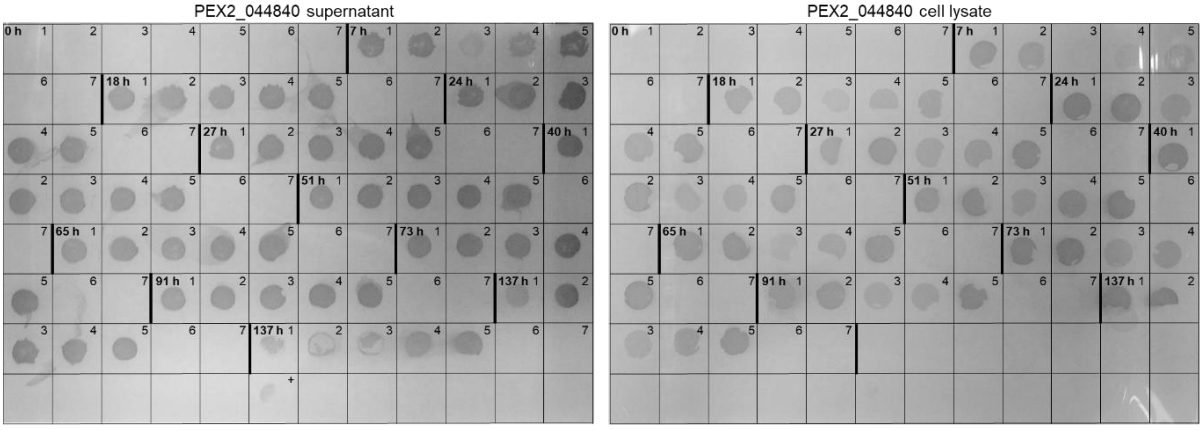


Figure 15: Expression experiments with *Pichia pastoris*_PEX2_044840 transformants
*Pichia pastoris*_PEX2_044840 transformants were cultivated in BMMY medium and supernatant as well as cell lysate samples were analyzed by dot blot with an Anti-6xHis-tag antibody after different incubation times. Samples were taken after 0 h, 7 h, 18 h, 24 h, 27 h, 40 h, 51 h, 65 h, 73 h, 91 h, and 137 h incubation at 30 °C under inducing conditions. A positive signal was detected by a coloration due to antibody binding. The tested transformants were: 1 = TMW 3.1088, 2 = TMW 3.1089, 3 = TMW 3.1090, 4 = TMW 3.1091, 5 = TMW 3.1092, 6 = negative control 1 (wild type *Pichia pastoris* TMW 3.0177), 7 = negative control 2 (empty pPICZαA vector TMW 3.1068), + = GFP with His-tag.

Five *Pichia pastoris*_PEX2_044840 transformants were tested together with two negative controls (the *Pichia pastoris* X33 wild type strain TMW 3.0177 and *Pichia pastoris* containing the empty pPICZαA vector TMW 3.1068). Transformants TMW 3.1088 (see 1 in Figure 15), TMW 3.1089 (2), and TMW 3.1092 (5) were multi-copy transformants. The negative controls showed no signal in samples from the supernatant as well as from the cell lysate at all time points. Also, at time point 0 h, no expression was detectable in transformants. After 7 h of incubation, all transformants showed expression of the recombinant protein in the supernatant and in the cell lysate, where a minor yield was found, especially for the non-multi-copy transformants (3 and 4). The signals did not vary much between different incubation times, but further analysis with diluted samples (results not shown) showed best signals in the supernatant after incubation for 24 h. Multi-copy transformants showed higher expression in the cell lysate samples as well as in the diluted supernatant samples than non-multi-copy transformants (results not shown). Therefore, two multi-copy transformants (TMW 3.1089 and TMW 3.1092) were selected for all further experiments using the culture supernatant with an incubation time of 24 h.

The results for *Pichia pastoris*_PDE_07106 transformants are shown in the following figure:

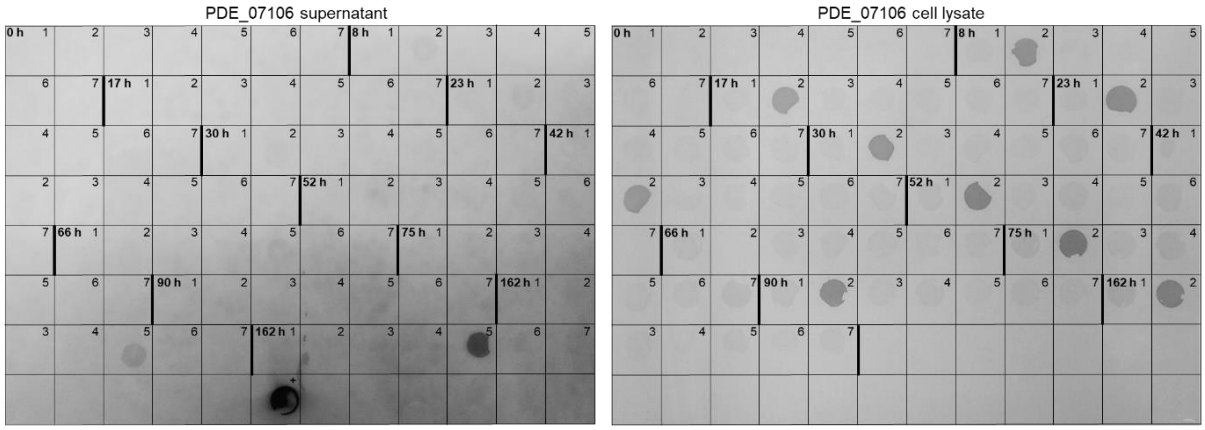


Figure 16: Expression experiments with *Pichia pastoris*_PDE_07106 transformants

*Pichia pastoris*_PDE_07106 transformants were cultivated in BMMY medium and supernatant as well as cell lysate samples were analyzed by dot blot with an Anti-6xHis-tag antibody after different incubation times. Samples were taken after 0 h, 8 h, 17 h, 23 h, 30 h, 42 h, 52 h, 66 h, 75 h, 90 h, and 162 h incubation at 30 °C under inducing conditions. A positive signal was detected by a coloration due to antibody binding. The tested transformants were: 1 = TMW 3.1076, 2 = TMW 3.1069, 3 = TMW 3.1070, 4 = TMW 3.1065, 5 = TMW 3.1066, 6 = negative control 1 (wild type *Pichia pastoris* TMW 3.0177), 7 = negative control 2 (empty pPICZαA vector TMW 3.1068), + = GFP with His-tag.

Five *Pichia pastoris*_PDE_07106 transformants were tested together with two negative controls (the *Pichia pastoris* X33 wild type strain TMW 3.0177 and *Pichia pastoris* containing the empty pPICZαA vector TMW 3.1068). Transformants TMW 3.1076 (see 1 in Figure 16), TMW 3.1069 (2), and TMW 3.1066 (5) were multi-copy transformants. The negative controls showed no signal in samples from the supernatant as well as from the cell lysate at all time points. Also, at time point 0 h, no expression was detectable in transformants. In the supernatant, no remarkable expression was detected for any of the transformants and time points. However, after 8 h of incubation, transformant TMW 3.1069 (2) showed expression of the recombinant protein in the cell lysate. All other transformants were negative at all time points. The signals of TMW 3.1069 did not vary much between different incubation times, but further analysis with diluted samples (results not shown) showed best signals in the cell lysate after incubation for 48 h. Therefore, the multi-copy transformant TMW 3.1069 was selected for all further experiments using the cell lysate with an incubation time of 48 h.

The results for *Pichia pastoris*_PDE_04519 transformants are shown in the following figure:

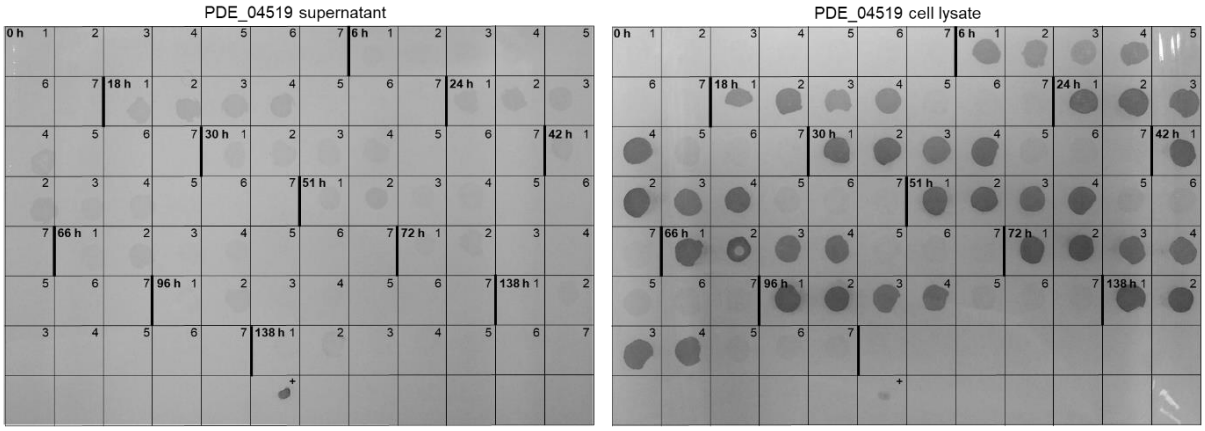


Figure 17: Expression experiments with *Pichia pastoris*_PDE_04519 transformants

*Pichia pastoris*_PDE_04519 transformants were cultivated in BMMY medium and supernatant as well as cell lysate samples were analyzed by dot blot with an Anti-6xHis-tag antibody after different incubation times. Samples were taken after 0 h, 6 h, 18 h, 24 h, 30 h, 42 h, 51 h, 66 h, 72 h, 96 h, and 138 h incubation at 30 °C under inducing conditions. A positive signal was detected by a coloration due to antibody binding. The tested transformants were: 1 = TMW 3.1078, 2 = TMW 3.1079, 3 = TMW 3.1081, 4 = TMW 3.1083, 5 = TMW 3.1086, 6 = negative control 1 (wild type *Pichia pastoris* TMW 3.0177), 7 = negative control 2 (empty pPICZαA vector TMW 3.1068), + = GFP with His-tag.

Five *Pichia pastoris*_PDE_04519 transformants were tested together with two negative controls (the *Pichia pastoris* X33 wild type strain TMW 3.0177 and *Pichia pastoris* containing the empty pPICZαA vector TMW 3.1068). Transformants TMW 3.1078 (see 1 in Figure 17), TMW 3.1079 (2), TMW 3.1081 (3), and TMW 3.1083 (4) were multi-copy transformants. The negative controls showed no signal in samples from the supernatant as well as from the cell lysate at all time points. Also, at time point 0 h, no expression was detectable in transformants. In the supernatant, no remarkable expression was detected for any of the transformants and time points. However, after 6 h of incubation, the four multi-copy transformants showed expression of the recombinant protein in the cell lysate. The non-multi-copy transformant (5) was negative at all time points. The signals of the multi-copy transformants did not vary much between different incubation times, but further analysis with diluted samples (results not shown) showed best signals in the cell lysate after incubation for 48 h for transformant TMW 3.1079. Therefore, the multi-copy transformant TMW 3.1079 was selected for all further experiments using the cell lysate with an incubation time of 48 h.

Moreover, the ability of strains to produce stable foam was assessed visually in transformants and the wild type strain because the gushing-inducing proteins are surface-active and can stabilize foams. Transformants *Pichia pastoris*_PEX2_044840 (TMW 3.1089), *Pichia pastoris*_PDE_07106 (TMW 3.1069), and *Pichia pastoris*_PDE_04519 (TMW 3.1079) were compared with the wild type strain X33 (TMW 3.0177), respectively (see Figure 18, A, B, C).

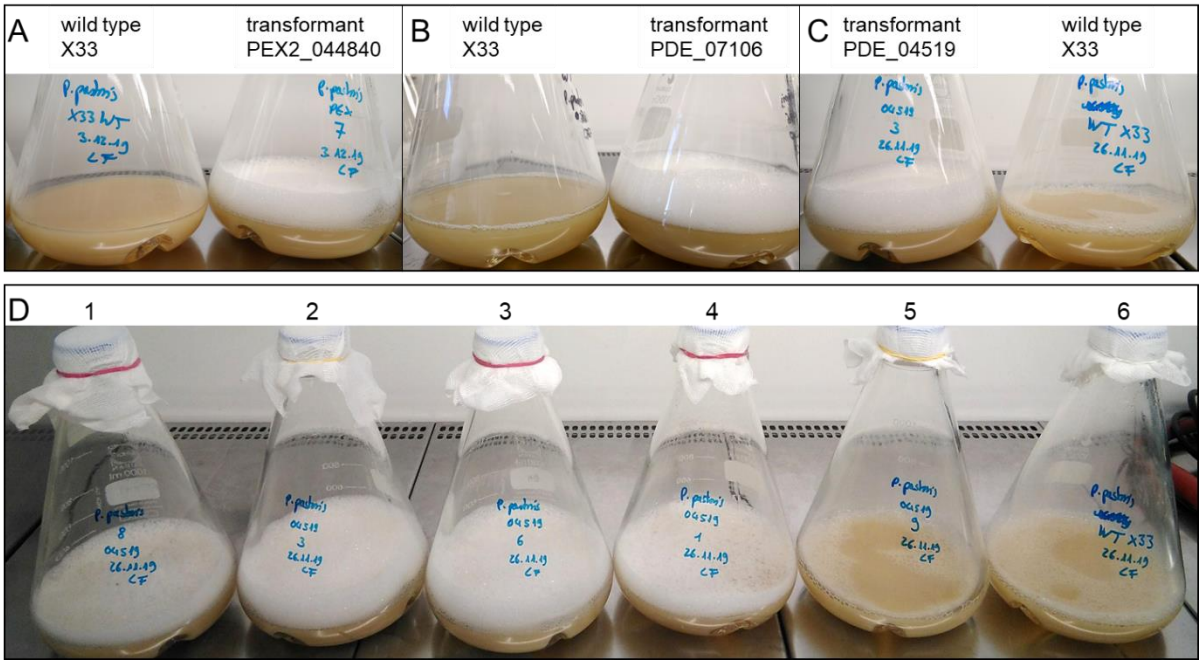


Figure 18: Foams of expression cultures

Expression cultures in BMMY medium were visually observed after 24 h incubation for foam production. A) Comparison of *Pichia pastoris* wild type X33 (TMW 3.0177) culture with *Pichia pastoris*_PEX2_044840 transformant (TMW 3.1089) culture. B) Comparison of *Pichia pastoris* wild type X33 (TMW 3.0177) culture with *Pichia pastoris*_PDE_07106 transformant (TMW 3.1069) culture. C) Comparison of *Pichia pastoris* wild type X33 (TMW 3.0177) culture with *Pichia pastoris*_PDE_04519 transformant (TMW 3.1079) culture. D) Comparison of *Pichia pastoris*_PDE_04519 multi-copy transformant cultures (1-4, TMW 3.1083, 3.1079, 3.1081, 3.1078) with *Pichia pastoris*_PDE_04519 non-multi-copy transformant (5, TMW 3.1086) culture and *Pichia pastoris* wild type X33 (6, TMW 3.0177) culture.

As shown in Figure 18 A, B, and C, the respective transformants produced stable foam, while the *Pichia pastoris* wild type did not. Also, in Figure 18 D, *Pichia pastoris*_PDE_04519 multi-copy transformants (1-4) were compared with a *Pichia pastoris*_PDE_04519 non-multi-copy transformant (5) and the *Pichia pastoris* wild type (6). Here, the multi-copy transformants produced stable foam, while the non-multi-copy transformant and the wild type did not.

In order to obtain optimal expression results and product yield, tests were performed with different growth media (BMGY/BMMY or BMG/BMM). The use of BMGY/BMMY medium led to higher yields than the use of BMG/BMM medium (results not shown). Therefore, all further experiments were conducted in BMGY/BMMY medium.

After selection of optimal expression conditions and transformants, the recombinant proteins secreted into the culture supernatant or in the cell lysate were analyzed. Therefore, proteins in culture supernatant or cell lysate were purified using Ni-NTA agarose resins for affinity purification or an ÄKTApure 25L1 FPLC system equipped with a 5 mL HisPur™ Ni-NTA Chromatography Cartridge for immobilized metal affinity chromatography (IMAC).

The results of supernatant analysis of *Pichia pastoris*_PEX2_044840 transformant and *Pichia pastoris* wild type by SDS-PAGE and Western blot analysis are shown in the following figure:

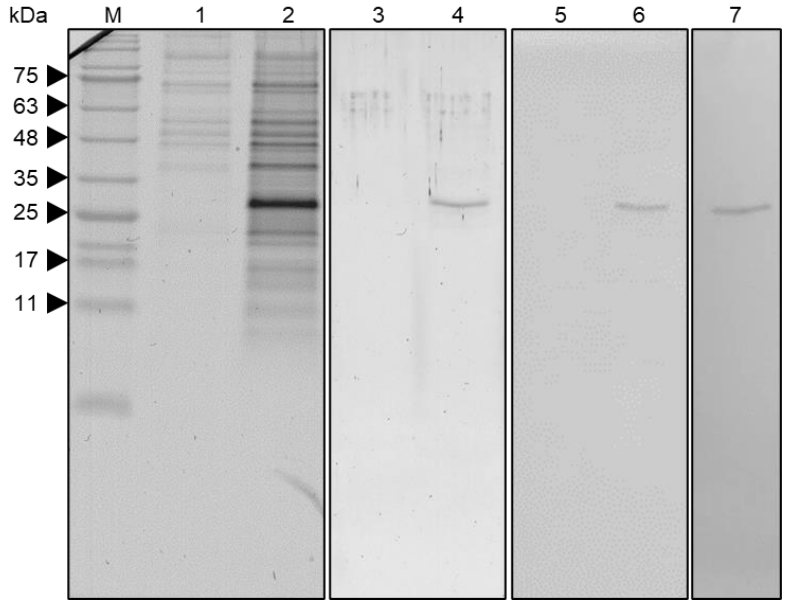


Figure 19: Protein analyses of *Pichia pastoris*_PEX2_044840 transformant and wild type
Pichia pastoris wild type strain X33 TMW 3.0177 and PEX2_044840 transformant TMW 3.1089 were analyzed by SDS-PAGE and Western blot. The different lanes show: Silver-stained SDS-PAGE of culture supernatant of the *Pichia pastoris* wild type strain X33 (lane 1) and transformant *Pichia pastoris*_PEX2_044840 (lane 2). Silver-stained SDS-PAGE of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 3) and transformant *Pichia pastoris*_PEX2_044840 (lane 4). Western blot analysis of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 5) and transformant *Pichia pastoris*_PEX2_044840 (lane 6) with an Anti-6xHis-tag antibody. Western blot analysis of ÄKTA purified proteins of transformant *Pichia pastoris*_PEX2_044840 (lane 7) with an Anti-PEX2_044840 antibody. M = Marker SERVA Triple Color Protein Standard III. Figure modified from Frisch et al. (2021a).

SDS-PAGE analysis of the culture supernatants of the transformant *Pichia pastoris*_PEX2_044840 TMW 3.1089 and *Pichia pastoris* wild type X33 TMW 3.0177 revealed a prominent band at approximately 29 kDa (see Figure 19, lane 2) that was absent in the wild type strain (lane 1). After purification, this band was exclusively present in the transformant sample (lane 4). Western blot analysis with an Anti-6xHis-tag antibody revealed the same pattern (lane 6), indicating that the secreted recombinant protein harbors a 6xHis-tag resulting in a 29 kDa band. The protein-containing band was excised and analyzed by nano-ESI-LC-MS/MS. The analysis confirmed the identity of the protein as PEX2_044840 with two peptides and a sequence coverage of 25 %. Analysis of individual sequences of analyzed peptides showed 100 % identity with the published PEX2_044840 sequence. Moreover, Western blot analysis of ÄKTA purified proteins of transformant TMW 3.1089 with an Anti-PEX2_044840 antibody detected the same band (lane 7), indicating that this protein is PEX2_044840. An amino acid sequence analysis of PEX2_044840 without the signal peptide but with a 6xHis-tag and an α-factor secretion signal cleaved at the first cleavage site was performed *in silico* using the ExPASy ProtParam tool. The analysis revealed a molecular weight of 29 kDa and a theoretical isoelectric point (pI) of 4.92. This theoretical analysis matched the results of the practical experiments. As the results confirmed the identity of the protein and the purification

using Ni-NTA beads and ÄKTA was successful, further experiments were conducted with the purified recombinant PEX2_044840 protein from *P. expansum*.

For the *Pichia pastoris*_PDE_07106 and *Pichia pastoris*_PDE_04519 transformants, no remarkable secretion of the recombinant proteins was detectable in the respective culture supernatants. Therefore, the cell pellets were used for preparation of cell lysates. Tests were performed with different cell pellet treatments (FastPrep or ultrasonication) for optimized cell lysis. The preparation in a FastPrep homogenizer resulted in cell lysates of higher purity than the preparation by ultrasonication (results not shown). Therefore, all further cell lysate preparations were conducted in a FastPrep homogenizer.

The results of cell lysate analysis of *Pichia pastoris*_PDE_07106 transformant and *Pichia pastoris* wild type by SDS-PAGE and Western blot are shown in the following figure:

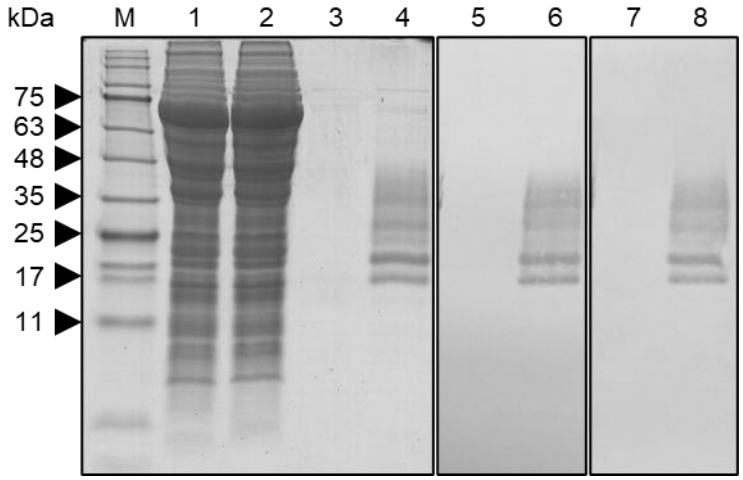


Figure 20: Protein analyses of *Pichia pastoris*_PDE_07106 transformant and wild type
Pichia pastoris wild type strain X33 TMW 3.0177 and PDE_07106 transformant TMW 3.1069 were analyzed by SDS-PAGE and Western blot. The different lanes show: Coomassie-stained SDS-PAGE of cell lysate of the *Pichia pastoris* wild type strain X33 (lane 1) and transformant *Pichia pastoris*_PDE_07106 (lane 2). Coomassie-stained SDS-PAGE of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 3) and transformant *Pichia pastoris*_PDE_07106 (lane 4). Western blot analysis of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 5) and transformant *Pichia pastoris*_PDE_07106 (lane 6) with an Anti-6xHis-tag antibody. Western blot analysis of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 7) and transformant *Pichia pastoris*_PDE_07106 (lane 8) with an Anti-PDE_07106 antibody.M=MarkerSERVATriple Color Protein Standard III.

SDS-PAGE analysis of the cell lysate of the transformant *Pichia pastoris*_PDE_07106 TMW 3.1069 and *Pichia pastoris* wild type X33 TMW 3.0177 showed no visible difference as a variety of proteins was present (see Figure 20, lanes 1 and 2). After purification, the transformant strain showed four prominent bands at approximately 17 kDa, 21 kDa, 27 kDa, and 36 kDa (lane 4) which were absent in the wild type strain (lane 3). Western blot analysis with an Anti-6xHis-tag antibody revealed the same pattern (lane 6). The four protein-containing bands were excised and analyzed by LC-MS/MS. The analysis confirmed the identity of the protein bands as PDE_07106 with 18 peptides and a sequence coverage of 54 %. Analysis of individual sequences of analyzed peptides showed 100 % identity with the published

PDE_07106 sequence. Moreover, Western blot analysis with an Anti-PDE_07106 antibody detected the same bands (lane 8), indicating that these are PDE_07106. An amino acid sequence analysis of PDE_07106 without the signal peptide but with a 6xHis-tag and an uncleaved α-factor secretion signal was performed *in silico* using the ExPASy ProtParam tool. The analysis revealed a molecular weight of 27 kDa and a theoretical pI of 5.06. This theoretical analysis matched one of the bands of the practical experiments. The sequence with α-factor secretion signal cleaved at the second cleavage site revealed a molecular weight of 17.7 kDa that also matched one band of the practical experiments.

The results of cell lysate analysis of *Pichia pastoris*_PDE_04519 transformant and *Pichia pastoris* wild type by SDS-PAGE and Western blot are shown in the following figure:

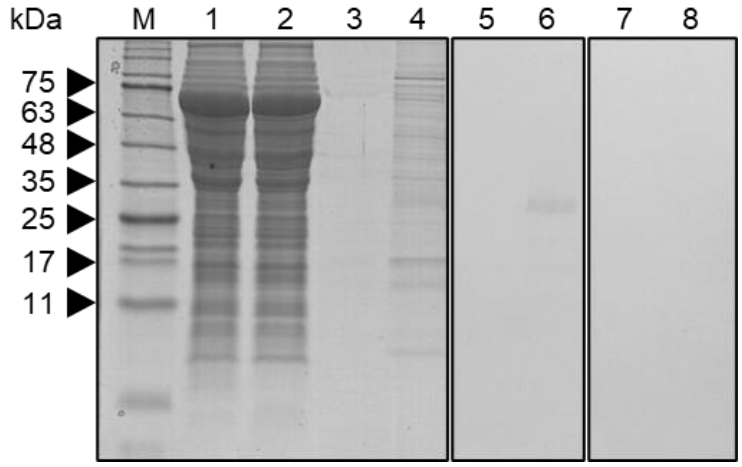


Figure 21: Protein analyses of *Pichia pastoris*_PDE_04519 transformant and wild type
Pichia pastoris wild type strain X33 TMW 3.0177 and PDE_04519 transformant TMW 3.1079 were analyzed by SDS-PAGE and Western blot. The different lanes show: Coomassie-stained SDS-PAGE of cell lysate of the *Pichia pastoris* wild type strain X33 (lane 1) and transformant *Pichia pastoris*_PDE_04519 (lane 2). Coomassie-stained SDS-PAGE of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 3) and transformant *Pichia pastoris*_PDE_04519 (lane 4). Western blot analysis of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 5) and transformant *Pichia pastoris*_PDE_04519 (lane 6) with an Anti-6xHis-tag antibody. Western blot analysis of Ni-NTA purified proteins of the *Pichia pastoris* wild type strain X33 (lane 7) and transformant *Pichia pastoris*_PDE_04519 (lane 8) with an Anti-PDE_04519 antibody. M = Marker SERVA Triple Color Protein Standard III.

SDS-PAGE analysis of the cell lysate of the transformant *Pichia pastoris*_PDE_04519 TMW 3.1079 and *Pichia pastoris* wild type X33 TMW 3.0177 showed no visible difference as a variety of proteins was present (see Figure 21, lanes 1 and 2). After purification, the transformant strain showed several diffuse bands at different molecular weights (lane 4) that were absent in the wild type strain (lane 3). Western blot analysis with an Anti-6xHis-tag antibody revealed one band at approximately 31 kDa (lane 6) that was absent in the wild type strain (lane 5). Western blot analysis of the same samples with an Anti-PDE_04519 antibody detected no bands (lanes 7 and 8). An amino acid sequence analysis of PDE_04519 without the signal peptide but with a 6xHis-tag and an uncleaved α-factor secretion signal was performed *in silico* using the ExPASy ProtParam tool. The analysis revealed a molecular weight of 31 kDa and a theoretical pI of 4.58. This theoretical analysis matched the band of

the practical experiments that was detected with the Anti-6xHis-tag antibody. As the purification of the PDE_04519 recombinant protein revealed several bands in the SDS-PAGE with only a faint band in the Western blot analysis with an Anti-6xHis-tag antibody and no band with the specific Anti-PDE_04519 antibody, further experiments regarding *P. oxalicum* were conducted only with the recombinant protein PDE_07106 from *P. oxalicum*.

All in all, the results showed that the cloning procedure was successful and all three gushing-inducing proteins were heterologously expressed in *Pichia pastoris*. Due to different expression and purification levels, further experiments were conducted with the purified proteins PEX2_044840 and PDE_07106.

3.2 Effect of recombinant proteins on gushing in sparkling wine

The effect of addition of recombinant proteins to sparkling wine was analyzed by gushing tests.

Gushing tests were performed with purified recombinant PEX2_044840 and PDE_07106 proteins and purified proteins from the *Pichia pastoris* wild type strain X33 TMW 3.0177 in sparkling wine. The results are shown in Figure 22.

In the gushing tests with protein PEX2_044840 purified from culture supernatant (see Figure 22 A), no gushing was observed for untreated sparkling wines to which no protein was added. A general loss of 0.18 g of CO_2 that was caused by opening of the bottles was subtracted from the weight losses in all experiments. Sparkling wine inoculated with proteins purified from the culture supernatant of the wild type strain (negative control) showed no gushing. Addition of 30 µg, 60 µg, and 120 µg purified PEX2_044840 protein resulted in gushing with weight losses of 14 g ± 10 g, 24 g ± 4 g, and 52 g ± 27 g per bottle, respectively. The addition of 15 µg did not result in gushing. No significant differences were found between the different treatments.

In the gushing tests with protein PDE_07106 purified from cell lysate (see Figure 22 B), no gushing was observed for untreated sparkling wines to which no protein was added. A general loss of 0.4 g of CO_2 that was caused by opening of the bottles was subtracted from the weight losses in all experiments. As negative control, sparkling wine was inoculated with 15 µg and 60 µg protein purified from the cell lysate of the wild type strain, respectively, which resulted in over-foaming with weight losses of 36 g ± 11 g and 92 g ± 4 g per bottle, respectively. Addition of 15 µg, 30 µg, 60 µg, and 120 µg purified PDE_07106 protein resulted in gushing with weight losses of 62 g ± 2 g, 110 g ± 8 g, 118 g ± 1 g, and 111 g ± 11 g per bottle, respectively. Significant differences were found between the different treatments depicted in Figure 22 B for the addition of 15 µg and 60 µg PDE_07106 protein (paired t-test, $p = 0.04$, $\alpha = 0.05$).

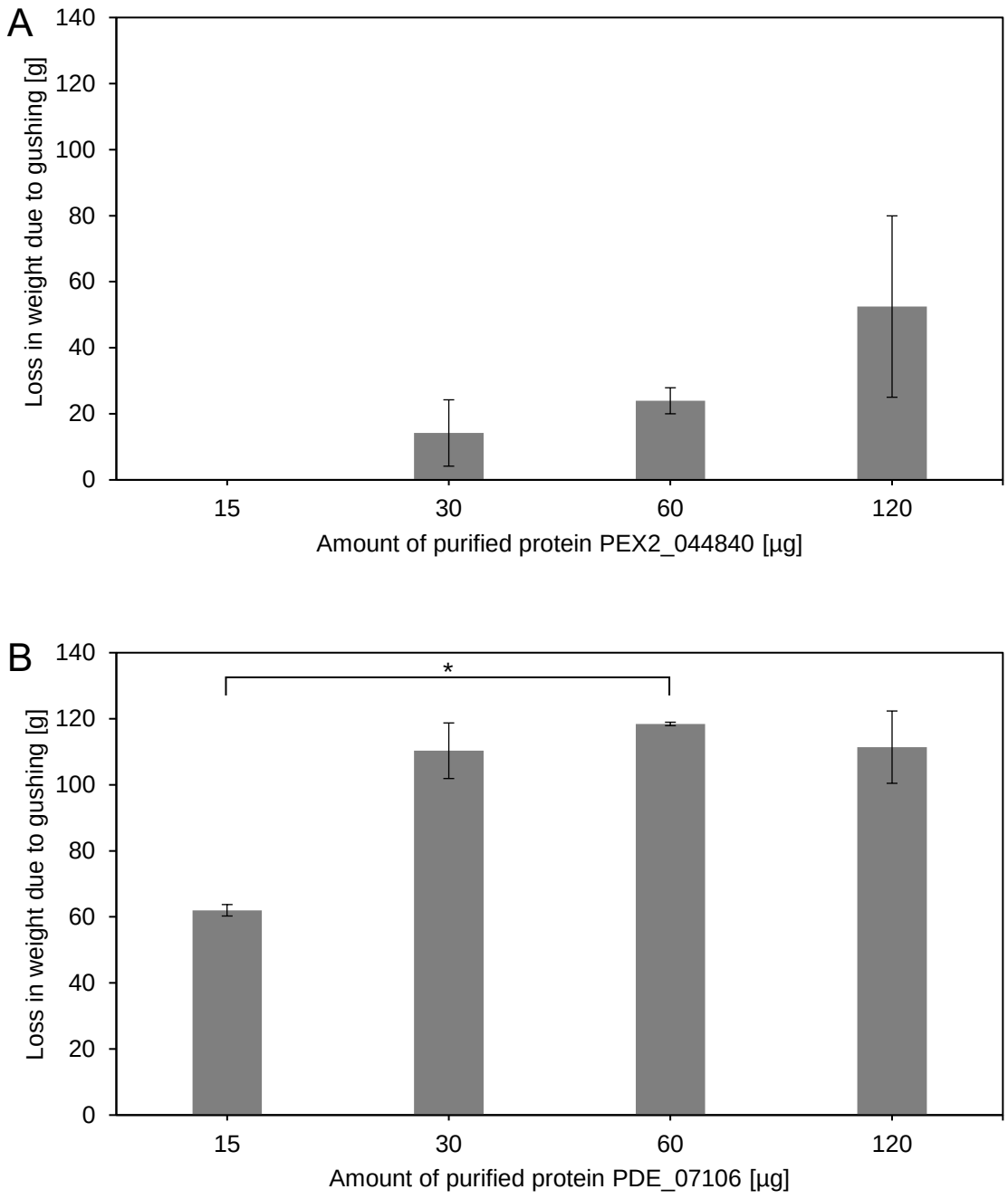


Figure 22: Gushing tests in sparkling wine with recombinant proteins

The average loss in weight due to gushing with different concentrations of protein PEX2_044840 purified from culture supernatant (A) and PDE_07106 purified from cell lysate (B) in sparkling wines is shown with standard error. An asterisk marks significance (α = 0.05). Figure A modified from Frisch et al. (2021a).

3.3 Development of immunochemical methods for the detection of gushing-inducing proteins

For the detection of the gushing-inducing proteins PEX2_044840 and PDE_07106, peptide antibodies were generated. A previously generated antibody for the detection of PDE_04519 (Anti-VOG-EFA-IgG) was used as it was shown to be highly specific (Vogt-Hrabak, 2017). These antibodies were used for Western blot and dot blot analyses and in case of PEX2_044840 for the development of an ELISA.

3.3.1 Antibody production and testing

The production of polyclonal peptide antibodies was performed by Davids Biotechnologie GmbH (Regensburg, Germany). For immunization, peptides were synthesized that derived from the amino acid sequences without signal peptide from proteins PEX2_044840 and PDE_07106, respectively (see appendix Figure 52 and Figure 55 for sequences, peptide marked in orange). The most suitable peptide regarding antigenicity, solubility, and epitope prediction was chosen for immunization of rabbits. Table 46 lists the chosen peptide sequences and their characteristics. Five immunizations were performed at days 1, 14, 28, 42, and 56. After day 63, the final bleed was received and affinity purified. The antibody titer was 300,000, respectively. The resulting antibodies were designated as Anti-PEX2_044840 and Anti-PDE_07106.

Table 46: Characteristics of the peptides used for antibody production
Peptides for antibody production are listed with their characteristics regarding antigenicity, solubility, and epitope prediction according to Davids Biotechnologie GmbH (Regensburg, Germany).

Target protein	Peptide sequence	Antigenicity	Solubility	Epitope prediction	Antibody name
PEX2_044840	ARQSKHFHLKSTGATNENHN	good	medium to good	good	Anti-PEX2_044840
PDE_07106	KSRQIAYPAYTDKQVEGGN	medium to good	medium to good	good	Anti-PDE_07106

For testing of the antibodies, Western blot analysis was conducted. The results are shown in Figure 23.

Grape must without fungal infection was tested with three different antibodies (newly generated Anti-PDE_07106 for detection of PDE_07106, previously generated Anti-VOG-APA-IgG for the detection of PDE_07106 (Vogt-Hrabak, 2017), previously generated Anti-VOG-EFA-IgG for the detection of PDE_04519 (Vogt-Hrabak, 2017)), and showed no bands in the Western blot (see lanes 1-4 in Figure 23 A, B, C). When the same antibodies were used in a Western blot analysis of grape must after artificial infection with *P. oxalicum* TMW 4.2553 (see lanes 5-8 in Figure 23 A, B, C), proteins were shown to be bound by the tested antibodies.

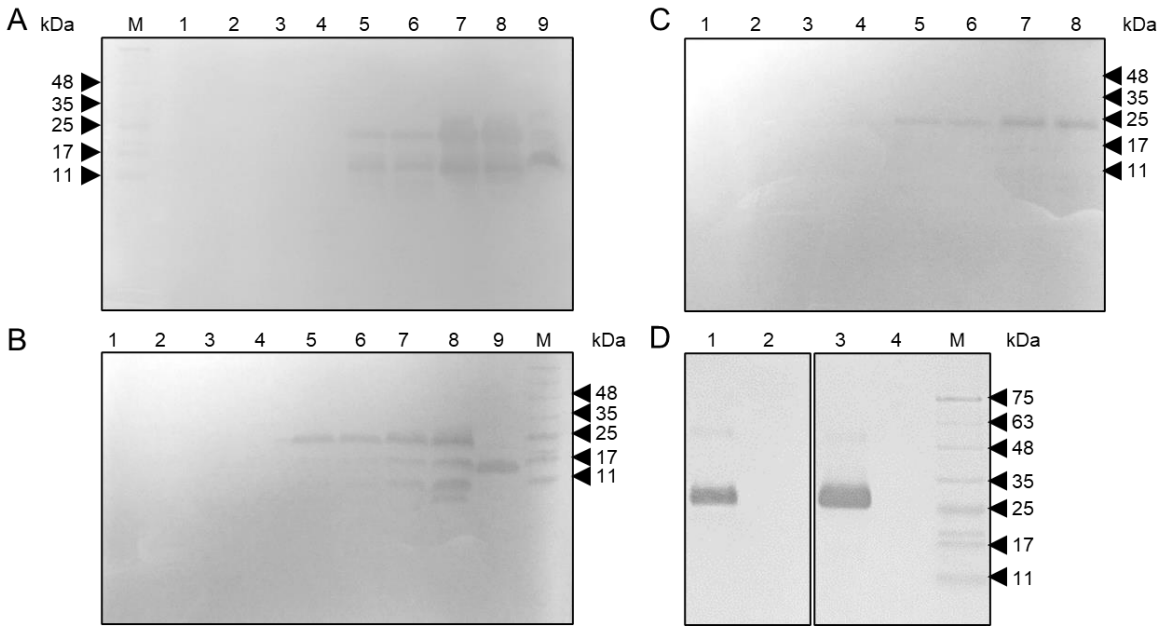


Figure 23: Testing of antibodies by Western blot analysis

Antibodies were tested by Western blot analysis. A) Western blot with newly generated Anti-PDE_07106 antibody for detection of PDE_07106 from *P. oxalicum*. B) Western blot with Anti-VOG-APA-IgG antibody (Vogt-Hrabak, 2017) for detection of PDE_07106 from *P. oxalicum*. C) Western blot with Anti-VOG-EFA-IgG antibody (Vogt-Hrabak, 2017) for detection of PDE_04519 from *P. oxalicum*. Lanes in A, B, and C: 1 and 2 = must A without *P. oxalicum* infection, 3 and 4 = must B without *P. oxalicum* infection, 5 and 6 = must A inoculated with spores from *P. oxalicum* TMW 4.2553, 7 and 8 = must B inoculated with spores from *P. oxalicum* TMW 4.2553, 9 = lyophilized spumate from *P. oxalicum* TMW 4.2539. D) Western blot with newly generated Anti-PEX2_044840 antibody for detection of PEX2_044840 from *P. expansum*. Lane 1 = 1 mg/mL ÄKTA purified recombinant PEX2_044840 protein in PBS buffer, 2 = 1 mg/mL *Pichia pastoris* wild type X33 TMW 3.0177 supernatant in PBS buffer, 3 = base wine (no. 41) inoculated with 5 mg/mL ÄKTA purified recombinant PEX2_044840 protein, 4 = uninoculated base wine (no. 41). M = Marker SERVA Triple Color Protein Standard III.

The Western blot shown in Figure 23 A lanes 5-8 revealed that the newly generated Anti-PDE_07106 antibody for the detection of PDE_07106 from *P. oxalicum* reacted with proteins of approximately 15 kDa and 23 kDa, respectively. According to *in silico* performed sequence analysis, the PDE_07106 protein has a molecular weight of 15 kDa which matches the obtained results. The band at approximately 23 kDa could be a dimer of the protein. Lane 9 in Figure 23 A shows the reaction of Anti-PDE_07106 with the lyophilized spumate from a *P. oxalicum* liquid culture as a positive control. The Western blot showed a prominent band at 15 kDa which matches the molecular weight of PDE_07106 and demonstrates that the newly generated antibody detects the protein both in pure culture supernatants and artificially infected grape must samples.

The Western blot shown in Figure 23 B with a previously generated antibody (Anti-VOG-APA-IgG) for the detection of PDE_07106 from *P. oxalicum* (Vogt-Hrabak, 2017) revealed that this antibody reacted with proteins of approximately 9 kDa, 11 kDa, 17 kDa, and 23 kDa, respectively. The results indicated that this antibody has several cross-reactions with other proteins and is not suited for specific detection of PDE_07106 in must samples. In comparison, the newly generated Anti-PDE_07106 antibody for the detection of PDE_07106 worked fine and was used for all further analyses.

The Western blot shown in Figure 23 C lanes 5-8 with the Anti-VOG-EFA-IgG antibody (Vogt-Hrabak, 2017) for the detection of PDE_04519 from *P. oxalicum* revealed that the antibody reacted with a protein of approximately 22 kDa. According to *in silico* performed sequence analysis, the PDE_04519 protein has a molecular weight of 20 kDa which matches the obtained results. This antibody was therefore used for further analyses.

In the Western blot shown in Figure 23 D, the newly generated Anti-PEX2_044840 antibody for the detection of PEX2_044840 from *P. expansum* was used. By testing lyophilized recombinant PEX2_044840 protein, the antibody reacted highly specifically with a protein of 29 kDa (see lane 1 in Figure 23). In comparison, the antibody did not react with proteins of the *Pichia pastoris* wild type supernatant (lane 2). Base wine that was tested uninoculated (lane 4) and inoculated with lyophilized recombinant PEX2_044840 protein (lane 3) revealed a specific detection of the target in the inoculated sample. This antibody demonstrated its ability to detect the target protein in sample material and could therefore be used for further analyses, especially for the development of an ELISA.

3.3.2 Development of an ELISA for the detection of PEX2_044840

For the quantitative determination of the amount of the gushing-inducing protein PEX2_044840 in grape-derived beverages, a detection assay was developed. Because of its high sensitivity and specificity, ELISA was the method of choice.

A competitive ELISA was developed that makes use of the competition between solid phase bound antigen and the PEX2_044840 protein present in a sample for binding to the Anti-PEX2_044840 antibody. The more antigen is present in the sample, the less antibody binds to the solid phase bound antigen and can be detected by an enzyme-linked secondary antibody (Anti-Rabbit-IgG). Therefore, extinction values resulting from the enzymatic color reaction are inversely proportional to the antigen concentration in the sample.

In a first attempt, the solid phase (microtiter plate) was coated with the PEX2_044840 peptide as antigen against which the antibody is directed. After optimization of the concentrations of coating antigen and primary and secondary antibody by checkerboard titrations, a serial dilution of lyophilized purified PEX2_044840 protein as the standard was measured with the ELISA. The obtained standard curve is shown in Figure 24.

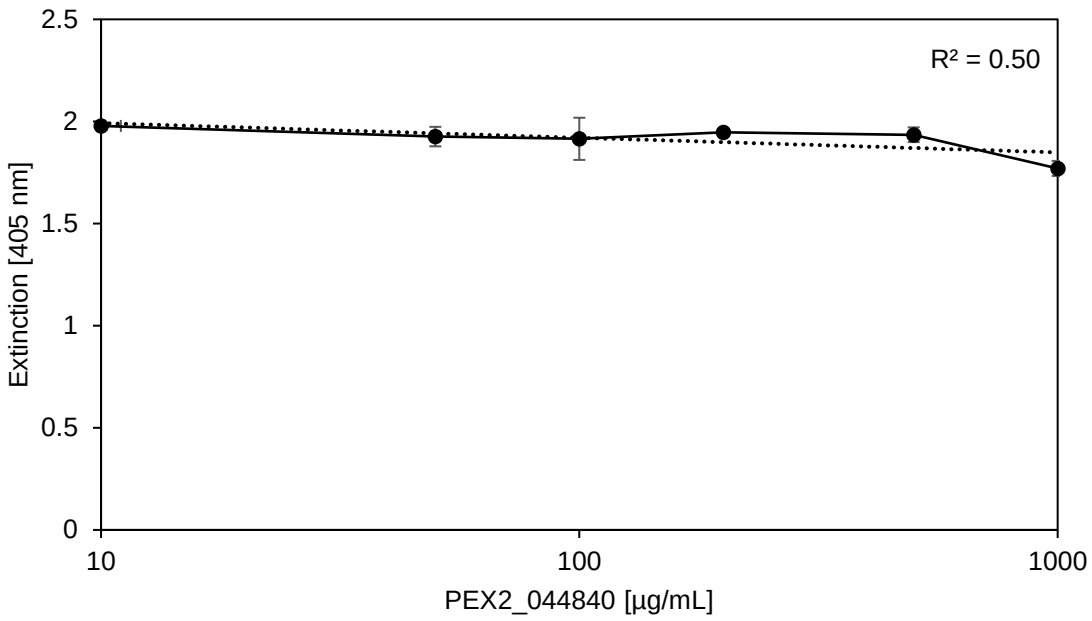


Figure 24: ELISA standard curve by coating with peptide
ELISA standard curve with PEX2_044840 protein concentrations ranging from 10 µg/mL to 1,000 µg/mL plotted on logarithmic scale against the mean extinction at 405 nm. The solid phase was coated with 100 ng/mL PEX2_044840 peptide. Coefficient of determination was $R^2 = 0.50$. A trendline and standard errors are shown.

As shown in Figure 24, the standard curve ranging from 10 µg/mL to 1,000 µg/mL PEX2_044840 protein was without any dynamic range about an extinction value of 2. No difference in extinction values between a low and high concentration of the protein was measurable. A change of the concentration range of the standard samples did not improve the results either (results not shown).

In a second attempt, the solid phase was coated with lyophilized PEX2_044840 protein to provide the same competition partners for antibody binding on the solid phase and in the standard solution. Again, several checkerboard titrations were examined to assess the optimal concentrations of reaction partners in the assay. Moreover, the effect of different buffers with different pH, different types of solid phases, and different incubation times on assay performance was analyzed (results not shown). Again, a serial dilution of lyophilized purified PEX2_044840 protein as the standard was measured with the ELISA. The obtained standard curve is shown in Figure 25.

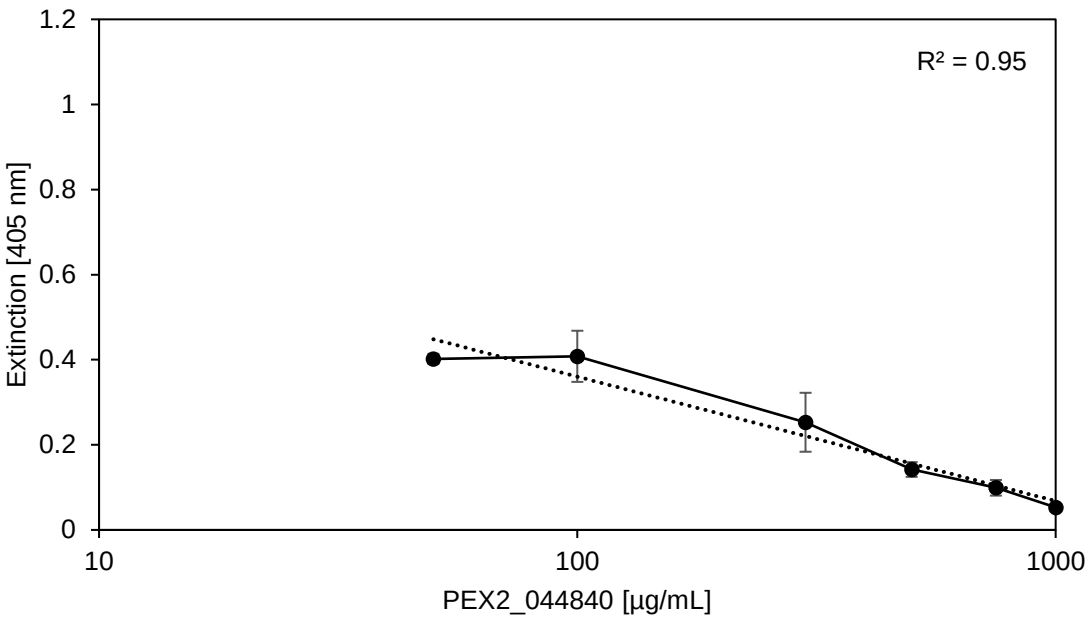


Figure 25: ELISA standard curve by coating with PEX2_044840 protein
ELISA standard curve with PEX2_044840 protein concentrations ranging from 50 µg/mL to 1,000 µg/mL plotted on logarithmic scale against the mean extinction at 405 nm. The solid phase was coated with 1,000 ng/mL PEX2_044840 protein. Coefficient of determination was $R^2 = 0.95$. A trendline and standard errors are shown.

As shown in Figure 25, the standard curve ranging from 50 µg/mL to 1,000 µg/mL PEX2_044840 protein showed a correlation between antigen concentration and extinction. However, the overall extinction values were low, ranging from 0.4 to 0.05. Again, a very low level of dynamic was found between low and high protein concentrations. The use of different buffers, solid phases, or different incubation times did not improve the results (results not shown).

In order to further examine the reason for the weak signal intensity obtained so far in the ELISA, the correlation between protein concentration and signal intensity was analyzed by dot blot analysis. The standard curve samples of recombinant PEX2_044840 used in the ELISA were blotted. The protein was analyzed untreated and after heat denaturation (95 °C for 10 min in application buffer, see Table 7) before application. The same Anti-PEX2_044840 antibody was used as in the ELISA. The results are shown in Figure 26.

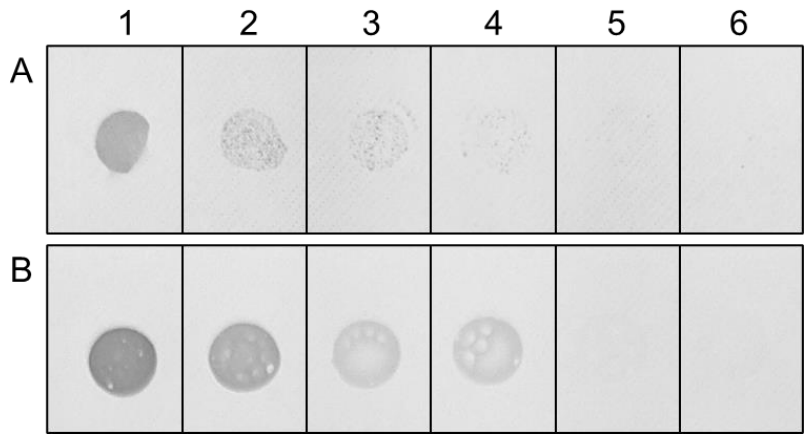


Figure 26: Dot blot analysis of PEX2_044840 protein untreated and denatured
Different concentrations of purified PEX2_044840 protein were analyzed by dot blot with Anti-PEX2_044840 antibody. A) Untreated: Samples were blotted untreated before application. B) Denatured: Samples were heated to 95 °C for 10 min in application buffer (SDS-PAGE) before application. 1 = 1,000 µg/mL PEX2_044840 protein in PBS buffer, 2 = 200 µg/mL PEX2_044840 protein in PBS buffer, 3 = 100 µg/mL PEX2_044840 protein in PBS buffer, 4 = 50 µg/mL PEX2_044840 protein in PBS buffer, 5 = 10 µg/mL PEX2_044840 protein in PBS buffer, 6 = 1 µg/mL PEX2_044840 protein in PBS buffer.

The signal intensity was much higher in the samples that contained the target protein in its heat denatured form (see Figure 26 B) than in the samples that contained the untreated protein (see Figure 26 A). In A, there is a clear signal in samples containing 1,000 µg/mL to 100 µg/mL PEX2_044840 protein, while in B also a signal occurred for the sample containing 50 µg/mL PEX2_044840 protein. The results showed that the used antibody had a higher affinity to its target protein after heat denaturation than to the untreated protein.

In a follow-up experiment, the solid phase was coated with denatured protein and the denatured protein was also used to set up the serial dilution for the standard curve. Different temperatures of 60 °C, 70 °C, 80 °C, and 95 °C, respectively, were applied for protein denaturation for 10 min to assess the optimal denaturation temperature. Optimal concentrations of coating antigen, primary and secondary antibody, substrate, standard curve range, and incubation times were assessed by several checkerboard titrations (results not shown). The results of the optimized assay are shown in Figure 27.

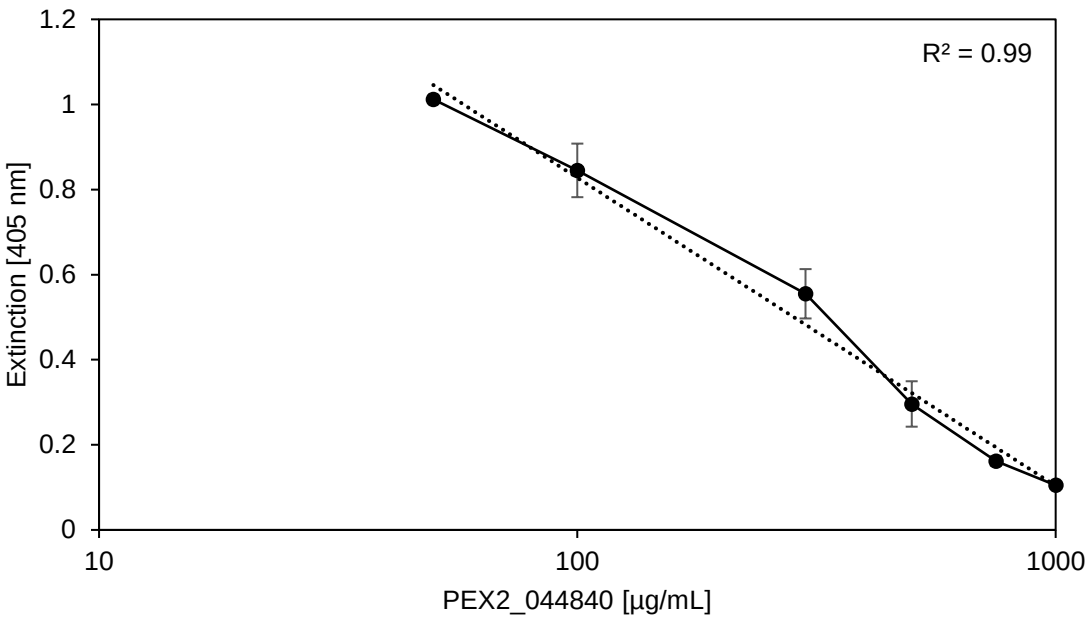


Figure 27: ELISA standard curve with denatured protein for solid phase coating and as standard
ELISA standard curve with PEX2_044840 protein heated to 60 °C for 10 min with concentrations ranging from 50 µg/mL to 1,000 µg/mL plotted on logarithmic scale against the mean extinction at 405 nm. The solid phase was coated with 1,000 ng/mL PEX2_044840 protein heated to 60 °C for 10 min. Coefficient of determination was R^2 = 0.99. A trendline and standard errors are shown.

As shown in Figure 27, the standard curve ranging from 50 µg/mL to 1,000 µg/mL PEX2_044840 protein showed extinction values ranging from 1 to 0.1 with a great dynamic range. As expected, low protein concentrations in the samples had higher extinction values than high protein concentrations. The coefficient of determination calculated from the regression (R^2 = 0.99) indicated a very strong correlation between parameters. The various applied measures used to optimize the assay revealed best results for heating of the PEX2_044840 containing sample to 60 °C for 10 min. The best coating concentration with denatured PEX2_044840 protein was 1,000 ng/mL in bicarbonate buffer. The optimal concentration of the primary antibody was 20 µg/mL in PBS buffer and the secondary antibody worked best when diluted 1:5,000 in blocking solution. The enzyme substrate was diluted to 1 mg/mL in diethanolamine buffer (see 2.2.3.3.1. for detailed protocol).

3.3.2.1 Evaluation of the PEX2_044840 ELISA

The optimizations regarding the standard curve were necessary to get a strong correlation between extinction value and PEX2_044840 concentration. Especially the heating of the standard and coating protein to 60 °C improved the standard curve. When PEX2_044840 concentrations ranging from 25 µg/mL to 1,000 µg/mL were plotted on logarithmic scale against the extinction at 405 nm, an applicable standard curve was obtained to calibrate the assay.

To further characterize the newly developed assay, the intraspecific reproducibility was determined by measuring a triplicate of the standard samples within the same microtiter plate, while the interspecific reproducibility was determined by measuring a triplicate in parallel in three separate microtiter plates. The results are shown in Figure 28.

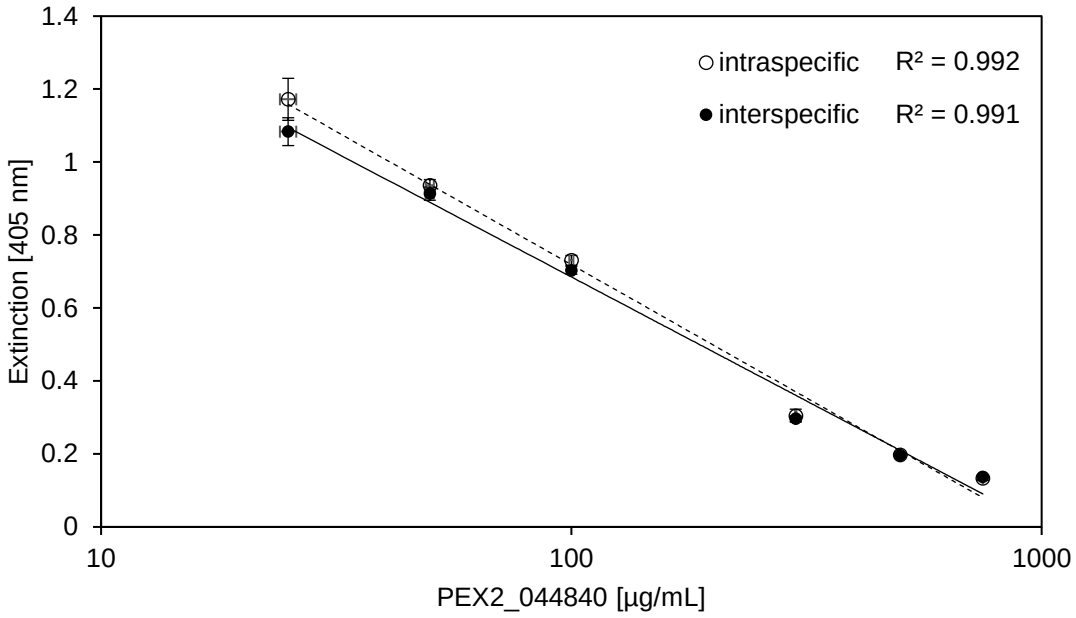


Figure 28: ELISA reproducibility

ELISA standard curves of intra- and interspecific tests with PEX2_044840 protein heated to 60 °C for 10 min with concentrations ranging from 25 µg/mL to 750 µg/mL plotted on logarithmic scale against the extinction at 405 nm. The solid phase was coated with 1,000 ng/mL PEX2_044840 protein heated to 60 °C for 10 min. The intraspecific standard curve was obtained from testing triplicates of each standard concentration within one plate. The interspecific standard curve was obtained from testing triplicates of each standard concentration in parallel in three separate plates. Standard errors of the mean of the triplicates are shown. Coefficient of determination was R^2 = 0.992 for the intraspecific test and R^2 = 0.991 for the interspecific test. Trendlines are shown.

As shown in Figure 28, both the intra- and interspecific tests revealed high reproducibility of the respective standard curves. The curve of the intraspecific test had a coefficient of determination of R^2 = 0.992, while for the interspecific test the coefficient of determination was R^2 = 0.991. For a protein concentration of 25 µg/mL as the lowest detectable concentration, the standard error in the intraspecific test was 0.06, while in the interspecific test it was 0.04. The standard errors for all other concentrations ranged between 0.00 and 0.02.

The recovery of the developed ELISA was determined by applying known concentrations of PEX2_044840 and calculating the PEX2_044840 concentration according to the results in the ELISA. The results are shown in Figure 29.

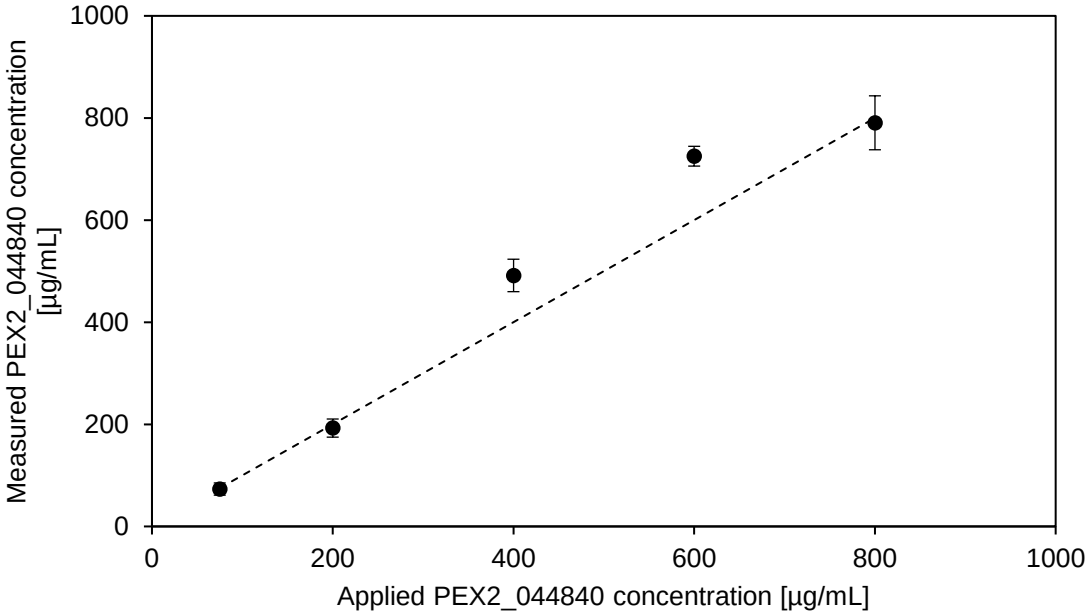


Figure 29: ELISA recovery
Different concentrations of PEX2_044840 (75 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL) were applied and measured with the ELISA based on a standard curve with PEX2_044840 protein heated to 60 °C for 10 min with concentrations ranging from 50 µg/mL to 1,000 µg/mL. The solid phase was coated with 1,000 ng/mL PEX2_044840 protein heated to 60 °C for 10 min. Standard errors of the mean of the triplicates are shown. A trendline is shown according to applied concentrations.

The trendline given in Figure 29 shows the actual PEX2_044840 concentration that was applied and which should be measured. The recovery rates were best for applied PEX2_044840 concentrations of 75 µg/mL, 200 µg/mL, and 800 µg/mL (96-99 %). Recovery rates of 121-123 % were found for concentrations 400 µg/mL and 600 µg/mL.

In order to apply the newly developed ELISA to the testing of sample materials, a sample preparation protocol was set up and optimized for the analysis of base wines in sparkling wine production. Base wines were tested in untreated form, after dialysis, as well as after lyophilization of dialyzed wines. The experiments showed the best results for non-dialyzed base wines (results not shown).

Whether or not a heat denaturation step at 60 °C was necessary for sample analysis was tested by dot blot analysis. The results are shown in Figure 30.

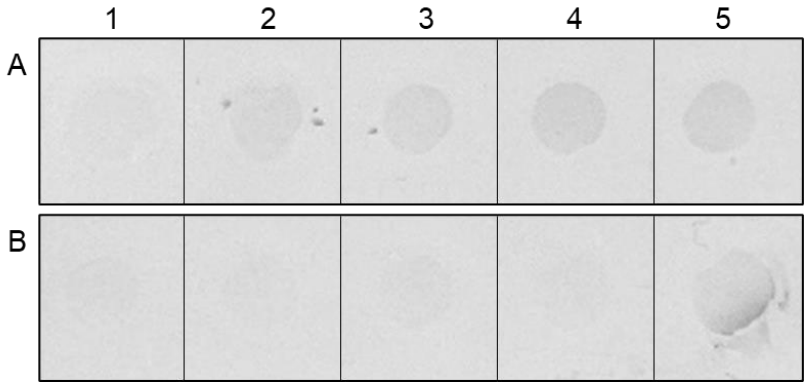


Figure 30: Dot blot with purified PEX2_044840 protein in base wine
Base wine (no. 43) was mixed with different amounts of purified PEX2_044840 protein and analyzed by dot blot with Anti-PEX2_044840 antibody. A) Samples were untreated before application. B) Samples were heated to 60 °C for 10 min before application. 1 = base wine containing 0 µg/mL PEX2_044840 protein, 2 = base wine containing 0.02 µg/mL PEX2_044840 protein, 3 = base wine containing 0.04 µg/mL PEX2_044840 protein, 4 = base wine containing 0.08 µg/mL PEX2_044840 protein, 5 = base wine containing 0.16 µg/mL PEX2_044840 protein.

Higher signal intensities were found when untreated base wine was used as sample material (see Figure 30 A). Concentrations of 0.02 µg/mL PEX2_044840 protein and more were detected in the dot blot with higher signal intensities in the higher concentrated samples. In heated base wine (see Figure 30 B), only a concentration of 0.16 µg/mL could be detected. Further tests were therefore performed with non-dialyzed and unheated base wines.

By testing different dilutions of untreated base wines, the optimal sample dilution was assessed. The results showed that the dilution that was necessary to hit the concentration range of the standard curve differed strongly between samples (results not shown). In general, red wines needed a higher dilution as white wines. Moreover, tests with optimally diluted base wines that were spiked with known concentrations of PEX2_044840 protein showed that the measured concentrations were far beyond the spiked amounts of the protein. The calculated concentrations did not seem reasonable even when the value for the unspiked control was subtracted. Therefore, correct absolute quantification of PEX2_044840 concentrations in base wine samples was not possible with the newly developed ELISA. Nevertheless, the calculated concentrations showed a correlation with the extinction values measured at 405 nm. Instead of absolute quantification, the assay was therefore applied to compare samples relatively with respect to their PEX2_044840 protein content, while the calculated protein content of each sample was normalized to its total protein content measured by BCA assay. For comparison of samples, it was necessary to dilute the wines with the same dilution factor because otherwise the calculated protein concentrations were not comparable.

Lyophilized white sparkling wines were tested with the ELISA and the percentage of PEX2_044840 in the total protein content was calculated. For the analysis, only those sparkling wines were used that had been declared as gushing-positive or gushing-negative by the manufacturer and the gushing potential of which was confirmed after opening of the bottles in the author's laboratory. The results are shown in Figure 31.

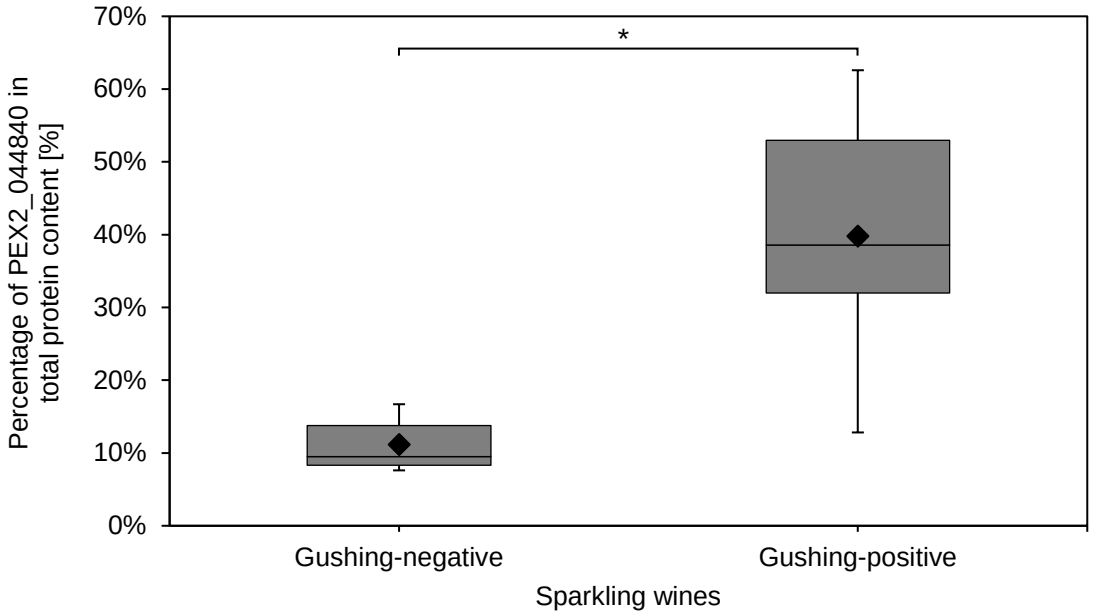


Figure 31: ELISA results by testing gushing-negative and gushing-positive sparkling wines
Five gushing-negative and 5 gushing-positive white sparkling wines were tested with the ELISA using an antibody against PEX2_044840. The box plot shows the minimum, 25th percentile, median, 75th percentile, and maximum of the percentage of PEX2_044840 in the total protein content measured by BCA assay for the tested gushing-negative and gushing-positive sparkling wines, respectively. The diamond shows the mean percentage of PEX2_044840 in the total protein content. An asterisk marks significance ($\alpha = 0.05$).

By testing 5 gushing-negative and 5 gushing-positive white sparkling wines, the calculated percentage of PEX2_044840 in the total protein content was higher for the gushing-positive sparkling wines than for the gushing-negative ones as shown in Figure 31. The mean percentage of PEX2_044840 in the total protein content was 11 % for gushing-negative sparkling wines and 40 % for gushing-positive ones. The difference in means between the two groups was found to be significant (unpaired t-test, $p = 0.03$, $\alpha = 0.05$).

The results showed that the developed ELISA can be applied to compare samples relatively with respect to their PEX2_044840 protein content. Moreover, the PEX2_044840 protein was found in higher concentrations in gushing-positive sparkling wines indicating its gushing-inducing effect.

3.3.3 Dot blot for immunochemical detection of PDE_07106

The development of an ELISA for the determination of the amount of the gushing-inducing protein PDE_07106 in grape-derived beverages was not feasible during the current study due to too low amounts of purified recombinant protein to be used as standard (see section 3.1.2). Therefore, it was tested whether a dot blot analysis that requires lower purified protein amounts than an ELISA can be used as an alternative assay to detect critical amounts of this protein in base wines.

The tested concentrations of purified protein PDE_07106 that were used in gushing tests (see section 3.2) were added to base wine (no. 43), followed by dot blot analysis with the Anti-PDE_07106 antibody. The results are shown in Figure 32.

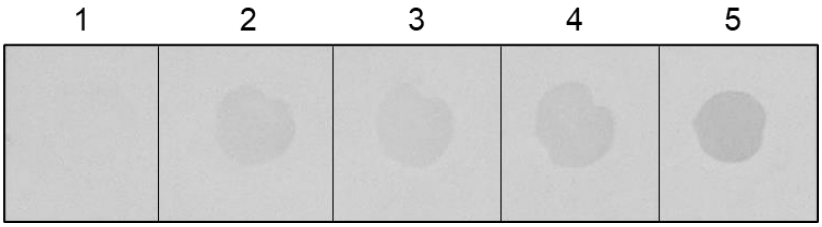


Figure 32: Dot blot with purified PDE_07106 protein in base wine

Base wine (no. 43) was mixed with different amounts of purified PDE_07106 protein and analyzed by dot blot with Anti-PDE_07106 antibody. 1 = base wine containing 0 µg/mL PDE_07106 protein, 2 = base wine containing 0.02 µg/mL PDE_07106 protein, 3 = base wine containing 0.04 µg/mL PDE_07106 protein, 4 = base wine containing 0.08 µg/mL PDE_07106 protein, 5 = base wine containing 0.16 µg/mL PDE_07106 protein.

Concentrations of 0.02 µg/mL PDE_07106 protein and more were detected in base wine by dot blot analysis with higher signal intensities in the higher concentrated samples as shown in Figure 32. The tested concentrations corresponded to the tested concentrations in the gushing tests (e.g., 0.02 µg/mL = 15 µg/750 mL).

The results showed that the dot blot can be used due to its sensitivity for analyses of base wines for the gushing-inducing *P. oxalicum* protein PDE_07106. In addition, qualitative Western blot analysis was shown to be applicable for detection of both *P. oxalicum* proteins (see section 3.3.1).

3.4 Detection of gushing-relevant fungi on sample materials from European vineyards

The filamentous fungi *P. expansum* and *P. oxalicum* were shown to be involved in the induction of gushing in sparkling wine by the results of this study as well as by previous studies (Frisch, 2018; Vogt et al., 2017b). On the other hand, *B. cinerea* was demonstrated to reduce the concentration of proteins that are discussed to have a gushing-reducing effect (Kupfer et al., 2017b). As a result, the occurrence of these fungi in vineyards was assumed to increase the risk of gushing in sparkling wines produced from infected grapes. Early and sensitive detection of these fungal species on sample materials from vineyards can help to determine and reduce the risk of a gushing problem in later production steps. Therefore, LAMP assays were developed and optimized during the current study for testing of grape, soil, and must samples.

3.4.1 Development of a LAMP assay for the detection of *P. expansum*

P. expansum was shown to be involved in the induction of gushing due to its protein PEX2_044840 (see section 3.2) causing economic losses and reputational damages to the beverage industries. Moreover, it is known as one of the major producers of the mycotoxin patulin and as a widespread plant pathogen causing considerable economic losses due to postharvest rot of fruits, including grapes (König et al., 2009; Vico et al., 2014). Therefore, a specific, sensitive, and rapid detection and identification of this fungus is an important tool for quality control. Hence, a DNA amplification assay based on the LAMP technology was developed and optimized for the detection of *P. expansum* for on-site applications, especially for winemakers.

3.4.1.1 Primer design and confirmation of LAMP product

The LAMP primers were designed based on the nucleotide sequence of the gene coding for the gushing-inducing protein PEX2_044840 from *P. expansum* (GenBank accession number NW_015971172.1: c457334-456670). The position and orientation of the primers is shown in Figure 33. The specificity of the designed primers was confirmed *in silico* by the nucleotide BLAST search tool on the NCBI website (Altschul et al., 1990).

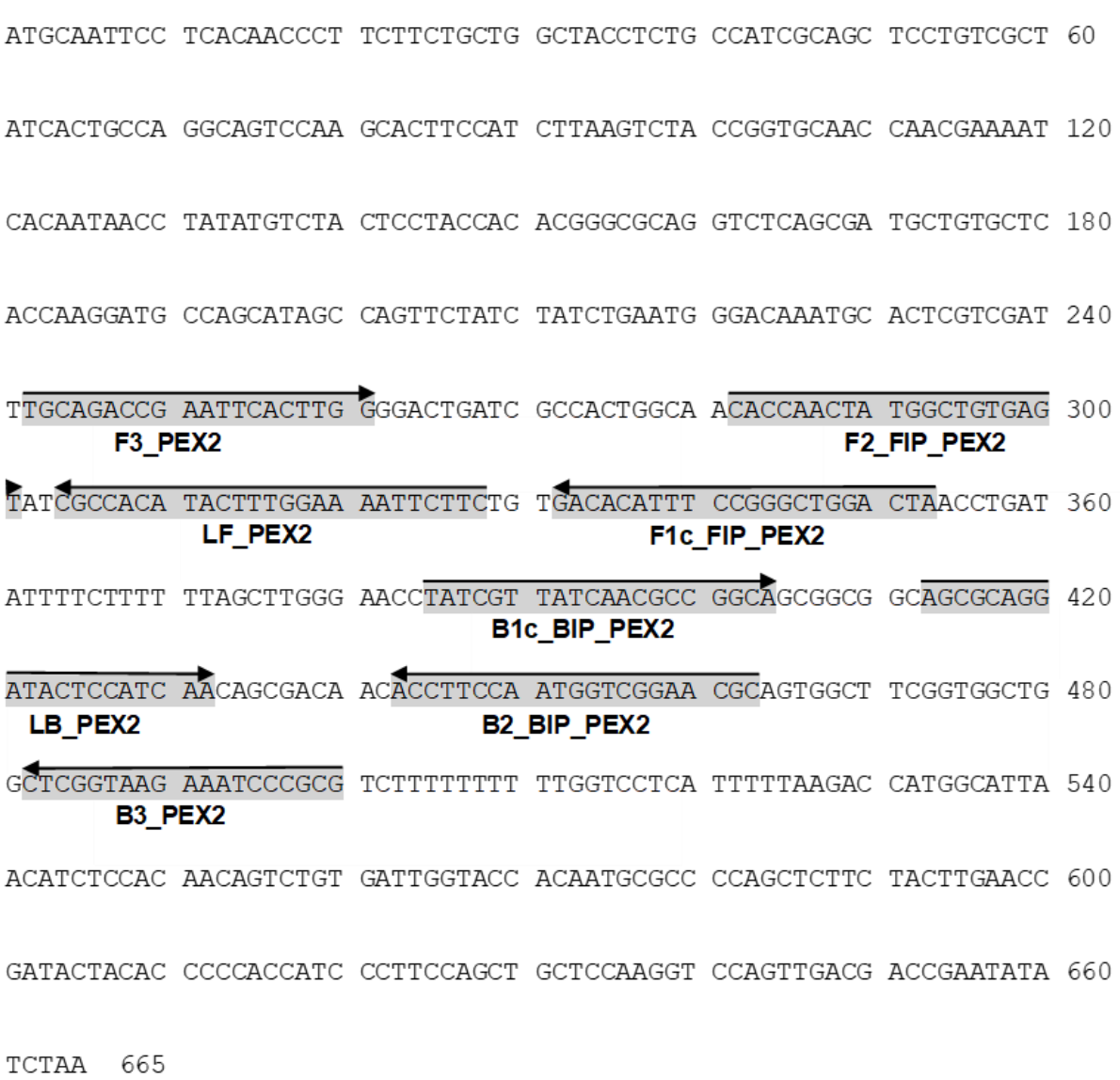


Figure 33: Position and orientation of LAMP primers for detection of *P. expansum*

The nucleotide sequence of the gene coding for the protein PEX2_044840 from *P. expansum* is shown. The position and orientation of designed LAMP primers are highlighted in grey and with arrowheads. Figure modified from Frisch et al. (2021b).

For confirmation of DNA amplification in positive LAMP reactions, the LAMP products of a tenfold serial dilution of genomic DNA from *P. expansum* TMW 4.2805 were applied onto an agarose gel. The results are shown in Figure 34.

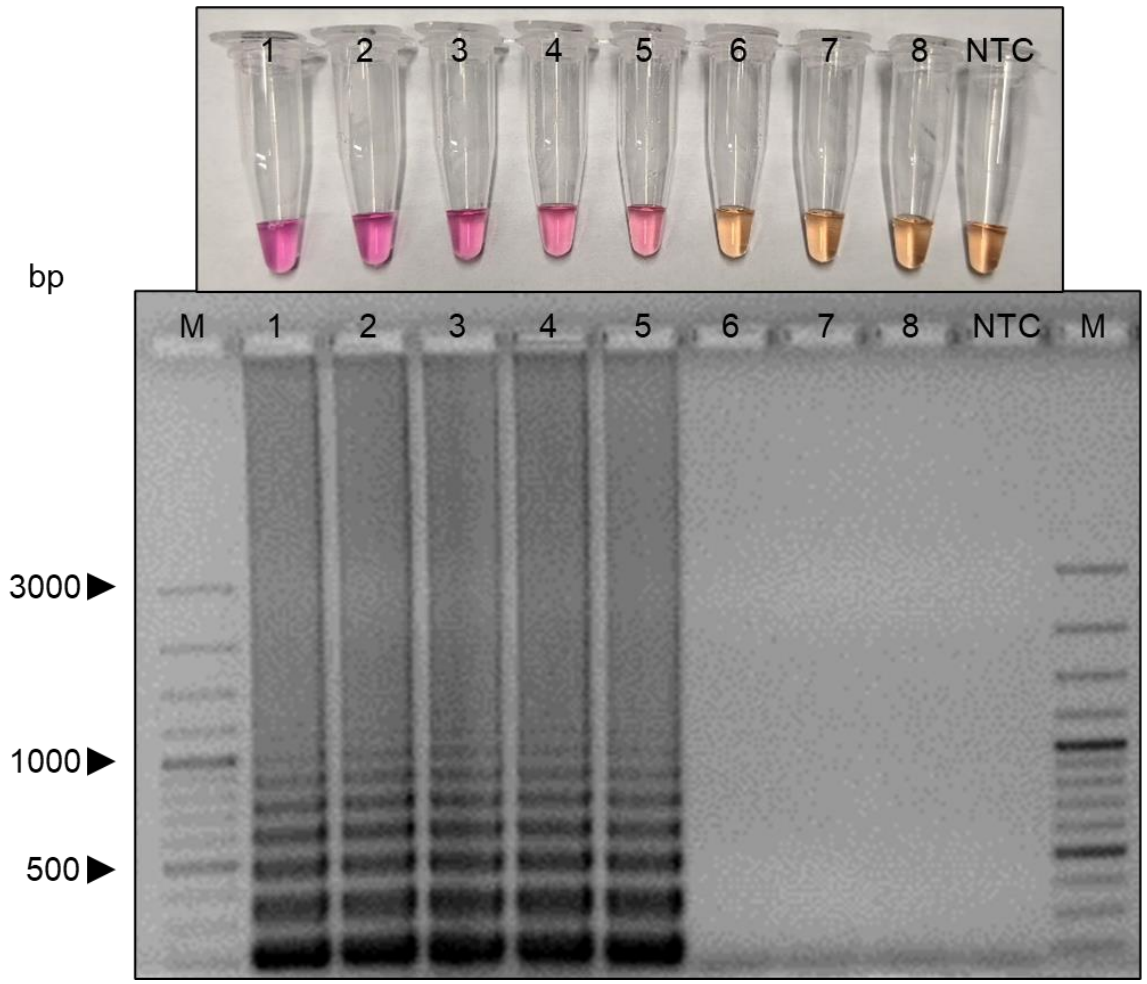


Figure 34: Analysis of *P. expansum* LAMP amplification products by agarose gel electrophoresis
The results of the *P. expansum* LAMP assay with a tenfold serial dilution of genomic DNA of *P. expansum* TMW 4.2805 ranging from 250 ng/reaction (rxn) to 25 fg/rxn are shown. Pink = positive reaction, orange = negative reaction. 1 = 250 ng/rxn, 2 = 25 ng/rxn, 3 = 2.5 ng/rxn, 4 = 250 pg/rxn, 5 = 25 pg/rxn, 6 = 2.5 pg/rxn, 7 = 250 fg/rxn, 8 = 25 fg/rxn, NTC = no template control with sterile $_{d}H_2O$ instead of DNA, M = GeneRuler 100 bp Plus DNA ladder. LAMP products were applied in the same order on an agarose gel. Figure modified from Frisch et al. (2021b).

Positive LAMP reactions (see Figure 34, lane 1-5) that were indicated by a color change from orange to pink showed a ladder-like pattern of DNA fragments in the agarose gel, while the negative reactions (lane 6-8, NTC) showed no bands because no DNA amplification had occurred under the reaction conditions. The smallest DNA fragments from the positive reactions were excised from the gel and pooled together. In order to prove the identity of the amplified product, the fragments were purified and used as template in a PCR with primers F2_PEX2 and B2_PEX2. Sequencing of the resulting PCR product revealed 100 % identity with a partial sequence of the *PEX2_044840* gene from *P. expansum* (GenBank accession number XM_016741759.1) as shown in Figure 35.

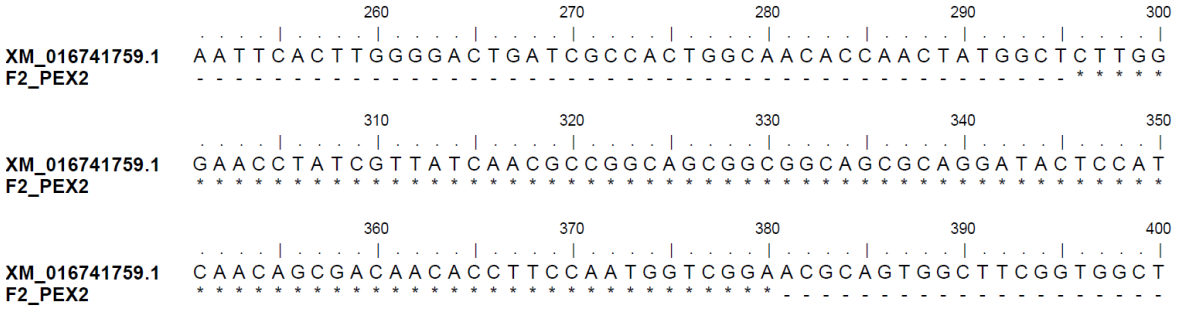


Figure 35: Sequence alignment of *P. expansum* LAMP product after PCR

The PCR product with primers F2_PEX2 and B2_PEX2 of DNA from positive LAMP reactions was aligned with a partial sequence of the *PEX2_044840* gene from *P. expansum* (GenBank accession number XM_016741759.1). Positions marked with an asterisk show identical nucleotides in both sequences.

3.4.1.2 Optimization of the *P. expansum* LAMP assay

LAMP reactions were conducted using neutral red as indicator dye indicating a positive reaction by a color change from orange to pink after isothermal incubation of the reaction mix for 60 min.

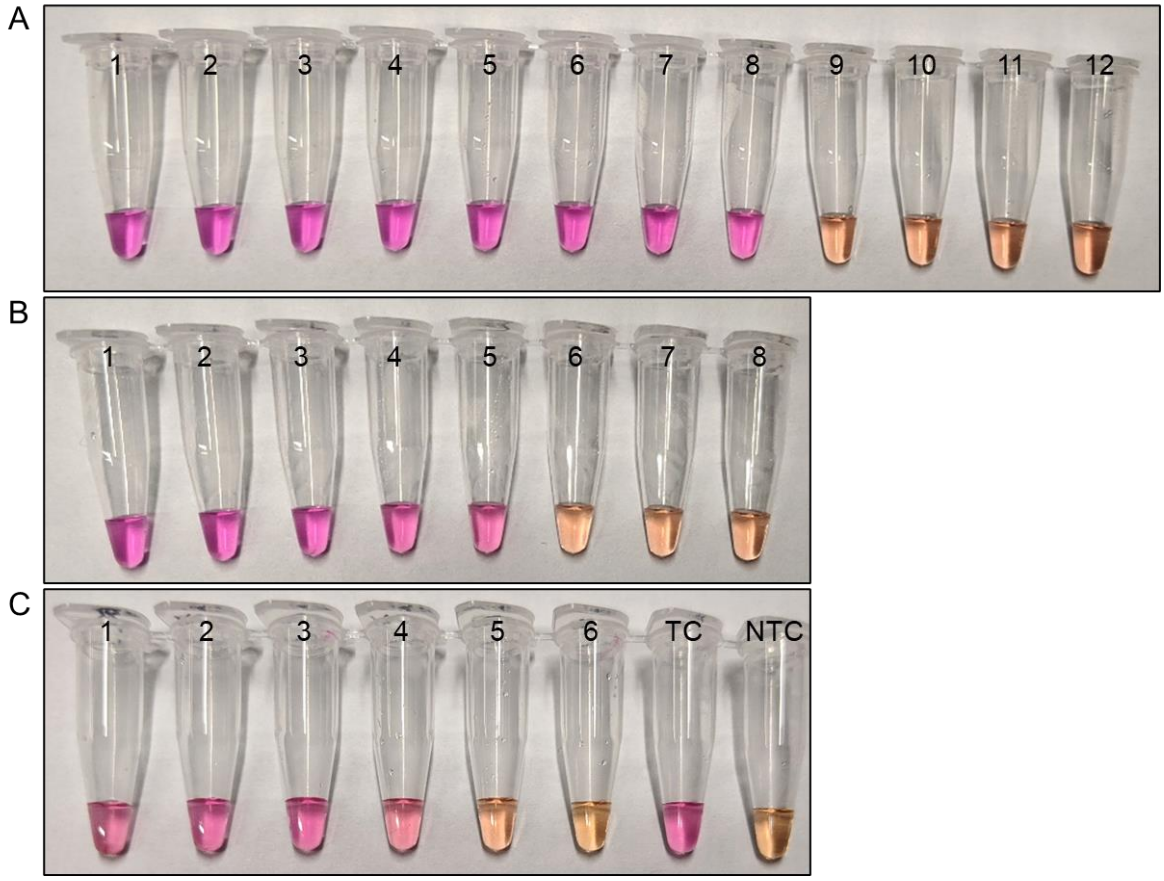


Figure 36: Determination of *P. expansum* LAMP assay characteristics

Determination of optimal reaction temperature (A) and sensitivity of the *P. expansum* LAMP assay using gDNA (B) and conidial suspension (C). Pink = positive reaction, orange = negative reaction. A) LAMP assay with a temperature gradient from 60 °C to 72.5 °C with gDNA of *P. expansum* TMW 4.2805 (50 ng/rxn) incubated at 1 = 60 °C, 2 = 60.2 °C, 3 = 60.9 °C, 4 = 62 °C, 5 = 63.3 °C, 6 = 64.9 °C, 7 = 66.6 °C, 8 = 68.2 °C, 9 = 69.7 °C, 10 = 71 °C, 11 = 72 °C; 12 = 72.5 °C. B) LAMP assay with a tenfold serial dilution of gDNA of *P. expansum* TMW 4.2805 ranging from 250 ng/rxn to 25 fg/rxn. 1 = 250 ng/rxn, 2 = 25 ng/rxn, 3 = 2.5 ng/rxn, 4 = 250 pg/rxn, 5 = 25 pg/rxn, 6 = 2.5 pg/rxn, 7 = 250 fg/rxn, 8 = 25 fg/rxn. C) LAMP assay with a tenfold serial dilution of a conidial suspension of *P. expansum* TMW 4.2808 ranging from $1x10^6$ spores/rxn to $1x10^1$ spores/rxn. 1 = $1x10^6$ spores/rxn, 2 = $1x10^5$ spores/rxn, 3 = $1x10^4$ spores/rxn, 4 = $1x10^3$ spores/rxn, 5 = $1x10^2$ spores/rxn, 6 = $1x10^1$ spores/rxn, TC = template control with gDNA of *P. expansum* TMW 4.2808 (20 ng/µL), NTC = no template control with sterile $_dH_2O$. Figure modified from Frisch et al. (2021b).

The optimal incubation temperature was assessed by incubating reactions in a gradient of different constant temperatures ranging from 60 °C to 72.5 °C (see Figure 36 A). Positive reactions were detected between 60 °C (1) and 68.2 °C (8), while no amplification occurred at higher temperatures. An incubation temperature of 68 °C was chosen for all further experiments as preliminary experiments showed unspecific reactions with a few non-target fungi at lower temperatures (results not shown).

Sensitivity of the assay was determined. The LAMP assay with genomic DNA of *P. expansum* (see Figure 36 B) showed positive results for reactions containing 250 ng/rxn (1) to 25 pg/rxn (5). No positive result was found for reactions containing 2.5 pg/rxn (6) to 25 fg/rxn (8) genomic DNA. Hence, the detection limit of the *P. expansum* LAMP assay for genomic DNA was 25 pg/rxn. The LAMP assay with a conidial suspension after glass bead treatment of *P. expansum* (Figure 36 C) showed positive results for reactions containing 1 x 10^6 spores/rxn (1) to 1 x 10^3 spores/rxn (4), and for the template control with genomic DNA of *P. expansum* (TC). No positive result was found for reactions containing 1 x 10^2 spores/rxn (5) or less, and for the no template control with sterile $_dH_2O$ instead of DNA (NTC). Hence, the detection limit of the *P. expansum* LAMP assay for conidia after glass bead treatment was 1 x 10^3 spores/rxn.

Specificity of the assay was assessed by testing genomic DNA of 188 fungal strains representing 32 genera and 132 species. The results are listed in Table 47.

Table 47: Determination of *P. expansum* LAMP assay specificity
Purified DNA (100 ng/rxn) of 188 fungal strains was tested as template in the *P. expansum* LAMP assay. Species name, strain and clone number, and the result in the LAMP assay are listed. **+** = positive result, - = negative result. Positive species are marked in bold. Table modified from Frisch et al. (2021b).

Species	Strain	Clone	LAMP result
Alternaria alternata	[n]TMW 4.0438	TMW 4.0438	-
Alternaria mali	[c]CBS 106.24	TMW 4.1406	-
Alternaria spp.	TMW 4.1428	TMW 4.1428	-
Aspergillus aculeatus	TMW 4.1776	TMW 4.1776	-
Aspergillus alliaceus	[e]DSM 813	TMW 4.1077	-
Aspergillus arachidicola	[f]IBT 27128	TMW 4.2204	-
Aspergillus auricomus	CBS 467.65	TMW 4.1631	-
Aspergillus awamori	CBS 101.704	TMW 4.1066	-
Aspergillus bombycis	IBT 23536	TMW 4.2210	-
Aspergillus bridgeri	CBS 350.81	TMW 4.1632	-
Aspergillus caelatus	IBT 29700	TMW 4.2209	-
Aspergillus carbonarius	TMW 4.1512	TMW 4.1512	-
Aspergillus clavatus	CBS 513.65	TMW 4.1086	-
Aspergillus clavatus	IBT 12362	TMW 4.1976	-
Aspergillus clavatus	IBT 12778	TMW 4.1977	-
Aspergillus clavatus	IBT 18790	TMW 4.1978	-

Table 47 (continued)

Species	Strain	Clone	LAMP result
Aspergillus clavatus	IBT 21704	TMW 4.1979	-
Aspergillus clavatus	IBT 21863	TMW 4.1980	-
Aspergillus elegans	CBS 310.80	TMW 4.1633	-
Aspergillus ellipticus	CBS 707.79	TMW 4.1629	-
Aspergillus flavus	TMW 4.1859	TMW 4.1859	-
Aspergillus foetidus	CBS 114.49	TMW 4.1628	-
Aspergillus fumigatus	CBS 113.55	TMW 4.0623	-
Aspergillus helicothrix	CBS 677.79	TMW 4.1630	-
Aspergillus heteromorphus	CBS 117.55	TMW 4.1626	-
Aspergillus insulicola	CBS 382.75	TMW 4.1634	-
Aspergillus japonicus	CBS 114.51	TMW 4.1627	-
Aspergillus minisclerotigenes	IBT 27177	TMW 4.2205	-
Aspergillus niger	CBS 101.698	TMW 4.1068	-
Aspergillus nomius	CBS 260.88	TMW 4.1960	-
Aspergillus ochraceoroseus	CBS 101.887	TMW 4.1772	-
Aspergillus ochraceus	CBS 263.67	TMW 4.0706	-
Aspergillus oryzae	IBT 28103	TMW 4.2208	-
Aspergillus parasiticus	CBS 126.62	TMW 4.1768	-
Aspergillus parvisclerotigenes	IBT 3850	TMW 4.2205	-
Aspergillus petrakii	CBS 105.57	TMW 4.1087	-
Aspergillus pseudotararii	IBT 21092	TMW 4.2212	-
Aspergillus rambellii	IBT 14580	TMW 4.2211	-
Aspergillus sclerotiorum	CBS 549.65	TMW 4.1089	-
Aspergillus sojae	IBT 21643	TMW 4.2207	-
Aspergillus sulfureus	CBS 550.65	TMW 4.1067	-
Aspergillus tamarii	CBS 591.68	TMW 4.1771	-
Aspergillus terreus	CBS 377.64	TMW 4.1060	-
Aspergillus toxicarius	CBS 822.72	TMW 4.1766	-
Aspergillus tubingensis	[g]ITEM 4496	TMW 4.2008	-
Aspergillus usami var. *shiro-usami*	CBS 101.700	TMW 4.1072	-
Aureobasidium pullulans	TMW 4.2253	TMW 4.2253	-
Beltraniella portoricensis	CBS 856.70	TMW 4.0402	-
Bipolaris sorokiniana	CBS 311.64	TMW 4.0509	-
Botrytis cinerea	CBS 121.39	TMW 4.2527	-
Botrytis cinerea	TMW 4.2743	TMW 4.2743	-
Byssochlamys nivea	TMW 4.1565	TMW 4.1565	-
Byssochlamys nivea	CBS 100.11	TMW 4.1594	-
Cladobotryum dendroides	[j]NRRL 2903	TMW 4.0467	-
Cladosporium macrocarpum	TMW 4.2371	TMW 4.2371	-
Cladosporium sphaerospermum	TMW 4.2370	TMW 4.2370	-
Colletotrichum acutatum	CBS 295.67	TMW 4.0652	-
Colletotrichum fragariae	CBS 142.31	TMW 4.0651	-
Colletotrichum gloeosporioides	CBS 285.50	TMW 4.0650	-
Cryptomela acutispora	CBS 157.33	TMW 4.1620	-

Table 47 (continued)

Species	Strain	Clone	LAMP result
Drechslera teres	CBS 378.59	TMW 4.0558	-
Drechslera tricici-repentis	CBS 265.80	TMW 4.0559	-
Emericella astellata	IBT 21903	TMW 4.2202	-
Emericella olivicola	IBT 26499	TMW 4.2201	-
Emericella venezuelensis	IBT 20956	TMW 4.2203	-
Epicoccum nigrum	TMW 4.1407	TMW 4.1407	-
Fusarium acuminatum	CBS 485.94	TMW 4.0701	-
Fusarium avenaceum	DSM 62161	TMW 4.0140	-
Fusarium beomiforme	[a]BBA 69406	TMW 4.0513	-
Fusarium cerealis	CBS 589.93	TMW 4.0406	-
Fusarium compactum	CBS 466.92	TMW 4.0433	-
Fusarium culmorum	DSM 62191	TMW 4.0149	-
Fusarium dimerum	CBS 175.31	TMW 4.0626	-
Fusarium dlamini	[h]MRC 3024	TMW 4.0571	-
Fusarium eumartii	DSM 62809	TMW 4.0303	-
Fusarium heterosporum	DSM 62231	TMW 4.0224	-
Fusarium longipes	CBS 739.79	TMW 4.0350	-
Fusarium melanochlorum	CBS 202.65	TMW 4.0625	-
Fusarium napiforme	BBA 67629	TMW 4.0510	-
Fusarium oxysporum	DSM 62292	TMW 4.0163	-
Fusarium proliferatum	DSM 62261	TMW 4.0236	-
Fusarium scirpi	CBS 448.84	TMW 4.0410	-
Fusarium solani	DSM 62416	TMW 4.0255	-
Fusarium subglutinans	BBA 63621	TMW 4.0947	-
Fusarium sublunatum var. *sublunatum*	CBS 189.34	TMW 4.0417	-
Fusarium torulosum	BBA 64465	TMW 4.0437	-
Geomyces auratus	BBA 66873	TMW 4.0905	-
Geomyces auratus	BBA 66886	TMW 4.0906	-
Geomyces pannorum	TMW 4.2340	TMW 4.2340	-
Geotrichum candidum	TMW 4.0508	TMW 4.0508	-
Gliocephalotrichum spec. nov.	NRRL 2993	TMW 4.0468	-
Hypomyces rosellus	CBS 521.81	TMW 4.0400	-
Memnoniella echinata	CBS 627.61	TMW 4.0711	-
Microdochium majus	TMW 4.0496	TMW 4.0496	-
Microdochium nivale	TMW 4.0495	TMW 4.0495	-
Monascus ruber	TMW 4.1426	TMW 4.1426	-
Mucor hiemalis	TMW 4.2319	TMW 4.2319	-
Mucor mucedo	DSM 809	TMW 4.0441	-
Myrothecium roridum	CBS 331.51	TMW 4.0668	-
Paecilomyces saturatus	TMW 4.2614	TMW 4.2614	-
Penicillium aurantiogriseum	CBS 225.90	TMW 4.1603	-
Penicillium brevicompactum	TMW 4.2279	TMW 4.2279	-
Penicillium brevicompactum	TMW 4.2613	TMW 4.2613	-
Penicillium brevicompactum	[m]SP 831	TMW 4.2816	-

Table 47 (continued)

Species	Strain	Clone	LAMP result
Penicillium brevicompactum	TMW 4.2921	TMW 4.2921	-
Penicillium camembertii	DSM 1233	TMW 4.0442	-
Penicillium canescens	[i]MUM 14.55	TMW 4.2544	-
Penicillium chrysogenum	CBS 573.68	TMW 4.1958	-
Penicillium clavigerum	TMW 4.1973	TMW 4.1973	**+**
Penicillium clavigerum	TMW 4.1974	TMW 4.1974	**+**
Penicillium clavigerum	TMW 4.1975	TMW 4.1975	**+**
Penicillium commune	CBS 311.48	TMW 4.1088	-
Penicillium commune	TMW 4.2270	TMW 4.2270	-
Penicillium coprophilum	SP 817	TMW 4.2815	-
Penicillium corylophilum	CBS 321.48	TMW 4.1598	-
Penicillium crustosum	CBS 499.73	TMW 4.1080	-
Penicillium digitatum	DSM 62840	TMW 4.1083	-
Penicillium expansum	DSM 62841	TMW 4.0466	**+**
Penicillium expansum	TMW 4.1363	TMW 4.1363	**+**
Penicillium expansum	TMW 4.1605	TMW 4.1605	**+**
Penicillium expansum	TMW 4.2495	TMW 4.2495	**+**
Penicillium expansum	TMW 4.2496	TMW 4.2496	**+**
Penicillium expansum	ITEM 6801	TMW 4.2577	**+**
Penicillium expansum	ITEM 7015	TMW 4.2578	**+**
Penicillium expansum	ITEM 7545	TMW 4.2579	**+**
Penicillium expansum	ITEM 9590	TMW 4.2580	**+**
Penicillium expansum	TMW 4.2778	TMW 4.2778	**+**
Penicillium expansum	TMW 4.2779	TMW 4.2779	**+**
Penicillium expansum	TMW 4.2780	TMW 4.2780	**+**
Penicillium expansum	TMW 4.2781	TMW 4.2781	**+**
Penicillium expansum	MUM 17.87	TMW 4.2802	**+**
Penicillium expansum	MUM 17.88	TMW 4.2803	**+**
Penicillium expansum	MUM 17.86	TMW 4.2804	**+**
Penicillium expansum	TMW 4.2805	TMW 4.2805	**+**
Penicillium expansum	TMW 4.2806	TMW 4.2806	**+**
Penicillium expansum	TMW 4.2807	TMW 4.2807	**+**
Penicillium expansum	TMW 4.2808	TMW 4.2808	**+**
Penicillium expansum	TMW 4.2820	TMW 4.2820	**+**
Penicillium expansum	TMW 4.2821	TMW 4.2821	**+**
Penicillium expansum	TMW 4.2822	TMW 4.2822	**+**
Penicillium expansum	TMW 4.2823	TMW 4.2823	**+**
Penicillium expansum	TMW 4.2824	TMW 4.2824	**+**
Penicillium expansum	TMW 4.2825	TMW 4.2825	**+**
Penicillium expansum	TMW 4.2826	TMW 4.2826	**+**
Penicillium expansum	TMW 4.2827	TMW 4.2827	**+**
Penicillium expansum	TMW 4.2829	TMW 4.2829	**+**

Table 47 (continued)

Species	Strain	Clone	LAMP result
Penicillium expansum	TMW 4.2830	TMW 4.2830	**+**
Penicillium expansum	TMW 4.2831	TMW 4.2831	**+**
Penicillium expansum	TMW 4.2832	TMW 4.2832	**+**
Penicillium expansum	TMW 4.2833	TMW 4.2833	**+**
Penicillium expansum	TMW 4.2834	TMW 4.2834	**+**
Penicillium expansum	TMW 4.2835	TMW 4.2835	**+**
Penicillium expansum	TMW 4.2836	TMW 4.2836	**+**
Penicillium expansum	TMW 4.2837	TMW 4.2837	**+**
Penicillium glabrum	TMW 4.2027	TMW 4.2027	-
Penicillium glandicola	TMW 4.1543	TMW 4.1543	-
Penicillium glandicola	TMW 4.2500	TMW 4.2500	-
Penicillium griseofulvum	TMW 4.2914	TMW 4.2914	-
Penicillium italicum	DSM 62846	TMW 4.1084	-
Penicillium jensenii	TMW 4.2316	TMW 4.2316	-
Penicillium nalgiovense	TMW 4.1371	TMW 4.1371	-
Penicillium nalgiovense	MUM 14.34	TMW 4.2532	-
Penicillium nordicum	[b]BFE 487	TMW 4.2213	-
Penicillium nordicum	TMW 4.2271	TMW 4.2271	-
Penicillium olsonii	TMW 4.1362	TMW 4.1362	-
Penicillium oxalicum	MUM 14.41	TMW 4.2539	-
Penicillium oxalicum	MUM 17.82	TMW 4.2799	-
Penicillium oxalicum	MUM 17.81	TMW 4.2800	-
Penicillium paneum	MUM 14.47	TMW 4.2542	-
Penicillium purpurescens	CBS 223.28	TMW 4.1082	-
Penicillium purpurogenum	CBS 286.36	TMW 4.1079	-
Penicillium roqueforti	CBS 221.30	TMW 4.1599	-
Penicillium roseopurpureum	TMW 4.1770	TMW 4.1770	-
Penicillium rugulosum	TMW 4.1902	TMW 4.1902	-
Penicillium stoloniferum	TMW 4.2280	TMW 4.2280	-
Penicillium variabile	CBS 385.48	TMW 4.1081	-
Penicillium verrucosum	CBS 603.74	TMW 4.1073	-
Penicillium vulpinum	TMW 4.2399	TMW 4.2399	-
Pseudogymnoascus destructans	[k]OT-29-2010	TMW 4.2511	-
Pseudogymnoascus roseus	[d]CCF 3426	TMW 4.2421	-
Scopulariopsis acremonioides	TMW 4.2366	TMW 4.2366	-
Stachybotrys chartarum	[l]Sp 2682	TMW 4.0523	-
Trichoderma harzianum	TMW 4.1502	TMW 4.1502	-
Trichoderma virens	CBS 344.47	TMW 4.0710	-
Trichothecium roseum	CBS 567.50	TMW 4.0691	-
Zygosaccharomyces bailii	DSM 70834	TMW 3.058	-
Zygosaccharomyces bisporus	TMW 3.062	TMW 3.062	-
Zygosaccharomyces rouxii	DSM 2531	TMW 3.057	-

Table 47 (continued)

[a] BBA	= Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, Germany
[b] BFE	= Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Karlsruhe, Germany
[c] CBS	= Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands
[d] CCF	= Culture Collection of Fungi, Charles University Prague, Czech Republic
[e] DSM	= Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, Germany
[f] IBT	= Culture collection of Center for Microbial Biotechnology (CMB), Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark
[g] ITEM	= Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy
[h] MRC	= South African Medical Research Council, Tygerberg, South Africa
[i] MUM	= Micoteca da Universidade Minho, University of Minho, Braga, Portugal
[j] NRRL	= Northern Regional Research Laboratory, Peoria (Illinois), USA
[k] OT	= Leibniz-Institut für Zoo- und Wildtierforschung, Berlin, Germany
[l] Sp	= Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Kulmbach, Germany
[m] SP	= Lehrstuhl für Lebensmittelsicherheit, Ludwig-Maximilians-University, Munich, Germany
[n] TMW	= Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, Germany

As shown in Table 47, all 37 *P. expansum* strains tested positive in the LAMP assay. In addition, three strains of *P. clavigerum* (TMW 4.1973, TMW 4.1974, TMW 4.1975) tested positive, while all other tested species showed negative results.

3.4.1.3 Application of the *P. expansum* LAMP assay

The applicability of the LAMP assay for food analyses was assessed by testing artificially contaminated apples, grapes, apple juice, grape juice, and apple puree over a period of five days.

Simple washing steps and mechanical treatment (see 2.2.4.1.2) were sufficient to obtain detectable amounts of DNA from grapes and apples for LAMP analysis. However, DNA extraction (see 2.2.4.1.1) was necessary prior to LAMP analysis of apple juice, grape juice, and apple puree. Optimization work (results not shown) showed that DNA prepared from grape washings and mechanical treatment needed to be diluted 1:5 with $_dH_2O$ before addition to the assay in order to reduce inhibitive effects of the matrix. DNA obtained from washings and mechanical treatment of apples as well as DNA extracted from grape juice, apple juice, and apple puree could be directly used in the LAMP reaction. All matrices inoculated with *P. brevicompactum* TMW 4.2921 (negative control) or $_dH_2O$ (no template control), respectively, were tested negative over the entire test period. Immediately after inoculation at day 0 and at successive days, the LAMP assay was positive for grapes, apple juice, and grape juice. Positive results were obtained at day 2 and following days in apples and at day 5 in apple puree.

The results showed that the newly developed assay for the detection of *P. expansum* is a useful tool for the screening of sample materials. Therefore, samples of grapes, soil, and must were analyzed during the harvesting season 2020 (see 3.4.3).

3.4.2 Optimization of LAMP assays for *P. oxalicum* and *B. cinerea*

LAMP assays for the detection of *P. oxalicum* (Vogt et al., 2017a) and *B. cinerea* (Tomlinson et al., 2010) were adapted from the literature and optimized for the use of ammonium sulfate buffer and neutral red instead of the originally described buffers and detection methods. As with the *P. expansum* assay described previously, the optimal incubation temperatures were assessed by incubation for 60 min in a temperature gradient. The results are shown in Figure 37 A and B.

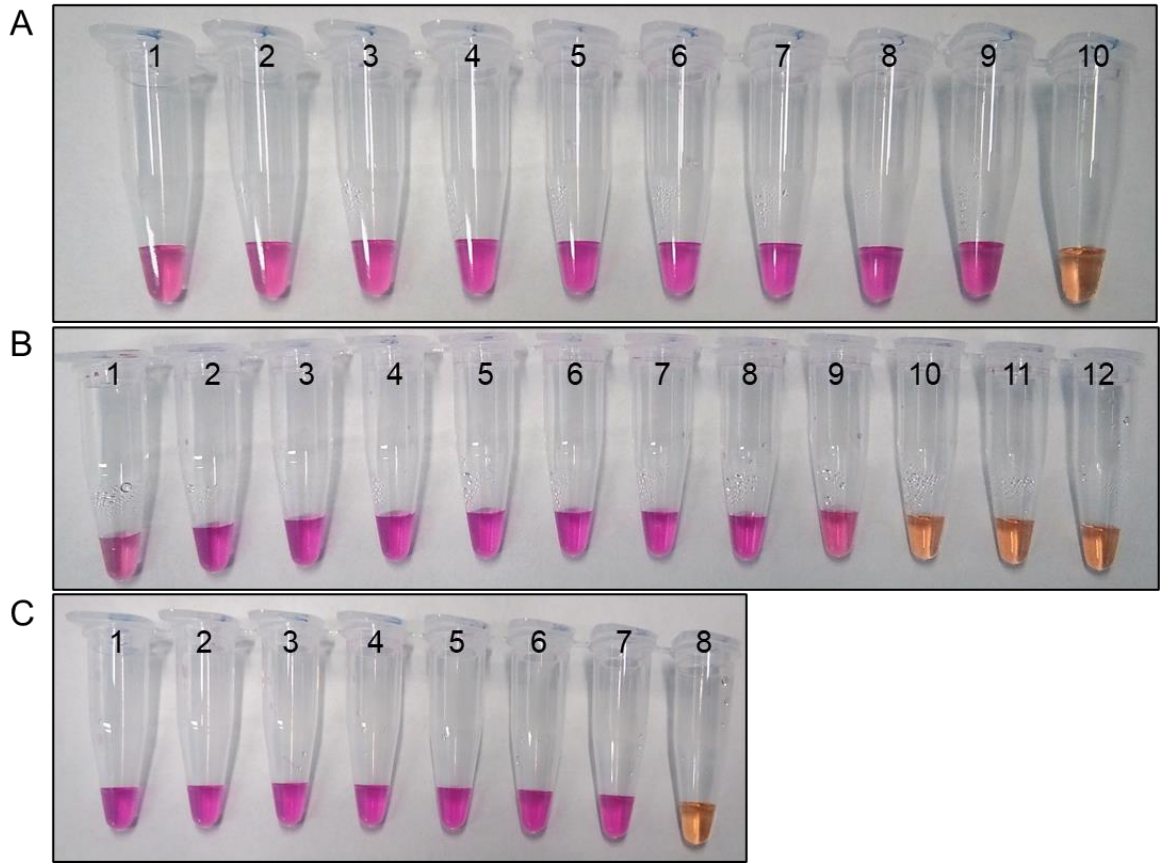


Figure 37: Optimization of LAMP assays for the detection of *P. oxalicum* and *B. cinerea*
Determination of optimal reaction temperature of the optimized LAMP assays for the detection of *P. oxalicum* (A) and *B. cinerea* (B) and determination of sensitivity of the *B. cinerea* LAMP assay (C) using gDNA. Pink = positive reaction, orange = negative reaction. A) LAMP assay with a temperature gradient from 59 °C to 70 °C with gDNA of *P. oxalicum* TMW 4.2553 (50 ng/rxn) incubated at 1 = 59 °C, 2 = 59.2 °C, 3 = 59.9 °C, 4 = 60.9 °C, 5 = 62.3 °C, 6 = 63.9 °C, 7 = 65.6 °C, 8 = 67.2 °C, 9 = 68.7 °C, 10 = 70 °C. B) LAMP assay with a temperature gradient from 59 °C to 71.5 °C with gDNA of *B. cinerea* TMW 4.2527 (20 ng/rxn) incubated at 1 = 59 °C, 2 = 59.2 °C, 3 = 59.9 °C, 4 = 60.9 °C, 5 = 62.3 °C, 6 = 63.9 °C, 7 = 65.6 °C, 8 = 67.2 °C, 9 = 68.7 °C, 10 = 70 °C, 11 = 71 °C; 12 = 71.5 °C. C) LAMP assay with a tenfold serial dilution of gDNA of *B. cinerea* TMW 4.2527 ranging from 100 ng/rxn to 10 fg/rxn. 1 = 100 ng/rxn, 2 = 10 ng/rxn, 3 = 1 ng/rxn, 4 = 100 pg/rxn, 5 = 10 pg/rxn, 6 = 1 pg/rxn, 7 = 100 fg/rxn, 8 = 10 fg/rxn.

Positive reactions in the LAMP assay for the detection of *P. oxalicum* (see Figure 37 A) were detected between 59 °C (1) and 68.7 °C (9), while a higher temperature resulted in no DNA amplification. An incubation temperature of 65 °C was chosen for all further experiments.

Positive reactions in the LAMP assay for the detection of *B. cinerea* (see Figure 37 B) were detected between 59.2 °C (2) and 67.2 °C (8). Also, temperatures at 59 °C (1) and 68.7 °C (9) showed a positive reaction but with lower intensities, while a higher temperature resulted in no DNA amplification. An incubation temperature of 63 °C was chosen for all further experiments.

Sensitivity of the *B. cinerea* LAMP assay with ammonium sulfate buffer and neutral red was assessed by testing genomic DNA of *B. cinerea* TMW 4.2527 (see Figure 37 C). The LAMP assay showed positive results for reactions containing 100 ng/rxn (1) to 100 fg/rxn (7). No positive result was found for the reaction containing 10 fg/rxn (8) genomic DNA. Hence, the detection limit of the optimized *B. cinerea* LAMP assay for genomic DNA was 100 fg/rxn.

Specificity of the optimized *B. cinerea* LAMP assay was assessed by testing genomic DNA of 132 fungal strains. The results are listed in Table 48.

Table 48: Determination of *B. cinerea* LAMP assay specificity
Purified DNA (100 ng/rxn) of 132 fungal strains was tested as template in the *B. cinerea* LAMP assay. Species name, strain and clone number, and the result in the LAMP assay are listed. **+** = positive result, - = negative result. Positive species are marked in bold.

Species	Strain	Clone	LAMP result
Alternaria alternata	[l]TMW 4.0438	TMW 4.0438	-
Alternaria mali	[c]CBS 106.24	TMW 4.1406	-
Alternaria spp.	TMW 4.1428	TMW 4.1428	-
Aspergillus aculeatus	TMW 4.1776	TMW 4.1776	-
Aspergillus alliaceus	[e]DSM 813	TMW 4.1077	-
Aspergillus arachidicola	[f]IBT 27128	TMW 4.2204	-
Aspergillus auricomus	CBS 467.65	TMW 4.1631	-
Aspergillus awamori	CBS 101.704	TMW 4.1066	-
Aspergillus bombycis	IBT 23536	TMW 4.2210	-
Aspergillus bridgeri	CBS 350.81	TMW 4.1632	-
Aspergillus caelatus	IBT 29700	TMW 4.2209	-
Aspergillus carbonarius	TMW 4.1512	TMW 4.1512	-
Aspergillus clavatus	CBS 513.65	TMW 4.1086	-
Aspergillus elegans	CBS 310.80	TMW 4.1633	-
Aspergillus ellipticus	CBS 707.79	TMW 4.1629	-
Aspergillus flavus	TMW 4.1859	TMW 4.1859	-
Aspergillus foetidus	CBS 114.49	TMW 4.1628	-
Aspergillus fumigatus	CBS 113.55	TMW 4.0623	-
Aspergillus helicothrix	CBS 677.79	TMW 4.1630	-
Aspergillus heteromorphus	CBS 117.55	TMW 4.1626	-
Aspergillus insulicola	CBS 382.75	TMW 4.1634	-
Aspergillus japonicus	CBS 114.51	TMW 4.1627	-
Aspergillus minisclerotigenes	IBT 27177	TMW 4.2205	-
Aspergillus niger	CBS 101.698	TMW 4.1068	-

Table 48 (continued)

Species	Strain	Clone	LAMP result
Aspergillus nomius	CBS 260.88	TMW 4.1960	-
Aspergillus ochraceoroseus	CBS 101.887	TMW 4.1772	-
Aspergillus ochraceus	CBS 263.67	TMW 4.0706	-
Aspergillus oryzae	IBT 28103	TMW 4.2208	-
Aspergillus parasiticus	CBS 126.62	TMW 4.1768	-
Aspergillus parvisclerotigenes	IBT 3850	TMW 4.2205	-
Aspergillus petrakii	CBS 105.57	TMW 4.1087	-
Aspergillus pseudotararii	IBT 21092	TMW 4.2212	-
Aspergillus rambellii	IBT 14580	TMW 4.2211	-
Aspergillus sclerotiorum	CBS 549.65	TMW 4.1089	-
Aspergillus sojae	IBT 21643	TMW 4.2207	-
Aspergillus sulfureus	CBS 550.65	TMW 4.1067	-
Aspergillus tamarii	CBS 591.68	TMW 4.1771	-
Aspergillus terreus	CBS 377.64	TMW 4.1060	-
Aspergillus toxicarius	CBS 822.72	TMW 4.1766	-
Aspergillus tubingensis	[g]ITEM 4496	TMW 4.2008	-
Aspergillus usami var. *shiro-usami*	CBS 101.700	TMW 4.1072	-
Aureobasidium pullulans	TMW 4.2253	TMW 4.2253	-
Beltraniella portoricensis	CBS 856.70	TMW 4.0402	-
Bipolaris sorokiniana	CBS 311.64	TMW 4.0509	-
Botrytis cinerea	CBS 121.39	TMW 4.2527	+
Botrytis cinerea	TMW 4.2743	TMW 4.2743	+
Cladobotryum dendroides	[i]NRRL 2903	TMW 4.0467	-
Cladosporium macrocarpum	TMW 4.2371	TMW 4.2371	-
Cladosporium sphaerospermum	TMW 4.2370	TMW 4.2370	-
Colletotrichum acutatum	CBS 295.67	TMW 4.0652	-
Colletotrichum fragariae	CBS 142.31	TMW 4.0651	-
Colletotrichum gloeosporioides	CBS 285.50	TMW 4.0650	-
Cryptomela acutispora	CBS 157.33	TMW 4.1620	-
Drechslera teres	CBS 378.59	TMW 4.0558	-
Drechslera tricici-repentis	CBS 265.80	TMW 4.0559	-
Emericella astellata	IBT 21903	TMW 4.2202	-
Emericella olivicola	IBT 26499	TMW 4.2201	-
Emericella venezuelensis	IBT 20956	TMW 4.2203	-
Epicoccum nigrum	TMW 4.1407	TMW 4.1407	-
Fusarium acuminatum	CBS 485.94	TMW 4.0701	-
Fusarium avenaceum	DSM 62161	TMW 4.0140	-
Fusarium beomiforme	[a]BBA 69406	TMW 4.0513	-
Fusarium cerealis	CBS 589.93	TMW 4.0406	-
Fusarium chlamydosporum	CBS 145.25	TMW 4.0404	-
Fusarium compactum	CBS 466.92	TMW 4.0433	-
Fusarium culmorum	DSM 62191	TMW 4.0149	-

Table 48 (continued)

Species	Strain	Clone	LAMP result
Fusarium dimerum	CBS 175.31	TMW 4.0626	-
Fusarium dlamini	[h]MRC 3024	TMW 4.0571	-
Fusarium equiseti	CBS 406.86	TMW 4.0477	-
Fusarium eumartii	DSM 62809	TMW 4.0303	-
Fusarium heterosporum	DSM 62231	TMW 4.0224	-
Fusarium longipes	CBS 739.79	TMW 4.0350	-
Fusarium melanochlorum	CBS 202.65	TMW 4.0625	-
Fusarium napiforme	BBA 67629	TMW 4.0510	-
Fusarium oxysporum	DSM 62292	TMW 4.0163	-
Fusarium proliferatum	DSM 62261	TMW 4.0236	-
Fusarium scirpi	CBS 448.84	TMW 4.0410	-
Fusarium solani	DSM 62416	TMW 4.0255	-
Fusarium subglutinans	BBA 63621	TMW 4.0947	-
Fusarium sublunatum var. *sublunatum*	CBS 189.34	TMW 4.0417	-
Fusarium torulosum	BBA 64465	TMW 4.0437	-
Geomyces auratus	BBA 66836	TMW 4.0904	-
Geomyces auratus	BBA 66873	TMW 4.0905	-
Geomyces auratus	BBA 66886	TMW 4.0906	-
Geomyces pannorum	BBA 66108	TMW 4.0902	-
Geomyces pannorum	BBA 66120	TMW 4.0903	-
Geomyces pannorum	BBA 69656	TMW 4.1028	-
Geomyces pannorum	TMW 4.2340	TMW 4.2340	-
Geotrichum candidum	TMW 4.0508	TMW 4.0508	-
Gliocephalotrichum spec. nov.	NRRL 2993	TMW 4.0468	-
Hypomyces rosellus	CBS 521.81	TMW 4.0400	-
Memnoniella echinata	CBS 627.61	TMW 4.0711	-
Microdochium majus	TMW 4.0496	TMW 4.0496	-
Microdochium nivale	TMW 4.0495	TMW 4.0495	-
Monascus ruber	TMW 4.1426	TMW 4.1426	-
Mucor hiemalis	TMW 4.2319	TMW 4.2319	-
Mucor mucedo	DSM 809	TMW 4.0441	-
Myrothecium roridum	CBS 331.51	TMW 4.0668	-
Penicillium aurantiogriseum	CBS 225.90	TMW 4.1603	-
Penicillium brevicompactum	TMW 4.2279	TMW 4.2279	-
Penicillium camembertii	DSM 1233	TMW 4.0442	-
Penicillium chrysogenum	CBS 573.68	TMW 4.1958	-
Penicillium commune	CBS 311.48	TMW 4.1088	-
Penicillium corylophilum	CBS 321.48	TMW 4.1598	-
Penicillium crustosum	CBS 499.73	TMW 4.1080	-
Penicillium digitatum	DSM 62840	TMW 4.1083	-
Penicillium expansum	DSM 62841	TMW 4.0466	-
Penicillium glabrum	TMW 4.2027	TMW 4.2027	-

Table 48 (continued)

Species	Strain	Clone	LAMP result
Penicillium glandicola	TMW 4.1543	TMW 4.1543	-
Penicillium italicum	DSM 62846	TMW 4.1084	-
Penicillium jensenii	TMW 4.2316	TMW 4.2316	-
Penicillium nalgiovense	TMW 4.1371	TMW 4.1371	-
Penicillium nordicum	[b]BFE 487	TMW 4.2213	-
Penicillium olsonii	TMW 4.1362	TMW 4.1362	-
Penicillium purpurescens	CBS 223.28	TMW 4.1082	-
Penicillium purpurogenum	CBS 286.36	TMW 4.1079	-
Penicillium roqueforti	CBS 221.30	TMW 4.1599	-
Penicillium roseopurpureum	TMW 4.1770	TMW 4.1770	-
Penicillium rugulosum	TMW 4.1902	TMW 4.1902	-
Penicillium stoloniferum	TMW 4.2280	TMW 4.2280	-
Penicillium variabile	CBS 385.48	TMW 4.1081	-
Penicillium verrucosum	CBS 603.74	TMW 4.1073	-
Pseudogymnoascus destructans	[j]OT-29-2010	TMW 4.2511	-
Pseudogymnoascus roseus	[d]CCF 3426	TMW 4.2421	-
Scopulariopsis acremonioides	TMW 4.2366	TMW 4.2366	-
Stachybotrys chartarum	[k]Sp 2682	TMW 4.0523	-
Trichoderma harzianum	TMW 4.1502	TMW 4.1502	-
Trichoderma virens	CBS 344.47	TMW 4.0710	-
Trichothecium roseum	CBS 567.50	TMW 4.0691	-
Zygosaccharomyces bailii	DSM 70834	TMW 3.058	-
Zygosaccharomyces bisporus	TMW 3.062	TMW 3.062	-
Zygosaccharomyces rouxii	DSM 2531	TMW 3.057	-

[a] BBA = Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, Germany
[b] BFE = Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Karlsruhe, Germany
[c] CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands
[d] CCF = Culture Collection of Fungi, Charles University Prague, Czech Republic
[e] DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, Germany
[f] IBT = Culture collection of Center for Microbial Biotechnology (CMB), Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark
[g] ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy
[h] MRC = South African Medical Research Council, Tygerberg, South Africa
[i] NRRL = Northern Regional Research Laboratory, Peoria (Illinois), USA
[j] OT = Leibniz-Institut für Zoo- und Wildtierforschung, Berlin, Germany
[k] Sp = Max Rubner-Institut, Bundesforschungsinstitut für Ernährung und Lebensmittel, Kulmbach, Germany
[l] TMW = Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technical University of Munich, Freising, Germany

All species tested negative in the LAMP assay, except for both *B. cinerea* strains (TMW 4.2527, 4.2743).

3.4.3 Screening of samples from the harvests 2018, 2019, and 2020

Grape, soil, and must samples obtained from different European vineyards were tested for the occurrence of the gushing-relevant fungi *P. expansum*, *B. cinerea*, and *P. oxalicum* over a period of three years. Therefore, the preparation of these samples was optimized for analysis with the developed and optimized LAMP assays.

The sample preparation was different for the several matrices: Grapes were washed and mechanically treated to obtain sufficient DNA. The obtained DNA was then diluted 1:5 with $_{d}H_2O$ when used as template in the LAMP assay to reduce inhibiting substances. DNA from soil and must samples needed to be extracted using the FastDNA™ Spin Kit for Soil. Soil DNA was used in a concentration of 20 ng/µL for the LAMP reaction. DNA that was isolated from must samples was used in a modified LAMP master mix which was amended with 200 mM ammonium sulfate instead of 100 mM as well as potassium chloride, respectively, to achieve a higher buffer capacity of the master mix.

The samples were tested with the optimized LAMP assays for the detection of *P. oxalicum*, *B. cinerea*, *P. expansum*, as well as an assay that detects patulin-producing *Penicillium* species (Frisch and Niessen, 2019). The latter assay was used for the screening 2018 and 2019, when the specific LAMP assay for *P. expansum* was not yet developed, and in 2020 as control of the newly developed *P. expansum* LAMP assay. The LAMP assay of Frisch and Niessen (2019) could be used to detect *P. expansum* as this fungus is the only patulin-producing *Penicillium* species occurring frequently on grapes. Positive samples with this assay were tested again to confirm the LAMP result and the identity as *P. expansum* was checked by microbiological identification in the screenings 2018 and 2019. The detailed results are shown in Table 50, Table 51, and Table 52 in the appendix.

In 2018, 56 grape and 17 soil samples from different vineyards in Germany were tested. Figure 38 shows the percentage of positive samples.

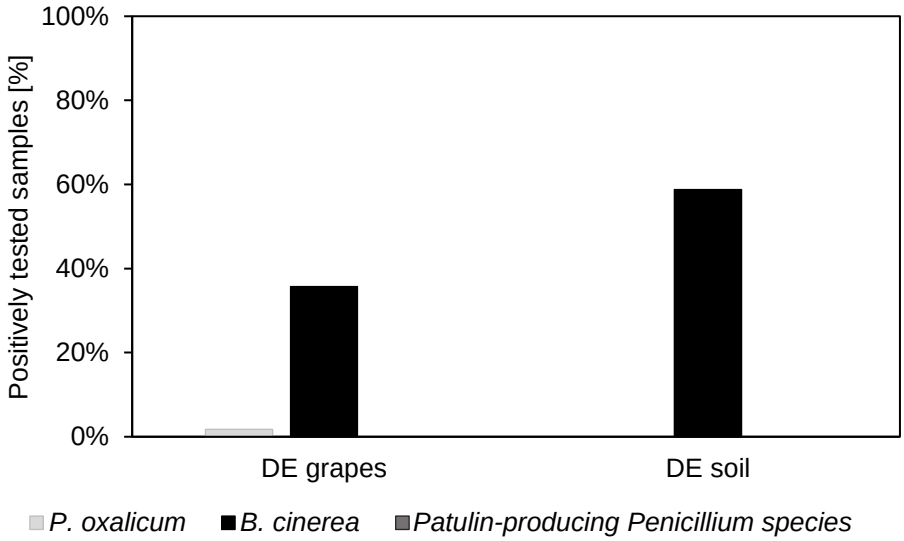


Figure 38: LAMP results of screening 2018

The results of LAMP-based screening of grape and soil samples of the 2018 harvest from vineyards in Germany (DE) with assays for the detection of *P. oxalicum*, *B. cinerea*, and patulin-producing *Penicillium* species are shown. The percentage on the y-axis indicates positively tested grape/soil samples from Germany in the respective LAMP assays.

As shown in Figure 38, none of the grape and soil samples of the 2018 harvest were positive in the LAMP assay for the detection of patulin-producing *Penicillium* species. In addition, none of the soil samples were positive in the LAMP assay for the detection of *P. oxalicum*, while 2 % of grape samples tested positive for the fungus. The LAMP assay for the detection of *B. cinerea* revealed 36 % positive grape and 59 % positive soil samples.

In 2019, 126 grape, 64 soil, and 19 must samples from vineyards in Germany, Italy, Portugal, Luxembourg, the Netherlands, and France were tested. Over all vineyards, a positive result in the LAMP assay for the detection of *P. oxalicum* was found in 5 % of grape, 33 % of soil, and 89 % of must samples. The LAMP assay for the detection of patulin-producing *Penicillium* species revealed positive results in 11 % of grape, 20 % of soil, and 16 % of must samples. A positive result in the LAMP assay for the detection of *B. cinerea* was found in 49 % of grape, 56 % of soil, and 95 % of must samples of the 2019 harvest. Figure 39 shows the percentage of positive samples for each country and species.

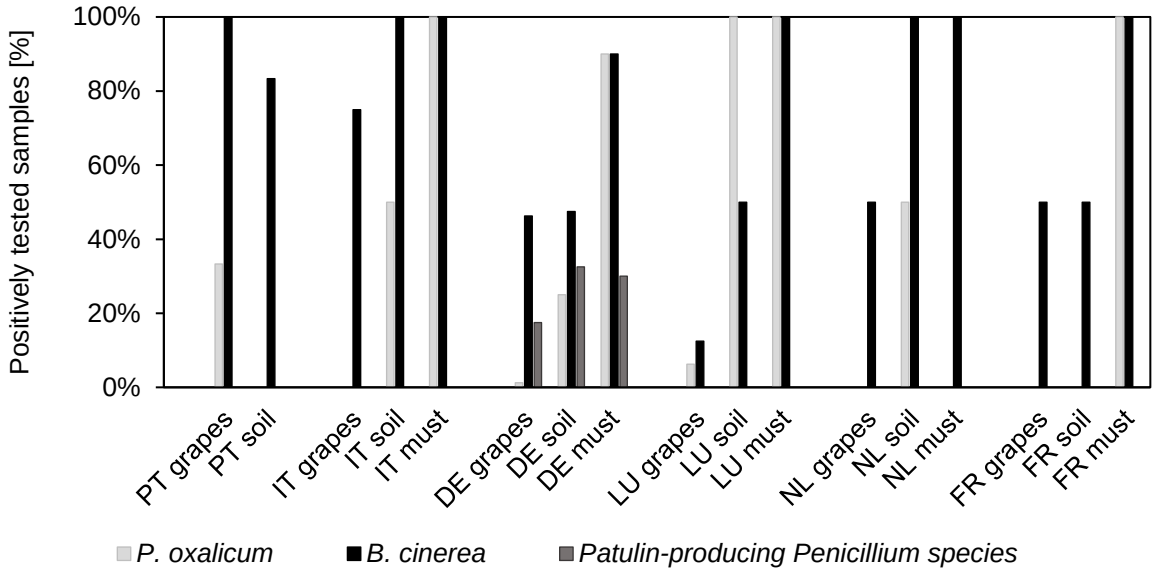


Figure 39: LAMP results of screening 2019

The results of LAMP-based screening of grape, soil, and must samples of the 2019 harvest from vineyards in Portugal (PT), Italy (IT), Germany (DE), Luxembourg (LU), the Netherlands (NL), and France (FR) with assays for the detection of *P. oxalicum*, *B. cinerea*, and patulin-producing *Penicillium* species are shown. The percentage on the y-axis indicates positively tested grape/soil/must samples from the respective countries in the respective LAMP assays.

As shown in Figure 39, the LAMP assay for the detection of patulin-producing *Penicillium* species revealed positive results only in samples from German vineyards of the 2019 harvest. Positively tested samples were examined microbiologically and *P. expansum* was identified based on morphological characteristics (Frisvad and Samson, 2004; Samson et al., 2019). The LAMP assay for the detection of *B. cinerea* revealed high numbers of positively tested samples in all tested vineyards. The fungus was especially abundant in must samples. The LAMP assay for the detection of *P. oxalicum* revealed the presence of this fungus in samples from all vineyards. On grapes, it was mostly detected on samples from Portugal, while in must, it showed a high presence in samples from Italy, Germany, Luxembourg, and France.

In 2020, 88 grape, 42 soil, and 13 must samples from vineyards in Germany, Italy, Luxembourg, and Greece were tested. Over all vineyards, a positive result in the LAMP assay for the detection of *P. oxalicum* was found in 6 % of all grape samples. Soil and must samples tested negative for the fungus. The LAMP assay for the detection of patulin-producing *Penicillium* species revealed positive results in 31 % of grape, 24 % of soil, and 15 % of must samples. Furthermore, 28 % of grape, 10 % of soil, and 8 % of must samples were tested positive with the LAMP assay for the detection for *P. expansum*. A positive result in the LAMP assay for the detection of *B. cinerea* was found in 67 % of grape, 69 % of soil, and 69 % of must samples of the 2020 harvest. Figure 40 shows the percentage of positive samples for each country and species.

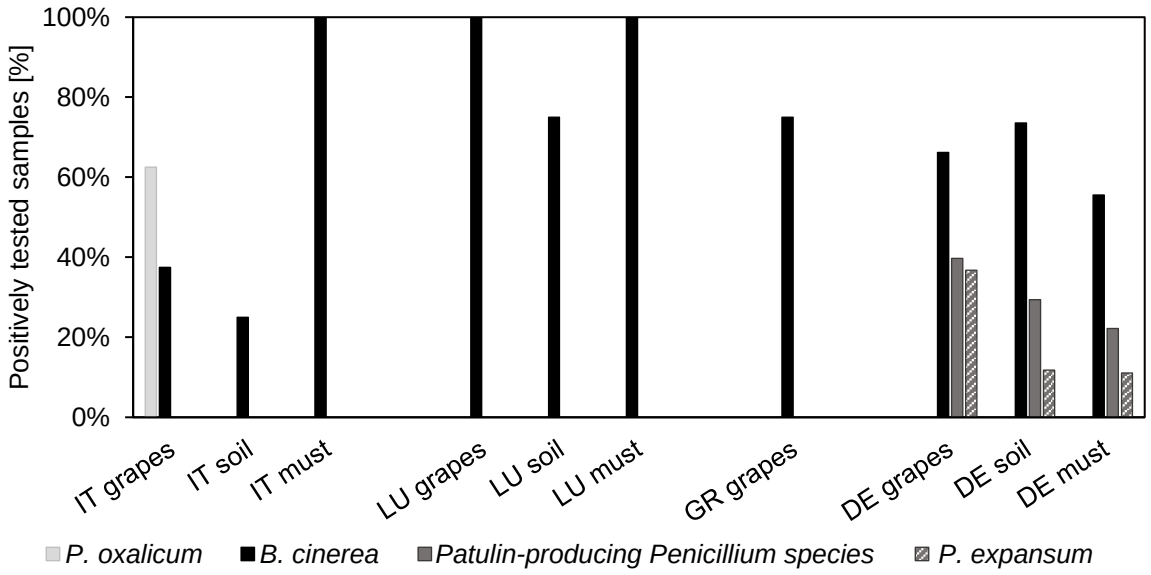


Figure 40: LAMP results of screening 2020

The results of LAMP-based screening grape, soil, and must samples of the 2020 harvest from vineyards in Italy (IT), Luxembourg (LU), Greece (GR), and Germany (DE) with assays for the detection of *P. oxalicum*, *B. cinerea*, patulin-producing *Penicillium* species, and *P. expansum* are shown. The percentage on the y-axis indicates positively tested grape/soil/must samples of the respective countries in the respective LAMP assays.

As shown in Figure 40, the LAMP assay for the detection of patulin-producing *Penicillium* species revealed positive results only in samples from German vineyards of the 2020 harvest, similar to the results obtained for screening the 2019 harvest. This assay was used as a control to verify positive results in the LAMP assay for the detection of *P. expansum* because the assay was used for the first time in samples from the 2020 harvest. Comparison of results that were obtained with both assays revealed slightly lower numbers of positive samples for *P. expansum* as compared to patulin-producing *Penicillium* species. Samples showing a positive LAMP reaction for *P. expansum* were confirmed microbiologically. The LAMP assay for the detection of *B. cinerea* revealed high numbers of positively tested samples in all tested vineyards. The LAMP assay for the detection of *P. oxalicum* revealed the presence of this fungus only on grape samples from Italy.

In the three-year screening, the highest total number of samples originated from German vineyards. Therefore, the results of the different harvesting years were compared for all German samples. Results are shown in Figure 41.

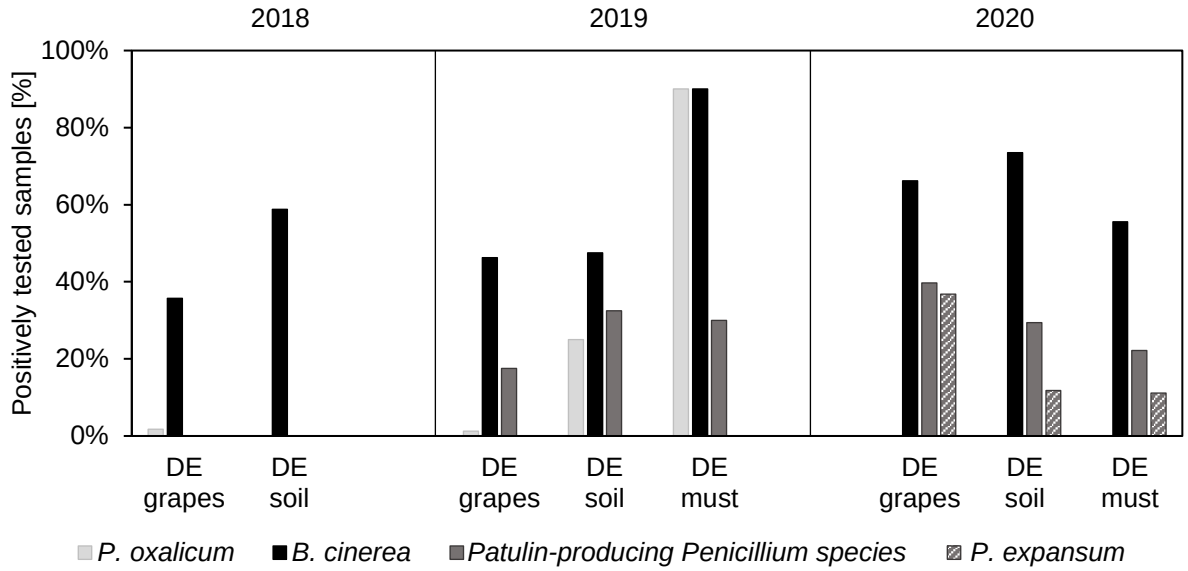


Figure 41: Comparison of LAMP results of screenings 2018, 2019, and 2020 in German vineyard samples
A comparison of the results of LAMP-based screening of grape, soil, and must samples from German vineyards (DE) from the 2018 harvest with the 2019 and 2020 harvests is shown. LAMP assays for the detection of *P. oxalicum*, *B. cinerea*, patulin-producing *Penicillium* species, and *P. expansum* (was only used in 2020) were applied. The percentage on the y-axis indicates positively tested grape/soil/must samples in the respective LAMP assays and harvest years.

As shown in Figure 41, *P. oxalicum* was detected mostly on sample materials from the 2019 harvest in Germany. Here, numbers were especially high in must samples. *B. cinerea* was detected in samples from all three years and in all sample types analyzed. Patulin-producing *Penicillium* species were not detected on samples from 2018 but were frequently found on samples from the 2019 and 2020 harvest and hereby in all sample types. *P. expansum* was only analyzed in samples from the 2020 harvest and was detected with similar frequencies as patulin-producing *Penicillium* species.

The presence of *P. oxalicum*, *B. cinerea*, and patulin-producing *Penicillium* species including *P. expansum* on grape, soil, and must samples was compared between samples from wine growing zones A and B and samples from wine growing zone C. Wine growing zone A includes vineyards in Germany, the Netherlands, and Luxembourg, while wine growing zone B includes vineyards in Baden (southwestern Germany). Wine growing zone C includes vineyards in Portugal, Italy, France, and Greece. The results are shown in Figure 42.

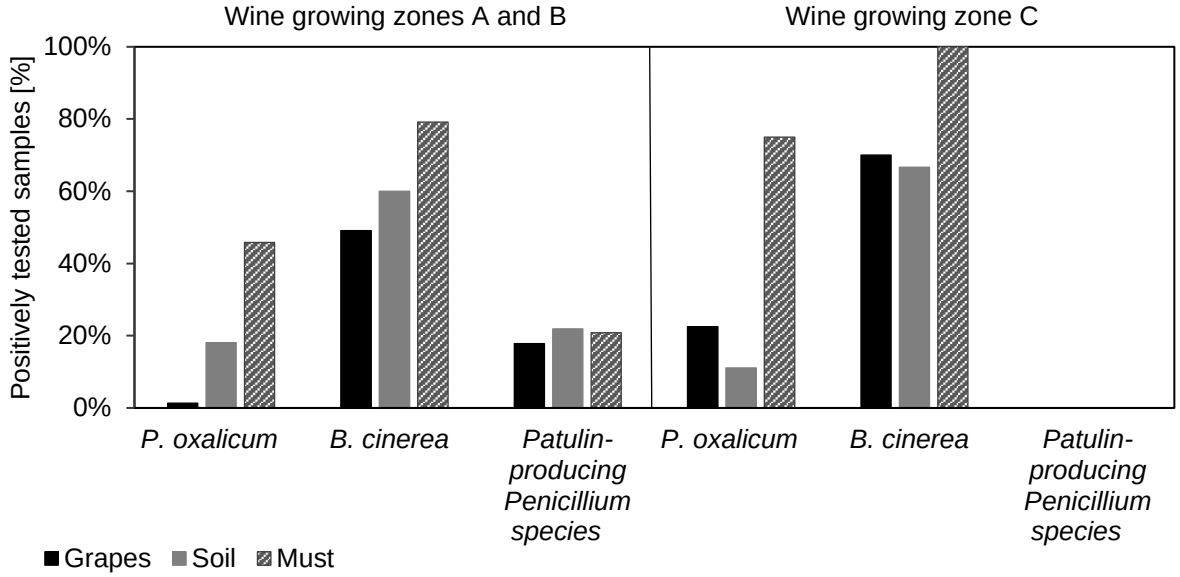


Figure 42: Comparison of detected fungi in different wine growing zones
A comparison of the results of screening grape, soil, and must samples from the 2018, 2019, and 2020 harvests from wine growing zones A and B (including DE, NL, LU) and from wine growing zone C (including PT, IT, FR, GR) by the LAMP assays for the detection of *P. oxalicum*, *B. cinerea*, and patulin-producing *Penicillium* species is shown. The percentage on the y-axis indicates positively tested grape/soil/must samples for the detection of the respective fungi.

As shown in Figure 42, patulin-producing *Penicillium* species did only occur on samples from wine growing zones A and B. Here, these fungi (primarily *P. expansum*) were found equally often on grape, soil, and must samples. *P. oxalicum* was detected more often on samples from wine growing zone C than from zones A and B. The fungus was especially frequent in must samples from zone C when compared to zones A and B. Also, on grape samples from zone C there was a higher presence of this fungus than on grape samples from zones A and B. *B. cinerea* did occur in all three wine growing zones, with the highest number of positively tested samples in must. A higher presence was found on samples from zone C. *B. cinerea* was the most frequently detected fungus on the samples using the three LAMP assays. The most positively tested samples were must samples, followed by soil samples in zones A and B, and grape samples in zone C.

Fungal infestation on grape and soil samples was compared between the three tested harvest years regarding different viticultural techniques and the application of pest control. The results are shown in Figure 43.

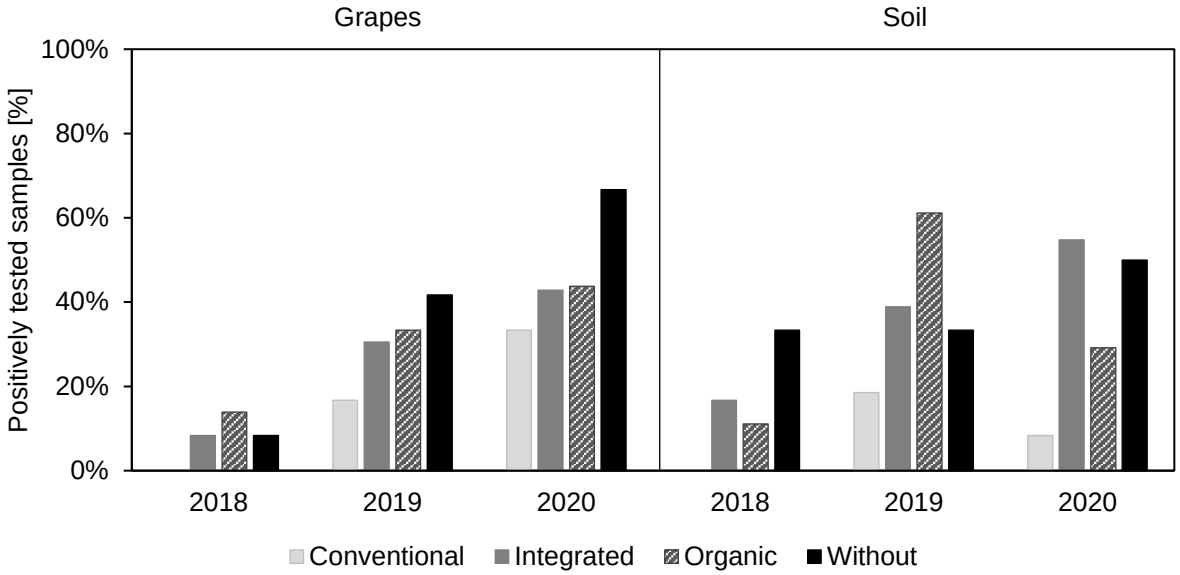


Figure 43: Comparison of fungal infestation on grapes and soil with different viticultural techniques and pest control applications

A comparison of the results of screening grape and soil samples from the 2018, 2019, and 2020 harvests from European vineyards for the presence of *P. oxalicum*, *B. cinerea*, and patulin-producing *Penicillium* species with different viticultural techniques and pest control applications (conventional viticulture, integrated viticulture, organic viticulture, viticulture without application of pest control). The presence of the analyzed fungi was cumulated over samples of the respective application type. Only the LAMP results of vineyards for which the method of pest control application had been indicated were used. The percentage on the y-axis indicates positively tested grape/soil samples in all used LAMP assays.

As shown in Figure 43, for grapes, the presence of *P. oxalicum*, *B. cinerea*, and *P. expansum* that was cumulated over samples of the respective technique and application type increased over the three tested years. In harvest years 2019 and 2020, the lowest number of positively tested samples was found for conventional viticulture, followed by integrated and organic viticulture, and highest for no application of pest control. In 2018, no samples from conventional viticulture were available for testing. In that year, the highest number of positively tested samples for the analyzed fungi was found for organic viticulture. For soil, the lowest number of positively tested samples in 2019 and 2020 was found for conventional viticulture. In 2018, no samples from conventional viticulture were available for testing. In that year, the lowest cumulated number of positively tested samples was found for samples grown under organic viticultural techniques. Highest infestation with the analyzed fungi was found for soil samples originating from vineyards with no application of pest control in 2018 as well as organic viticulture in 2019, and integrated viticulture in 2020.

3.5 Investigations of the role of ns-LTP1 *Vv* in gushing of sparkling wine

The role of the protein ns-LTP1 from *Vitis vinifera* in gushing of sparkling wine is not yet clear. It has been suspected to have a gushing-reducing effect so that a low concentration of this protein in sparkling wine could be a marker for an increased gushing risk. Investigations within the current study were intended to clarify whether this protein is involved in the mechanism of gushing.

3.5.1 Relative quantification of ns-LTP1 *Vv* in sparkling wines via ELISA against ns-LTP1 from *H. vulgare*

A competitive ELISA against ns-LTP1 from *H. vulgare* was used for the relative quantification of ns-LTP1 *Vv*. This assay was developed in a previous study by Specker (2014). Also, Kupfer (2018) used this assay to detect ns-LTP1 *Vv* because the amino acid sequence of the ns-LTP1 peptide from *H. vulgare* (see Figure 44, marked in orange) used for antibody generation as well as for coating of the solid phase in the ELISA showed almost 70 % identity with the amino acid sequence of ns-LTP1 *Vv* (see Figure 44, marked in orange). Kupfer (2018) suggested a cross-reaction of the antibody with both proteins due to this degree of sequence similarity.

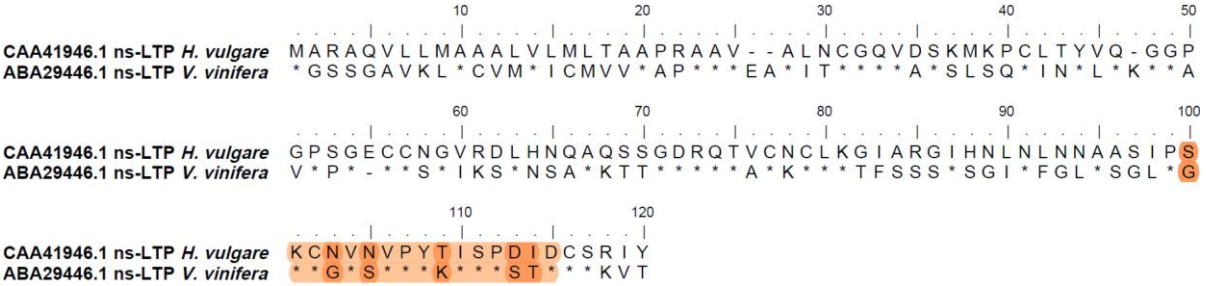


Figure 44: Alignment of the amino acid sequence of ns-LTP1 proteins from *H. vulgare* and *V. vinifera*
The amino acid sequences of ns-LTP1 from *H. vulgare* (GenBank accession number CAA41946.1) and *V. vinifera* (GenBank accession number ABA29446.1) were aligned. Positions marked with an asterisk show identical amino acids in both sequences. The peptide used for the generation of the antibody Anti-nsLtp1-P2-IgG against ns-LTP1 from *H. vulgare* and the respective aligned amino acids from ns-LTP1 from *V. vinifera* are marked in orange. The sequence identity of the orange marked amino acids in both sequences is 67 %. Amino acids that differ between the proteins in this section are marked in dark orange.

The optimized ELISA protocol using the Anti-nsLtp1-P2-IgG antibody is described in section 2.2.3.3.2 of the current study. For comparison of values, the relative intensity was calculated and normalized to the total protein content of samples that was measured by BCA assay.

With the optimized ELISA, 13 gushing-negative and 10 gushing-positive lyophilized sparkling wines were tested to investigate whether there is a correlation between the ns-LTP1 content and the occurrence of gushing. For the analysis, only those sparkling wines were used that had been declared as gushing-positive or gushing-negative by the manufacturer and the gushing potential of which was confirmed after opening of the bottles in the author's laboratory. The results are shown in Figure 45.

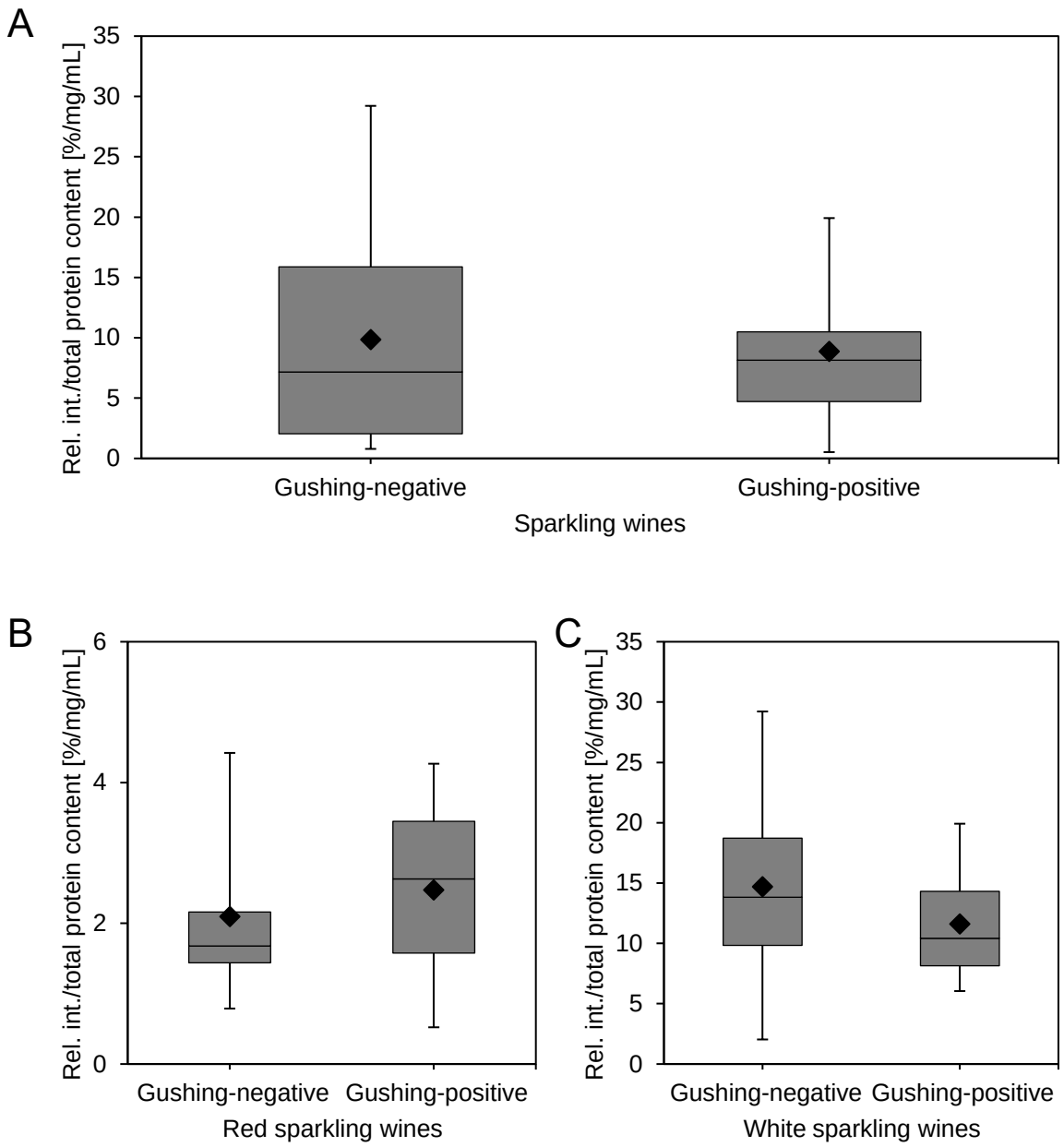


Figure 45: ELISA results for testing sparkling wines with antibody against ns-LTP1 from *H. vulgare*
Gushing-negative and gushing-positive sparkling wines were tested with the ELISA using an antibody against ns-LTP1 from *H. vulgare* (Specker, 2014). The box plot shows the minimum, 25th percentile, median, 75th percentile, and maximum of the relative (Rel.) intensity (int.) normalized to the total protein content measured by BCA assay. The diamond shows the mean of the normalized relative intensity. A) Results for 13 gushing-negative and 10 gushing-positive sparkling wines including both red and white varieties. In B and C, the results are shown for the sparkling wines tested in A separated into red sparkling wines (B, 5 gushing-negative, 3 gushing-positive) and white sparkling wines (C, 8 gushing-negative, 7 gushing-positive).

In Figure 45 A, the results for gushing-negative and gushing-positive sparkling wines including red and white varieties are depicted. The mean of the normalized relative intensity was slightly higher for gushing-negative sparkling wines (9.9 %/mg/mL) than for gushing-positive ones (8.9 %/mg/mL). The interquartile range for the gushing-negative sparkling wines was greater than that for the gushing-positive ones. No significant difference between the means was found for the two groups.

In Figure 45 B, the results for only red gushing-negative and gushing-positive sparkling wines tested in A are depicted. The mean of the normalized relative intensity was slightly higher for gushing-positive sparkling wines (2.5 %/mg/mL) than for gushing-negative ones (2.1 %/mg/mL). The interquartile range for the red gushing-positive sparkling wines was greater than that for the red gushing-negative ones. No significant difference between the means was found for the two groups.

In Figure 45 C, the results for only white gushing-negative and gushing-positive sparkling wines tested in A are depicted. The mean of the normalized relative intensity was slightly higher for gushing-negative sparkling wines (14.7 %/mg/mL) than for gushing-positive ones (11.6 %/mg/mL). No significant difference between the means was found for the two groups.

By comparing red (Figure 45 B) and white sparkling wines (Figure 45 C), the normalized relative intensities were higher for white sparkling wines than for red ones. The means of the red and white gushing-negative sparkling wines were significantly different (unpaired t-test, $p = 0.005$, $\alpha = 0.05$) as were the means of the red and white gushing-positive sparkling wines (unpaired t-test, $p = 0.004$, $\alpha = 0.05$).

Since there was no significant difference in the mean ns-LTP1 content between gushing-positive and gushing-negative sparkling wines, the asserted cross-reaction of the antibody against ns-LTP1 from *H. vulgare* used in the ELISA with ns-LTP1 *Vv* needed to be checked in order to validate the results. Western blot analysis of the sparkling wines with the highest relative intensity in the ELISA with the Anti-nsLtp1-P2-IgG antibody revealed no or only slight protein bands (results not shown, see Figure 46 B for comparison). In order to exclude non-specific binding of the antibody and to obtain a specific and more sensitive detection of ns-LTP1 *Vv*, the generation of a specific antibody against ns-LTP1 *Vv* was commissioned.

3.5.2 Development of an ELISA for the detection of ns-LTP1 *Vv*

In a second approach to develop an ELISA for the specific detection of ns-LTP1 *Vv* in sparkling wines, a specific antibody against ns-LTP1 *Vv* was generated.

3.5.2.1 Antibody production and testing

The production of a polyclonal peptide antibody was performed by Davids Biotechnologie GmbH (Regensburg, Germany). For immunization, peptides were synthesized that derived from the amino acid sequence without signal peptide of the ns-LTP1 *Vv* protein (see appendix Figure 60 for sequence, peptide marked in orange). The most suitable peptide regarding antigenicity, solubility, and epitope prediction was chosen for immunization of rabbits. Table 49 lists the chosen peptide sequence and its characteristics. Five immunizations were performed at days 1, 14, 28, 42, and 56. After day 63, the final bleed was received and affinity purified. The antibody titer was 300,000. The resulting antibody was designated as Anti-ns-LTP1 *Vv*.

Table 49: Characteristics of the peptide used for Anti-ns-LTP1 *Vv* antibody production
Peptide for Anti-ns-LTP1 *Vv* antibody production is listed with its characteristics regarding antigenicity, solubility, and epitope prediction according to Davids Biotechnologie GmbH (Regensburg, Germany).

Target protein	Peptide sequence	Antigenicity	Solubility	Epitope prediction	Antibody name
Ns-LTP1 *Vv*	KSLNSAAKTTGDRQTACK	good	medium	good	Anti-ns-LTP1 *Vv*

The newly generated antibody was tested by Western blot analysis. The results are shown in Figure 46 A and were compared to the Western blot results of the same samples with the antibody against ns-LTP1 from *H. vulgare* (Figure 46 B).

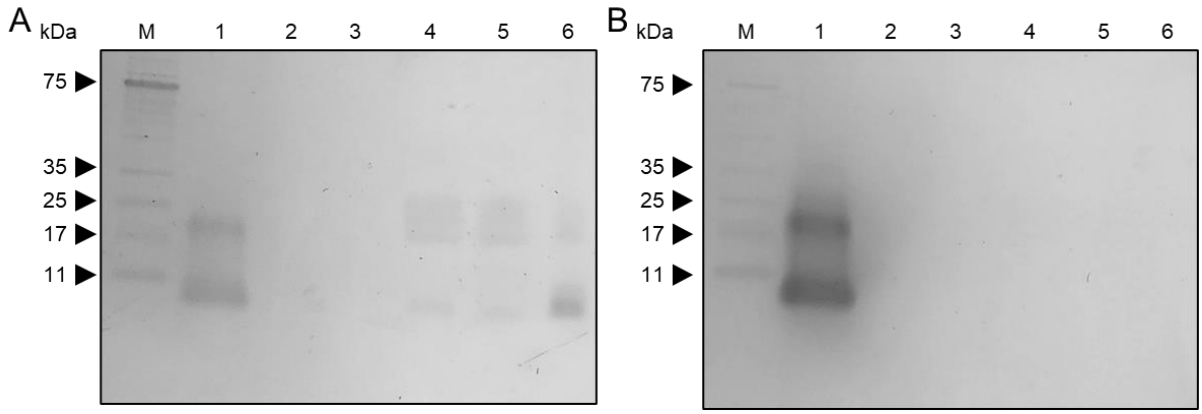


Figure 46: Comparison of Anti-ns-LTP1 antibodies
Anti-ns-LTP1 antibodies were tested by Western blot analysis. A) Western blot with the newly generated Anti-ns-LTP1 *Vv* antibody for detection of ns-LTP1 from *V. vinifera*. B) Western blot with Anti-nsLtp1-P2-IgG antibody (Specker, 2014) for detection of ns-LTP1 from *H. vulgare*. Lanes in A and B: 1 = positive control: lyophilized *Pichia pastoris* supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* (4 mg/mL in $_dH_2O$), 2 = gushing-negative sparkling wine (no. 29) lyophilized, 3 = gushing-negative sparkling wine (no. 29) after protein purification, 4 = gushing-negative sparkling wine (no. 39) lyophilized, 5 = gushing-negative sparkling wine (no. 39) after protein purification, 6 = grapes mashed, non-dialyzed, lyophilized. M = Marker SERVA Triple Color Protein Standard III.

In Figure 46 A, the newly generated antibody against ns-LTP1 *Vv* showed a cross-reaction with lyophilized *Pichia pastoris* culture supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* (lane 1) resulting in a protein band at approximately 10 kDa and a dimer at 20 kDa. Sparkling wine no. 29 (lanes 2 and 3) revealed no visible band in the Western blot. Sparkling wine no. 39 showed a protein band at approximately 10 kDa and a dimer at 20 kDa when lyophilized (lane 4) or purified (lane 5) proteins were applied. Lyophilized mashed grapes revealed a prominent protein band at 10 kDa with a dimer at 20 kDa. An amino acid sequence analysis of ns-LTP1 *Vv* was performed *in silico* using the ExPASy ProtParam tool. The analysis revealed a molecular weight of 12 kDa that matched the band of the practical experiments that was detected with the Anti-ns-LTP1 *Vv* antibody.

In contrast, in Figure 46 B, only the positive control (lane 1) showed the expected protein bands. The tested sparkling wines and mashed grapes had negative results in the Western blot with the Anti-nsLtp1-P2-IgG antibody that is directed against the ns-LTP1 protein of *H. vulgare*.

To confirm the identity of the visible protein bands in Figure 46 A, the prominent 10 kDa protein band from lane 6 was identified through protein sequencing by Edman degradation (see 2.2.2.8). The N-terminal sequencing of the sample revealed a sequence that showed 100 % homology to a partial sequence of ns-LTP1 from *V. vinifera* (GenBank accession number RVW40993.1). This RVW40993.1 sequence differed slightly from the sequence that was used for the Anti-ns-LTP1 *Vv* antibody generation (GenBank accession number ABA29446.1). A comparison of the two sequences is shown in Figure 47.

Figure 47: Alignment of two amino acid sequences of ns-LTP1 *Vv*

Two amino acid sequences of ns-LTP1 from *V. vinifera* were aligned. The sequence with GenBank accession number RVW40993.1 was the one that was derived from a BLAST search against the NCBI database with the obtained protein sequence from sequencing by Edman degradation. The sequence with GenBank accession number ABA29446.1 was the one that was used for Anti-ns-LTP1 *Vv* antibody generation. Positions marked with an asterisk show identical amino acids in both sequences. The amino acids that were identified by sequencing by Edman degradation and the respective aligned amino acids from ABA29446.1 are marked in grey. The peptide used for the generation of the antibody Anti-ns-LTP1 *Vv* against ns-LTP1 from *V. vinifera* and the respective aligned amino acids from RVW40993.1 are marked in orange. Different amino acids in colored sections are marked in dark color.

The grey marked sequence section in Figure 47 shows the amino acids that were identified by sequencing and the respective aligned amino acids from ABA29446.1. They share a homology of 75 %. The orange marked sequence section shows the peptide used for the generation of the antibody Anti-ns-LTP1 *Vv* against ns-LTP1 from *V. vinifera* and the respective aligned amino acids from RVW40993.1. Here, only one amino acid was different (alanine (A) instead of threonine (T)). As the newly developed antibody binds to the sequence of the peptide, it was checked whether the singular change in the amino acid sequence decreases the binding capacity of the antibody. Therefore, a peptide was generated by Davids Biotechnologie GmbH (Regensburg, Germany) that had an alanine on the mentioned position (peptide sequence: KSLNSAAKTTGDRQAACK). By testing the original and the newly generated peptide via Western blot, the Anti-ns-LTP1 *Vv* antibody detected both peptides equally well (results not shown).

All in all, the newly generated antibody showed its ability to detect the target in sample material and could therefore be used for the development of an ELISA for the detection of ns-LTP1 *Vv*.

3.5.2.2 Development of ns-LTP1 *Vv* ELISA

The newly generated Anti-ns-LTP1 *Vv* antibody showed a cross-reaction with lyophilized *Pichia pastoris* culture supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* in Western blot (see 3.5.2.1). As no purified ns-LTP1 *Vv* protein was available in the current study that could be used as calibration standard in an ELISA, the use of the recombinant barley ns-LTP1 as positive control in an ELISA for the relative quantification of ns-LTP1 *Vv* in samples was assessed. Therefore, the cross-reaction was tested in an ELISA with different concentrations of lyophilized *Pichia pastoris* supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* with the same ELISA protocol as used before with the Anti-nsLtp1-P2-IgG antibody. The result is shown in Figure 48.

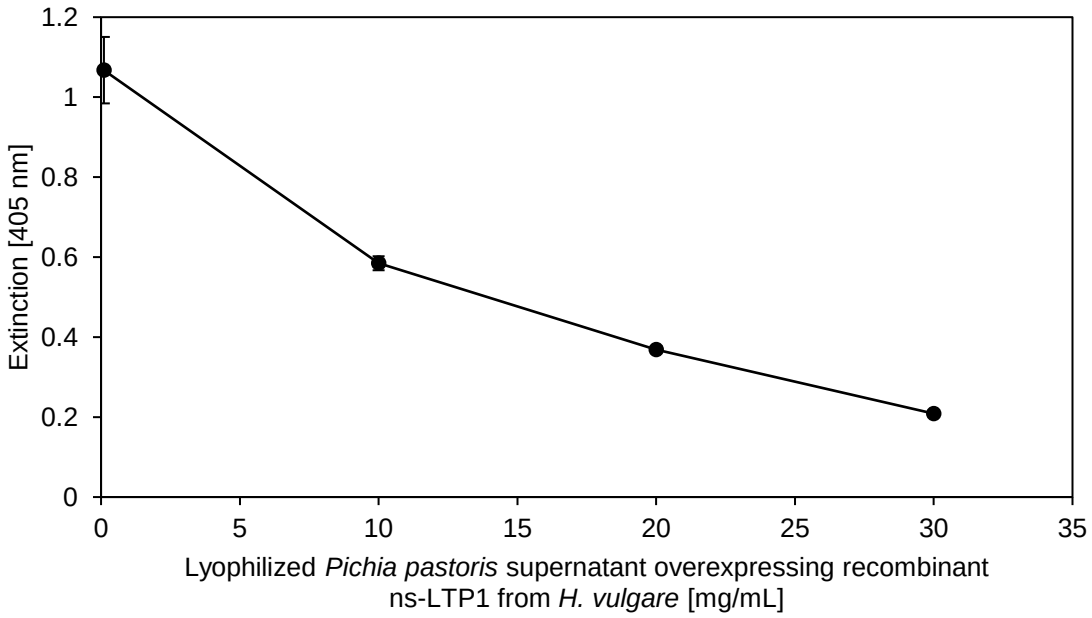


Figure 48: ELISA with Anti-ns-LTP1 *Vv* antibody
ELISA with lyophilized *Pichia pastoris* supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* [mg/mL] with concentrations ranging from 0.1 mg/mL to 30 mg/mL plotted against the mean extinction at 405 nm. Standard errors are shown.

As shown in Figure 48, the higher the applied amount of lyophilized *Pichia pastoris* culture supernatant (containing the recombinant barley ns-LTP1) was, the lower was the resulting extinction value in the ELISA. The correlation was linear between 10 mg/mL and 30 mg/mL. These results showed the antibody's ability to detect barley ns-LTP1 in the ELISA and that the lyophilized supernatant could be used as positive control.

The ns-LTP1 *Vv* ELISA protocol was optimized regarding coating concentration and concentration of positive control (results not shown). In contrast to the protocol used with the Anti-nsLtp1-P2-IgG antibody, the optimal concentration of the positive control was 15 mg/mL (see 2.2.3.3.2 for detailed protocol).

3.5.2.3 Evaluation of ELISA: Relative quantification of ns-LTP1 *Vv* in sparkling wines via ELISA against ns-LTP1 *Vv*

With the optimized ELISA, 14 gushing-negative and 11 gushing-positive lyophilized sparkling wines were tested (see Figure 49) to investigate a possible correlation between the ns-LTP1 *Vv* content and the gushing occurrence in the samples. For the analysis, only those sparkling wines were used that had been declared as gushing-positive or gushing-negative by the manufacturer and the gushing potential of which was confirmed after opening of the bottles in the laboratory.

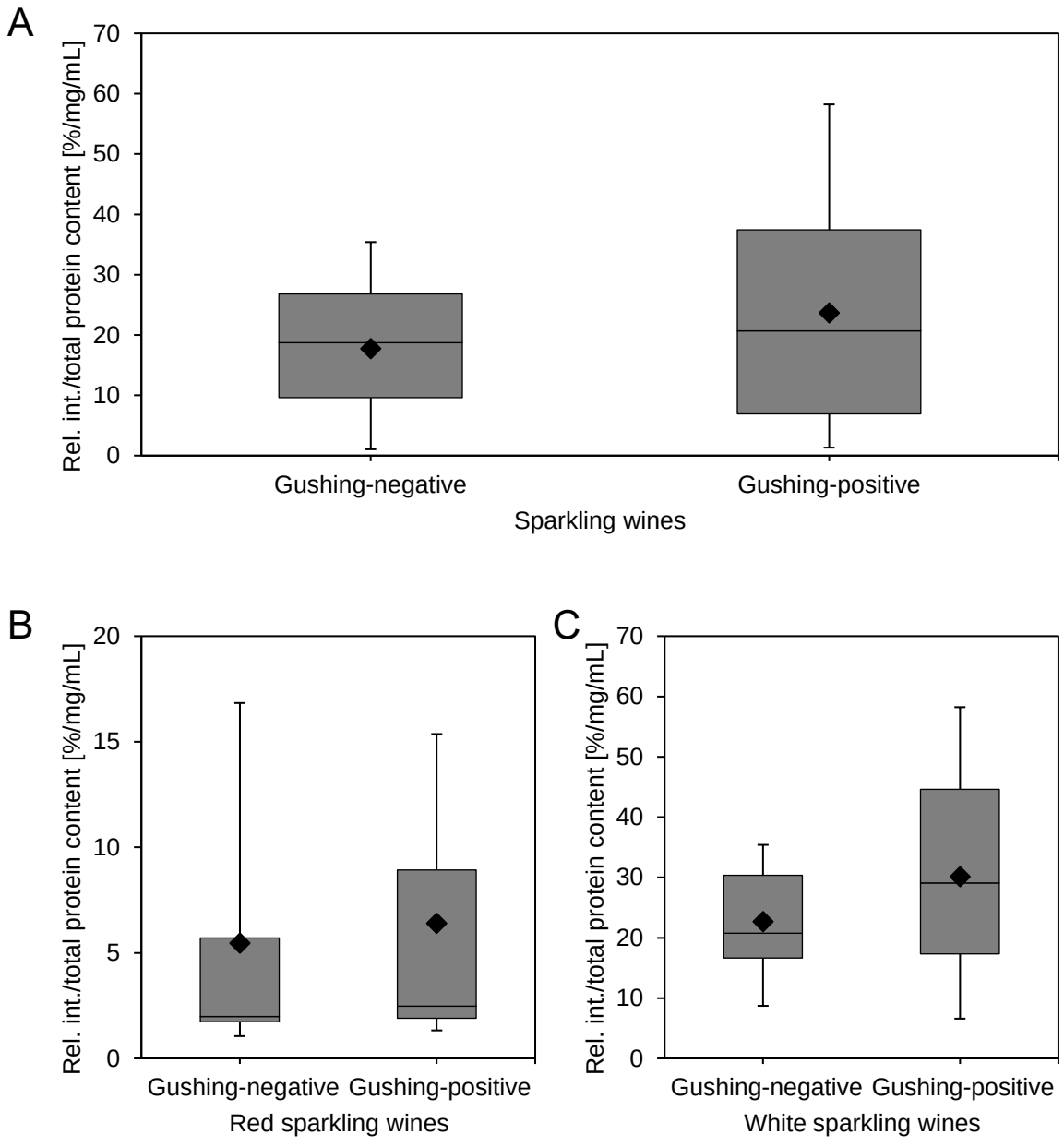


Figure 49: ELISA results for testing sparkling wines with antibody against ns-LTP1 *Vv*

Gushing-negative and gushing-positive sparkling wines were tested with the ELISA using an antibody against ns-LTP1 *Vv*. The box plot shows the minimum, 25th percentile, median, 75th percentile, and maximum of the relative (Rel.) intensity (int.) normalized to the total protein content measured by BCA assay. The diamond shows the mean of the normalized relative intensity. A) Results for 14 gushing-negative and 11 gushing-positive sparkling wines including both red and white varieties. In B and C, the results are shown for the wines tested in A separated into red (B, 4 gushing-negative, 3 gushing-positive) and white sparkling wines (C, 10 gushing-negative, 8 gushing-positive).

In Figure 49 A, the results for gushing-negative and gushing-positive sparkling wines including red and white varieties are depicted. The mean of the normalized relative intensity was slightly higher for gushing-positive sparkling wines (23.7 %/mg/mL) than for gushing-negative ones (17.8 %/mg/mL). The interquartile range for the gushing-positive sparkling wines was greater than that for the gushing-negative ones. No significant difference between the means was found for the two groups.

In Figure 49 B, the results for only red gushing-negative and gushing-positive sparkling wines tested in A are depicted. The mean of the normalized relative intensity was slightly higher for gushing-positive sparkling wines (6.4 %/mg/mL) than for gushing-negative ones (5.5 %/mg/mL). The interquartile range for the red gushing-positive sparkling wines was greater than that for the red gushing-negative ones. No significant difference between the means was found for the two groups.

In Figure 49 C, the results for only white gushing-negative and gushing-positive sparkling wines tested in A are depicted. The mean of the normalized relative intensity was higher for gushing-positive sparkling wines (30.1 %/mg/mL) than for gushing-negative ones (22.7 %/mg/mL). The interquartile range for the red gushing-positive sparkling wines was greater than that for the red gushing-negative ones. No significant difference between the means was found for the two groups.

By comparing red (Figure 49 B) and white sparkling wines (Figure 49 C), the normalized relative intensities were higher for white sparkling wines than for red ones. The means of the red and white gushing-negative sparkling wines were significantly different (unpaired t-test, $p = 0.009$, $\alpha = 0.05$) as well as the means of the red and white gushing-positive sparkling wines (unpaired t-test, $p = 0.016$, $\alpha = 0.05$).

In comparison to the results in the ELISA with the Anti-nsLtp1-P2-IgG antibody (see Figure 45), again no significant difference between the means of gushing-negative and gushing-positive sparkling wines was found. In the ELISA with the specific Anti-ns-LTP1 *Vv* antibody, a slightly higher normalized intensity was revealed for gushing-positive sparkling wines, while in the ELISA with the Anti-nsLtp1-P2-IgG antibody against ns-LTP1 from *H. vulgare*, a slightly higher normalized intensity was revealed for gushing-negative sparkling wines.

All in all, no correlation between the ns-LTP1 *Vv* content and the occurrence of gushing was found. A significantly higher abundance of the protein in gushing-negative sparkling wines was not observed.

4 Discussion

The aim of the current study was to develop and apply monitoring systems for filamentous fungi and their proteins that are involved in gushing of sparkling wine. Hereby, the direct effect of heterologously expressed and purified fungal proteins on gushing was investigated. Immunochemical and LAMP-based assays were developed and optimized to investigate the presence of gushing-inducing proteins and the producing fungi in sample materials which may facilitate early and rapid identification of a gushing risk during sparkling wine production. Moreover, a possible gushing-reducing effect of the protein ns-LTP1 *Vv* in the gushing phenomenon was analyzed.

The experiments performed during the current study aimed at evaluating the initial working hypotheses that formed the basis of the presented work. Examining the initial working hypotheses against the data obtained during the current study resulted in the following theses that will be further discussed in the subsequent chapters:

- Purified gushing-inducing proteins can be generated in high amounts.
  The proteins were produced by cloning and heterologous expression in *Pichia pastoris*. Sufficient amounts of purified protein were obtained of PEX2_044840 from *P. expansum* for gushing induction experiments and to serve as calibration standard for the development of an ELISA, and of PDE_07106 from *P. oxalicum* for gushing induction experiments.

- Purified proteins PEX2_044840 and PDE_07106 induce gushing in sparkling wine.
  Addition of the purified PEX2_044840 and PDE_07106 proteins, respectively, to sparkling wine induced gushing.

- Immunochemical assays detect gushing-inducing proteins in base wines.
  Specific antibodies were generated and applied in immunochemical analyses. An ELISA was developed for the detection of the PEX2_044840 protein from *P. expansum*, while Western blot and dot blot analyses enabled detection of gushing-inducing *P. oxalicum* proteins. The developed ELISA was applied for the relative determination of the PEX2_044840 protein content in wine samples. Significantly higher concentrations of PEX2_044840 were detected in samples of gushing-positive sparkling wine compared to gushing-negative samples indicating the gushing-inducing effect of this fungal protein and its suitability as an analytical marker for gushing.

- Rapid LAMP-based diagnostic assays are a useful tool for monitoring gushing-relevant fungi in the vineyard.

  A LAMP assay for the detection of *P. expansum* was developed and previously published assays were further optimized. The LAMP assays were applied to analyze grape, soil, and must samples from European vineyards for the presence of *P. expansum*, *P. oxalicum*, and *B. cinerea*, respectively. *B. cinerea* occurred ubiquitously, while the presence of *P. expansum* and *P. oxalicum* showed annual and regional variations.

- The ns-LTP1 *Vv* content in sparkling wine does not influence the occurrence of gushing.

  An ELISA was developed for the relative quantification of the protein ns-LTP1 *Vv* in sparkling wines. No correlation between the ns-LTP1 *Vv* content and the occurrence of gushing was detected.

4.1 Heterologous expression of gushing-inducing proteins in *Pichia pastoris* and their purification

During the current study, the gushing-inducing proteins PEX2_044840 from *P. expansum* and PDE_07106 and PDE_04519 from *P. oxalicum* were heterologously expressed in *Pichia pastoris* to obtain high quantities of the purified proteins for further experiments.

Pichia pastoris was chosen as expression system as it promises high yields of recombinant protein (Romanos, 1995) and several protocols for transformation are available from the literature. The expression of recombinant protein requires the insertion of the gene of interest into an expression vector, the integration of the expression vector into the *Pichia pastoris* genome, and a method for selection of transformants that express the recombinant gene product (Cereghino and Cregg, 2000). For the current study, pPICZαA was chosen as expression vector. This vector enables secreted expression of recombinant protein due to a *S. cerevisiae* α-factor secretion signal with a Zeocin™ resistance gene for selection in both the intermediate host *E. coli* and *Pichia pastoris*. In addition, the vector contains sequences encoding a 6xHis-tag for detection and purification of recombinant protein (Invitrogen, 2010b). The gene of interest was inserted into the vector by restriction digestion and ligation. These constructs were then transformed into *E. coli* that was used as an intermediate host for the propagation of the vector. *Pichia pastoris* wild type X33 was chosen as host strain since it has a Mut^+ (Methanol utilization plus) phenotype due to a native *AOX1* gene and therefore grows with methanol as carbon source (Invitrogen, 2010b). The *Pichia* transformation was conducted by electroporation. This method yields transformants at high frequencies and is commonly favored over spheroplast or polyethylene glycol based protocols (Higgins and Cregg, 1998).

Pichia pastoris cells were pretreated with lithium acetate and dithiothreitol prior to electroporation to enhance the transformation efficiency. Wu and Letchworth (2004) showed a 150-fold increased transformation efficiency compared to other protocols due to this kind of treatment. Moreover, the same authors revealed the influence of several factors on the efficiency, such as DNA concentration, cell density, or electroporation settings. After linearization of the vector by restriction digestion within the 5' *AOX1* region, the vector should be ready for integration into the *Pichia pastoris* genome at the *AOX1* locus (Invitrogen, 2010b). This integration occurs via homologous recombination between regions shared by the vector and the *Pichia pastoris* genome. Insertion within the native *AOX1* gene leads to a Mut^+ phenotype in transformants, while a disruption of the gene forces the transformant to use the weaker *AOX2* gene for growing on methanol resulting in a Mut^S (Methanol utilization slow) phenotype (Cereghino and Cregg, 2000; Cregg et al., 2000). *Pichia pastoris* transformants were sequenced and complete integration of the respective gene inserts with correct orientation and positioning in the *Pichia pastoris* genome was confirmed. Analysis of the transformants regarding their Mut phenotype revealed the Mut^+ phenotype for all analyzed transformants and confirmed their ability to metabolize methanol. The obtained transformants were further analyzed for the occurrence of multi-copy transformants. These contain multiple integrated copies of the expression cassette and can therefore yield higher amounts of recombinant protein (Cereghino and Cregg, 2000; Clare et al., 1991). According to Invitrogen (2010a), these "jack-pot" clones occur with a frequency of 1-10 % of total transformants. However, in the current study, such multi-copy transformants occurred with much higher frequencies: 46 % of *Pichia pastoris*_PDE_07106 transformants, 67 % of *Pichia pastoris*_PDE_04519 transformants, and 15 % of *Pichia pastoris*_PEX2_044840 transformants. Further experiments were conducted with multi-copy transformants only because a high yield of recombinant protein was wanted. Nevertheless, final yields of different proteins expressed by high copy number transformants can differ greatly as several other factors such as protein stability also affect protein production (Romanos, 1995). In the literature, further selection of multi-copy transformants based on the rate of resistance to Zeocin™ has been described since hyperresistance is correlated to the number of gene copies in a transformant strain (Higgins and Cregg, 1998).

Transformants were tested for their ability to express the recombinant protein by analyzing culture supernatants and cell lysates after different incubation times with an Anti-6xHis-tag antibody. For the *Pichia pastoris*_PDE_07106 and *Pichia pastoris*_PDE_04519 transformants, multi-copy transformants showed expression of recombinant protein in the cell lysate, while all tested *Pichia pastoris*_PEX2_044840 transformants showed expression in the culture supernatant as well as in the cell lysate. Due to the use of the *S. cerevisiae* α-factor secretion signal, secretion of the recombinant protein in the supernatant was expected for all

recombinant proteins. As *Pichia pastoris* in general secretes only low levels of endogenous proteins, the majority of proteins secreted into the medium are expected to be the recombinant ones (Cereghino and Cregg, 2000). Secreted proteins have the advantage of easier purification as compared to intracellular proteins. For the *Pichia pastoris*_PEX2_044840 transformants, this secretion worked with the highest expression in the supernatant. In contrast, the *Pichia pastoris*_PDE_07106 and *Pichia pastoris*_PDE_04519 transformants showed expression mainly in the cell lysate and only very little in the supernatant. A possible explanation for the observed difference is that the applied secretion signal in combination with the *P. oxalicum* proteins was not optimal. There are several different and improved signal sequences available, including native ones present on heterologous proteins, that have been applied with variable success (Barrero et al., 2018; Higgins and Cregg, 1998; Neiers et al., 2021). For instance, Barrero et al. (2018) showed up to 20-fold enhanced secretion levels of certain proteins by the use of a hybrid secretion signal consisting of a *S. cerevisiae* Ost1 signal sequence with the α-factor pro region as compared to the original α-factor secretion signal. Also, the processing of the used signal sequence could have been defective due to surrounding amino acids or tertiary structures in the recombinant protein which protect the cleavage sites of the signal sequence (Cereghino and Cregg, 2000). Other factors that were found to be influencing secretion are a suboptimal gene dosage or proteolysis of the secreted protein (Romanos, 1995). Another possible explanation is that the proteins have been secreted into the culture supernatant but were expressed with very low levels. This assumption is supported by the observation of stable foam produced by transformant strains compared to the *Pichia pastoris* wild type (see Figure 18). The proteins to be secreted are surface-active and were shown to stabilize foams (Vogt et al., 2017b). Assumingly, stable foams present in transformant cultures and absent in cultures of the wild type indicate the expression and secretion of these surface-active proteins into the culture supernatant. Depending on the surface-activity of such proteins, already small concentrations can be sufficient to form stable foam.

Purification of recombinant proteins from culture supernatants or cell lysates was followed by SDS-PAGE and Western blot analysis. Recombinant proteins were purified by the use of immobilized metal affinity chromatography (IMAC) taking advantage of the 6xHis-tag present at the C-termini. Due to the quite small size and charge of the tag, the protein's function or structure are supposed to be rarely affected by such manipulation (Bornhorst and Falke, 2000). The supernatant of the transformant strain *Pichia pastoris*_PEX2_044840 TMW 3.1089 revealed a prominent band at approximately 29 kDa that was absent in the *Pichia pastoris* wild type strain and was therefore deduced to be a result of the transformation. The generally low secretion rate of endogenous proteins by the *Pichia pastoris* wild type (Cereghino and Cregg, 2000) was confirmed in the experiment. Following purification, the 29 kDa protein band was

exclusively present in the SDS-PAGE of the transformant sample and was detected by Western blot analyses with an Anti-6xHis-tag antibody as well as with an Anti-PEX2_044840 antibody. Moreover, nano-ESI-LC-MS/MS analysis confirmed the identity of the protein as PEX2_044840. Theoretical analysis of the amino acid sequence of PEX2_044840 including a 6xHis-tag revealed a molecular weight of 29 kDa when the α-factor secretion signal was assumed to be cleaved improperly at the first cleavage site. This theoretical calculation suggests suboptimal processing of the signal sequence in the analyzed transformant. According to Cereghino et al. (2002) efficient secretion of proteins can occur despite improper processing of the signal sequence. Proper cleavage of the secretion signal at the second cleavage site would result in a theoretical molecular weight of 22 kDa indicating posttranslational modifications that might be responsible for the presence of a 29 kDa band of the protein visible in SDS-PAGE. The possibility of incomplete cleavage of the secretion signal or posttranslational modifications were also described by Stübner et al. (2010) for the heterologously expressed *F. culmorum* hydrophobin FcHyd5p.

The cell lysates of the *Pichia pastoris* wild type and the *Pichia pastoris*_PDE_07106 transformant contained high amounts of total protein. Purification of His-tagged proteins via IMAC revealed four protein bands in SDS-PAGE from the transformant sample that were absent in the wild type strain and were therefore deduced to be a result of the transformation. Surprisingly, all of the four bands were detected in Western blot analyses with both an Anti-6xHis-tag antibody and an Anti-PDE_07106 antibody indicating the intact 6xHis-tag and intact PDE_07106 sequence in all four proteins. Therefore, these different bands might result from different cleavage events in the α-factor secretion signal. This theory supports the previous assumption about the occurrence of stable foam in PDE_07106 transformant cultures despite no or very low detectable recombinant protein present in the supernatant: A very low secretion of the recombinant surface-active protein responsible for foam formation can be explained by several improper cleavage events in the secretion signal. Theoretical analysis of the amino acid sequence of PDE_07106 with a 6xHis-tag and an uncleaved α-factor secretion signal revealed a molecular weight of 27 kDa which matched one of the bands visible in the Western blot experiments. On the other hand, the sequence with α-factor secretion signal cleaved at the second cleavage site revealed a theoretical molecular weight of 17.7 kDa which also matched one of the bands in the Western blot experiments. Analysis of all of the four protein-containing bands by LC-MS/MS confirmed their identity as PDE_07106. Assumingly, an α-factor secretion signal cleaved at different sites results in different protein bands and very low secretion rates. Moreover, posttranslational modifications or a combination of these modifications with improper cleavage of the secretion signal might be responsible for the other two protein bands.

The cell lysates of the *Pichia pastoris* wild type and the *Pichia pastoris*_PDE_04519 transformant also contained high amounts of total protein. Purification of His-tagged proteins via IMAC revealed several bands in SDS-PAGE from the transformant sample, whereas no proteins were purified from the wild type cell lysate. SDS-PAGE showed several diffuse and faint bands in the preparation of the transformant sample indicating very low expression rates for the recombinant protein. Western blot analysis with an Anti-6xHis-tag antibody revealed the presence of a protein band at 31 kDa. This molecular weight was also obtained by theoretical amino acid sequence analysis of PDE_04519 with a 6xHis-tag and an uncleaved α-factor secretion signal which could explain secretion of the protein at very low rates. Nevertheless, Western blot analysis with an Anti-PDE_04519 antibody did not detect any protein band. As the Anti-PDE_04519 antibody was shown to detect PDE_04519 specifically in previous studies (Vogt-Hrabak, 2017), it can be assumed that the expression of the protein was too low for proper detection of the protein in cell lysates with this antibody.

As a result of the current study, the cloning procedure was successful and all three gushing-inducing proteins were heterologously expressed in *Pichia pastoris*, although at different levels: While *Pichia pastoris*_PEX2_044840 transformants secreted the recombinant protein at high levels and the protein could be purified via FPLC, both *P. oxalicum* proteins were secreted at negligible or very low level and were mainly expressed intracellularly. One critical factor possibly explaining these differences in expression is codon optimization. Among the genetic constructs used for *Pichia pastoris* transformation, only the PEX2_044840 nucleotide construct was codon-optimized for optimal expression in *Pichia pastoris*. The expression of recombinant proteins can be affected due to differences between the codon usage of the *Pichia* host genome and the protein-encoding sequence of the heterologous donor organism (Yu et al., 2013). Studies showed that a *Pichia* specific codon optimization of the heterologous protein-encoding sequence significantly increased protein production in *Pichia* transformants (Chang et al., 2006; Li et al., 2010; Outchkourov et al., 2002; Sinclair and Choy, 2002; Yu et al., 2013). Moreover, the cultivation conditions can be modified to further improve protein production in the transformants. During the current study, BMGY medium was used for the production of biomass and BMMY for the induction of expression in shake flask cultures according to the manual provided by Invitrogen (2010a) for secreted expression. These complex media are recommended to control the pH, to stabilize secreted proteins, and to decrease protease activity. Experiments with minimal media BMG/BMM resulted in low protein yields in the current study. As an alternative, an unbuffered medium could be used for proteins that are susceptible to neutral pH proteases by decreasing the pH. Such decrease would not affect *Pichia* growth which was observed in range of pH 3.0 to 7.0 (Cregg et al., 2000). Expression of recombinant protein can be improved by setting the optimal pH that can vary widely (Jiang et al., 2008) but also by optimizing temperature conditions: A temperature below

30 °C can stabilize proteins susceptible to proteolytic activity (Jiang et al., 2008) or proteins that are prone to aggregation (Li et al., 2007). Moreover, studies showed that the switch from shake flask to fermenter cultivation can increase expression levels and protein yields significantly because the influence of pH, dissolved oxygen, or methanol induction can easier be controlled (Li et al., 2007). Aeration of *Pichia* cultures is a highly critical factor for induction efficiency and the large increase in protein yield that occurs when switching to fermenter cultures can be explained by the tendency of especially Mut^+ strains to become oxygen-limited in shake flasks (Romanos, 1995). In the current study, baffled flasks closed with sterile gauze were used for shaking cultures at 240 rpm to provide sufficient aeration. However, oxygen levels were not measured during incubation in the performed experiments. Even though methanol can be used as carbon source, its concentration is an important factor for protein expression as *Pichia pastoris* is sensitive to the methanol concentration in the culture medium. Therefore, the compound needs to be added continuously to the growing culture (Trinh et al., 2003) to induce expression but to not exceed critical levels that inhibit cell growth (Lin et al., 2000). The use of a fermenter with its controlled environment for monitoring and controlling oxygen levels, pH, and methanol concentrations can hereby enable *Pichia pastoris* transformants to grow to higher cell densities (Cregg et al., 2000). As the concentration of protein in the medium is almost proportional to the concentration of cells in culture, high concentrations of secreted protein can be obtained in this way (Cregg et al., 2000). In general, it must be mentioned that all cultivation conditions strongly depend on the particular protein and need to be assessed individually.

In summary, the obtained *Pichia pastoris*_PEX2_044840 transformants expressed and secreted the recombinant protein at high levels which could be purified via IMAC. The *Pichia pastoris*_PDE_07106 and *Pichia pastoris*_PDE_04519 transformants showed expression of the respective proteins but did so in low levels and intracellularly. The expression levels of these transformants may be further improved in follow-up studies by adjustment of cultivation conditions and switch to fermenter cultivations but also by the use of alternative signal sequences and a *Pichia pastoris* codon-optimized protein-encoding sequence. The obtained amounts of purified PEX2_044840 protein were sufficient for application in further gushing experiments as well as its use as calibration standard for the development of an ELISA. The obtained amounts of purified PDE_07106 protein were sufficient for gushing experiments.

4.2 Gushing potential of the purified proteins

The proteins PEX2_044840 from *P. expansum* and PDE_04519 and PDE_07106 from *P. oxalicum* are supposed to be involved in gushing induction in sparkling wine, although gushing induction was shown so far only for the addition of culture supernatant that was foam fractionated (Frisch, 2018; Vogt et al., 2017b). Since also other proteins were present in the

gushing-active spumate fraction in the former studies, purified proteins were analyzed in the current study to investigate their gushing-inducing effect.

Sufficient amounts of purified recombinant protein were available of PEX2_044840 from the culture supernatant of *Pichia pastoris*_PEX2_044840 transformants as well as of PDE_07106 from the cell lysate of *Pichia pastoris*_PDE_07106 transformants.

Addition of a minimum of 30 µg of the purified PEX2_044840 protein to sparkling wine bottles (750 mL) resulted in gushing. Addition of higher amounts of the protein increased the gushing effect. Similar effects were observed by other authors who described a correlation between the amount of added gushing-inducing substances to carbonated beverages and the resulting volume loss (Lutterschmid et al., 2011; Lutterschmid et al., 2010; Sarlin, 2012; Stübner et al., 2010; Vogt et al., 2017b). In the current study, no gushing-inducing effect was shown for purified *Pichia pastoris* wild type culture supernatant which indicates that the induction of over-foaming can be attributed to the presence of the recombinant protein PEX2_044840. The observed volume losses varied between duplicates which was also shown in other studies (Lutterschmid et al., 2011; Stübner et al., 2010). Several factors influencing the gushing volume have not been determined during the current study, such as the content of gushing-reducing compounds like the protein Pau5p in the sparkling wines or the inner wall quality of bottles and carbon dioxide content (Lutterschmid et al., 2011). All of these factors may have contributed to the observed variance between the duplicates. Bach (2001) described gushing as being a more or less severe volume loss due to excessive over-foaming upon opening of a sparkling wine bottle indicating high variability from bottle to bottle. Also, during the current study, high variation in volume losses after bottle opening was observed between bottles that had been declared as being gushing-positive by the manufacturers: some of the bottles showed no or only slight over-foaming, while others had volume losses up to 600 g (results not shown). Such differences in the gushing tendency of different bottles even of the same sparkling wine lot are a commonly observed phenomenon (personal communication, project committee AiF 19952 N, 23.01.2019 and 28.01.2020). The volume losses obtained by the added amounts of recombinant PEX2_044840 were low to moderate in comparison to what has been observed in genuine gushing cases but fulfilled the definition of gushing because abrupt and excessive over-foaming occurred in bottles treated with the protein. However, it is difficult to assess whether the observed minimum level for gushing induction by the recombinant protein corresponds with the minimum level of the natural PEX2_044840 protein for gushing induction. Minimum levels in the current study were assessed with a recombinant PEX2_044840 protein which possesses a few additional amino acids that were unavoidable due to the used cloning protocol. Therefore, the protein slightly differs from the native PEX2_044840 protein that *P. expansum* produces in its natural environment. Nevertheless, the differences between the

recombinant and the native protein are not supposed to be of elemental importance because the core parts of the amino acid sequences are identical between both proteins and confer similar characteristics. Therefore, the obtained results enable a first estimation of the minimum PEX2_044840 protein level that is necessary for gushing induction. Results from the current study were supported by experiments performed by project partners at Hochschule Geisenheim University (Geisenheim, Germany) (results not shown). Grape must was inoculated with a conidial suspension of *P. expansum* and subsequently vinified, while uninoculated must served as control. The resulting wines were used as base wines for sparkling wine production under practical conditions. Gushing was assessed during disgorging of the bottles. The volume loss in bottles resulting from the inoculated must was 8-fold higher as compared to the untreated control. It was concluded that substances such as proteins produced by the fungus must have been responsible for the high volume loss which further emphasizes the impact of *P. expansum* on gushing.

Addition of a minimum of 15 µg of the purified PDE_07106 protein to sparkling wine bottles induced gushing. The induction level was lower and the volume losses were higher than for PEX2_044840. However, the results for the two proteins can hardly be compared because although the same sparkling wine brand was used, both gushing experiments were performed with two different production lots. Moreover, PDE_07106 was purified from *Pichia* cell lysate, whereas PEX2_044840 was isolated from *Pichia* culture supernatant. As proteins purified from the *Pichia pastoris* wild type cell lysate also provoked over-foaming in the experiment, it can be assumed that compounds other than the *P. oxalicum* protein present in the cell lysate can act as nucleation sites for bubble formation. However, since the volume loss due to addition of the PDE_07106 protein was higher compared to the wild type, a gushing-inducing effect of the PDE_07106 protein can still be claimed.

These results suggest that both proteins have a direct gushing-inducing effect in sparkling wine and are important factors in its gushing mechanism. Already low concentrations of these proteins in the beverage can induce gushing. Results indicate that an infection of grapes with *P. expansum* and/or *P. oxalicum* increases the gushing risk when such grapes are used for sparkling wine production. The occurrence of *P. oxalicum* in vineyards has rarely been described so far, while *P. expansum* occurs frequently in temperate regions (Abrunhosa et al., 2001; Bau et al., 2005; Bejaoui et al., 2006; Bragulat et al., 2008; Diguta et al., 2011; Felšöciová et al., 2021; Felšöciová et al., 2015; La Guerche et al., 2005; Lorenzini et al., 2016; Mikusová et al., 2010; Ostrý et al., 2007; Sage et al., 2002; Serra, 2003; Serra et al., 2005; Serra et al., 2006; Tančinová et al., 2015; Varga et al., 2007; Vogt et al., 2017a; Walter, 2008). Preliminary studies by the author of the current study revealed that from almost 40 *P. expansum* strains tested for their production of surface-active proteins, just under half of

the strains were able to do so with temporal dynamic (Frisch, 2018). Also Zapf (2006) observed strain differences in *F. graminearum* strains in a surface activity test. Moreover, the same author showed an influence of growth medium and incubation time on the production of surface-active proteins. Previous studies of the author of the current study showed that the spumate of some *P. expansum* strains resulted in no or inconsistent gushing behavior when added to carbonated water, while the spumate of others resulted in gushing (Frisch, 2018). These observations have led to the assumption that the production of gushing-inducing substances is dependent on *P. expansum* strains and optimal growth conditions. These factors can be responsible that not every *P. expansum* infection of grapes results in gushing equally, since due to the high occurrence of *P. expansum* in vineyards, gushing should be occurring more frequently as compared to what is reported in the industry. In general, gushing is known to be a multifactorial problem (Dachs and Nitschke, 1977; Draeger, 1996) so that the tendency of a sparkling wine bottle to over-foam is influenced by the interaction of multiple factors. The results of the current study have demonstrated that certain fungal proteins play an important role as inducing factors of gushing in sparkling wine. They should therefore be useful markers that can indicate the presence of a gushing risk during the production process and in the final product.

4.3 Immunochemical detection of the gushing-inducing proteins

The immunochemical detection of gushing-inducing proteins in base wines enables the possibility to monitor their levels and to determine base wines with a high risk for gushing in the corresponding sparkling wines due to high levels of the proteins. Therefore, specific antibodies were generated and applied in immunochemical analyses in the current study.

4.3.1 ELISA for the detection of PEX2_044840 from *P. expansum*

A competitive ELISA was developed for the detection and quantification of the PEX2_044840 protein of *P. expansum*. This assay type was chosen as it provides high sensitivity and only one specific antibody is required instead of a second specific antibody that is needed to set up a sandwich assay. An enzyme-linked secondary antibody (Anti-Rabbit-IgG) which is commercially available detects binding of the antigen-specific primary antibody to its antigen. The Anti-PEX2_044840 antibody was generated based on a peptide sequence that was calculated by Davids Biotechnologie GmbH (Regensburg, Germany) from the PEX2_044840 protein sequence. The resulting peptide had good antigenicity and good epitope prediction. The generated antibody detected PEX2_044840 specifically, also in base wine, and was therefore considered suitable for the ELISA development.

Several attempts were made to obtain a standard curve that could be used to calibrate the assay for the quantification of the target protein. In general, it was important to use glass tubes when handling solutions that contained PEX2_044840 or antibodies in order to prevent protein

binding to plastic tubes. Tubes made from polystyrene are not suitable for this purpose due to their high protein binding capacity (Luttmann et al., 2014). Sarlin et al. (2005b) supposed binding of hydrophobins to tube walls and pipette tips due to their hydrophobic nature as a reason for the errors that were found in their concentration measurements. Therefore, glass tubes were used in the current study for the handling of protein-containing solutions.

In a first attempt, the microtiter plate was coated with the PEX2_044840 peptide as the antigen against which the antibody is directed. Also, Specker (2014) used the FcHyd5p-P3 peptide successfully as solid phase coating in an ELISA for the relative quantification of the hydrophobin FcHyd5p. However, the standard curve obtained in the current study with lyophilized purified PEX2_044840 protein showed no correlation between antigen concentration and extinction. As a competitive ELISA format was used, high extinction values were expected for low protein concentrations and low extinction values for high concentrations. Though, extinction values in the first attempt were high for all samples indicating that most of the antibody must have been bound to the peptide on the solid phase of the plate without an interaction with the antigen in the liquid phase. Since the standard samples had a broad range of protein concentrations from 10 µg/mL to 1,000 µg/mL, it was assumed that the antibody has a higher affinity to the peptide than to the protein itself.

Modifications to the protocol were made in further experiments in which the solid phase was coated with the lyophilized PEX2_044840 protein instead of the peptide to provide the antibody with the same competition partners on the solid phase and in the liquid phase. This approach was similar to that performed successfully by Sarlin et al. (2005b) who used hydrophobins to coat the ELISA solid phase while using the same hydrophobins as standards in an ELISA for the detection of *Fusarium* hydrophobins in barley and malt samples. However, the standard curve obtained in the current study was again of poor quality showing a weak correlation between the concentration of the protein standard used and the extinction value measured. In further experiments, several different assay settings were changed including buffers, types of solid phases, incubation periods, and concentration of reagents, with no further improvement reached.

Western blot analysis of heat denatured and untreated PEX2_044840 protein revealed a much higher signal intensity in the heat-treated samples from which a higher binding capacity of the antibody was concluded. Hnasko et al. (2011) reported that many antibodies that detect their target protein in a Western blot show a poor binding capacity to the same target protein in an indirect ELISA. The authors hypothesized the inhibition of antibody binding to be caused by blocking of epitope binding sites which can be the result of protein conformation following attachment to the solid phase. To overcome the limitations in detection sensitivity, the authors suggested the use of chemical denaturation of the target protein. During the current study,

denaturation was attained by heating the solid phase coating protein and the liquid phase protein standards at 60 °C for 10 min. Also, Specker (2014) heated recombinant ns-LTP1 lyophilisate prior to its use as a standard in an ELISA for the detection of ns-LTP1 from *H. vulgare*. The used antibody only detected denatured ns-LTP1 and not the untreated protein. As a result of the heat denaturation step, the standard curve obtained in the current study covered a wide dynamic range and a strong correlation between protein concentrations and extinction values was found. Compared to the hydrophobin ELISAs described by Sarlin et al. (2005b) (5-100 µg/mL) and Specker (2014) (16-1,000 µg/mL), the PEX2_044840 ELISA (25-1,000 µg/mL) had a similarly high sensitivity. The intra- and interspecific reproducibility as well as the recovery were very high and revealed the suitability of the developed and optimized assay for further analyses of sample materials.

The ELISA was developed to analyze base wines for their concentration of gushing-inducing PEX2_044840 protein. A dialysis of base wines before ELISA analysis turned out to be unnecessary. This was also confirmed in other studies, in which non-dialyzed base wines were used in ELISA analyses (Dambrouck et al., 2005; Weber et al., 2009). In contrast to PBS buffer, base wine mixed with PEX2_044840 protein did not result in stronger binding of the antibody after heat treatment as compared to untreated wine. A possible explanation for this phenomenon may be the formation of wine haze at elevated temperatures. Hazy wine is an esthetic problem in white wines that is caused by protein heat instability (Ribéreau-Gayon et al., 2006; van Sluyter et al., 2015). Elevated temperatures lead to unfolding of wine proteins (e.g., chitinases, some thaumatin-like protein isoforms) in this matrix, followed by self-aggregation, and cross-linking (Gazzola et al., 2012; Marangon et al., 2011; van Sluyter et al., 2015). It is suggested here that due to such aggregation, the epitope in the PEX2_044840 protein is fully or partially masked so that the antibody cannot bind properly to its antigen.

Although a standard curve could be set up for the calibration of the optimized ELISA using PBS buffer as the liquid phase, attempts failed to quantify the PEX2_044840 protein in base wines and sparkling wines. Experiments with addition of known protein concentrations to base wines showed that the measured concentrations differed extremely from the added concentrations so that no reliable absolute quantification was possible. As a consequence, the ELISA was used to compare samples relatively to each other instead of absolutely. Also, Specker (2014) could not use a developed hydrophobin ELISA for absolute quantification of FcHyd5p but for its relative quantification in beer samples. Moreover, Koestel et al. (2016) applied an ELISA for the relative quantification of the allergen ovalbumin in several wines since the authors observed signal inhibition of the ELISA by the complexity of the wine matrix. Further studies are needed in the future to optimize the sample preparation enabling absolute determination of the PEX2_044840 protein concentrations in base wines. Nevertheless, the

developed ELISA was a useful tool for the comparison of sparkling wine samples relatively to each other in respect to their PEX2_044840 protein content when normalized to the total protein content of samples. The percentage of PEX2_044840 in the total protein content was significantly higher in gushing-positive sparkling wines than in gushing-negative ones indicating that the presence of this protein is positively correlated to the occurrence of gushing in sparkling wine.

The results show the great potential of the developed ELISA to evaluate the PEX2_044840 protein content in sample materials. In its current form, the assay can be used to compare samples relatively in respect to their PEX2_044840 content and therefore to enable further experiments in which a comparison of samples is needed. The significantly higher concentration of the protein in gushing-positive sparkling wines than in gushing-negative ones indicated its involvement in the gushing mechanism as gushing-inducing protein and its suitability as analytical marker. In order to prevent increased amounts of the PEX2_044840 protein in base wines intended for sparkling wine production, an infection of grapes in the vineyard with *P. expansum* should be avoided. In the future, optimization of the assay and the sample preparation protocol can facilitate absolute quantification of the protein content in base wines, including the establishment of threshold values, to allow the estimation of a possible gushing risk in the resulting sparkling wine. An analysis of base wines is especially important since many sparkling wine producers that import the used base wines have only little information about manufacturing and grape quality (personal communication, project committee AiF 19952 N, 28.01.2020).

4.3.2 Detection of *P. oxalicum* proteins

In contrast to high amounts of purified PEX2_044840 protein from *P. expansum* obtained by expression in *Pichia pastoris*, the expression of the two *P. oxalicum* proteins PDE_07106 and PDE_04519 was lower and did not provide sufficient amounts of the proteins to be useful as calibration standard for the development of ELISAs. Therefore, immunochemical analyses were conducted by Western blot and dot blot analyses instead.

The Anti-PDE_07106 antibody was newly generated based on a peptide that was calculated by Davids Biotechnologie GmbH (Regensburg, Germany) from the PDE_07106 protein amino acid sequence and had medium to good antigenicity and a good epitope prediction. This new antibody generation was necessary since a previously generated antibody for detection of PDE_07106 (Anti-VOG-APA-IgG) was shown to be unsuited due to several cross-reactivities (Vogt-Hrabak, 2017). The newly generated antibody detected PDE_07106 specifically together with a dimer of the protein in *P. oxalicum* inoculated must and was therefore considered suitable for further analyses in the current study.

In contrast to the previously mentioned Anti-VOG-APA-IgG antibody, the Anti-VOG-EFA-IgG antibody for the detection of PDE_04519 that was also generated in the study described by Vogt-Hrabak (2017) was shown to be highly specific for its target protein and could detect the protein in must samples after inoculation with *P. oxalicum* spores in the current study.

Detection of these proteins can therefore be performed by Western blot analysis to obtain a qualitative result about the protein's presence in a sample. Moreover, dot blot analysis was shown to be highly sensitive detecting critical protein amounts in base wine: The tested concentrations of purified protein PDE_07106 in the gushing tests given to base wine were analyzed. A concentration of 0.02 µg/mL which corresponds to 15 µg protein in a 750 mL sparkling wine bottle that induced gushing was detectable. By comparison of color intensities of the protein dots, a semi-quantitative determination of protein concentration can be made, especially when compared to a dilution of purified PDE_07106 protein.

In summary, the antibodies that were generated in the current study or used from previous work are highly suitable for the detection of the two gushing-inducing *P. oxalicum* proteins in base wines via immunochemical assays. Due to high sensitivity, a semi-quantitative determination of protein concentrations is possible and enables the determination of critical protein concentrations for gushing induction. Direct analysis of these gushing-influencing proteins is considered to provide the most reliable gushing prediction that can be achieved by immunochemical monitoring. An optimization of the expression levels of *Pichia pastoris* transformants to obtain sufficient amounts of purified *P. oxalicum* proteins as calibration standards can enable the development of a quantitative ELISA in future studies.

4.4 LAMP assays as rapid diagnostic tools for early detection of gushing-relevant fungi on sample materials

Early detection of gushing-relevant fungi on sample materials from vineyards enables an early determination of a possible gushing risk in the resulting sparkling wines. LAMP assays are suitable and rapid detection tools that were either newly developed in the current study for the detection of *P. expansum* or adopted and optimized based on previous publications (Frisch and Niessen, 2019; Tomlinson et al., 2010; Vogt et al., 2017a). The LAMP assays were applied for the detection of gushing-relevant fungi on grape, soil, and must samples to determine their occurrence in European vineyards.

4.4.1 LAMP assay for the detection of *P. expansum*

This chapter contains verbatim quotations from the following publication in which the author of the current thesis holds a first authorship: Frisch et al. (2021b).

P. expansum is known as a mycotoxin producer, a plant pathogen causing considerable economic losses due to postharvest rot, and due to the current study as well as preliminary work as a gushing-relevant fungus (Frisch, 2018; König et al., 2009; Vico et al., 2014). Since rapid and specific detection of *P. expansum* is supposed to be an important tool for quality control, a rapid and easy-to-use LAMP assay for the specific detection of *P. expansum* was developed and optimized.

The LAMP primers used target the gene coding for the gushing-inducing protein PEX2_044840 from *P. expansum*. Vogt et al. (2017a) used the gene sequence of the gushing-inducing protein PDE_07106 as target for the detection of *P. oxalicum* in a LAMP assay. Therefore, the use of the gene coding for the protein PEX2_044840 as target appears to be a logical approach since the LAMP assay can be applied for testing of grapes to assess the gushing risk in resulting sparkling wines. Other authors used primers based on the internal transcribed spacer (ITS) or intergenic spacer (IGS) sequence of their target in LAMP assays to detect typical fungal pathogens on vines such as *Plasmopara viticola* (Kong et al., 2016), *Erysiphe necator* (Thiessen et al., 2016), or *B. cinerea* (Tomlinson et al., 2010).

The assay showed positive results with high specificity at 68 °C after 60 min of isothermal incubation. This temperature is higher than the optimum temperature of 65 °C of the used *Bst* DNA polymerase recommended by the manufacturer. However, functional assays with higher temperature were also set up for the detection of fungal or bacterial targets in other studies (Ferrara et al., 2015; Nakano et al., 2015; Niessen et al., 2018). The LAMP assay using neutral red as pH-sensitive indicator dye facilitated indirect in-tube detection of DNA synthesis in positive reactions by a color change from orange to pink that was clearly visible to the naked eye. As an advantage of in-tube detection, no further post-reaction manipulations are necessary such as time-consuming agarose gel electrophoresis and cross-contamination of subsequent LAMP reactions with DNA can be avoided (Parida et al., 2008; Tomita et al., 2008). A visually detectable color change as indication for positive reactions was already obtained after 60 min of incubation demonstrating the rapidness and ease with which the DNA target is detected with the newly developed assay.

The sensitivity of the assay was demonstrated to be very high for both genomic DNA (25 pg/rxn) and conidia (1 x 10^3 spores/rxn) of *P. expansum*. In comparison to a PCR-based assay for the detection of *P. expansum* described by Tannous et al. (2015) with a detection limit of 100 pg/rxn, the developed LAMP assay had a 4-fold higher sensitivity.

The LAMP assay revealed a very high specificity for the detection of *P. expansum* during tests with purified genomic DNA of 188 fungal strains taken from the department's culture collection. Positive LAMP reactions resulted when genomic DNA from 37 tested *P. expansum* strains was

added as template. Strains had been isolated from a variety of sources, e.g., grape, apple, cherry, soil. Moreover, a positive result was found for three *P. clavigerum* strains (TMW 4.1973, TMW 4.1974, TMW 4.1975), whereas the other analyzed species were negative in the LAMP assay. *P. clavigerum* is a patulin-producing fungus known to occur in soil and animal dung and is very uncommon in foods and has so far not been isolated from grapes (Frisvad and Samson, 2004; Frisvad et al., 2004). Therefore, false-positive results in tested fruits are unlikely to appear. Nevertheless, *P. clavigerum* produces patulin (Frisvad and Samson, 2004; Frisvad et al., 2004; Svendsen and Frisvad, 1994) and (–)-geosmin (Larsen and Frisvad, 1994) such as *P. expansum* making a false-positive result acceptable as it would also give an indication of a risk of patulin contamination of fruits and unwanted off-flavors in wine. A comparison of the amino acid sequence of PEX2_044840 of *P. expansum* with annotated proteins of *P. clavigerum* revealed no significant similarities (results not shown). However, a cross-reaction with the coding sequence of similar but unannotated proteins seemed possible as only 17 annotated proteins of the fungus were available in the NCBI database for the comparison (September 2021). Therefore, the LAMP amplification products obtained with genomic DNA of all three positive *P. clavigerum* strains were analyzed by sequencing with primers F2_PEX2 and B2_PEX2 (results not shown). The results revealed 100 % sequence identity with a query coverage of < 65 % with the gene coding for the PEX2_044840 protein in *P. expansum*. From this result it can be concluded that there might be similar proteins in *P. clavigerum* that could have provoked a positive result in the *P. expansum* specific LAMP assay. *P. clavigerum* and *P. expansum* both belong to *Penicillium* subgenus *Penicillium* but to different series – *P. clavigerum* to series *Clavigera* and *P. expansum* to series *Penicillium* (Houbraken et al., 2020). Moreover, they share several features such as the production of ellipsoidal conidia, synnemata, and patulin indicating that both fungi share a common dung-borne ancestor (Frisvad and Samson, 2004) and therefore the presence of similar proteins seems obvious.

The specificity of the LAMP assay was confirmed by sequencing of the nucleotide sequence of the smallest LAMP product obtained with genomic DNA of *P. expansum* TMW 4.2805. The results revealed a 100 % sequence identity with a partial sequence of the gene coding for the PEX2_044840 protein in *P. expansum*. Other authors have used restriction digestion of complete LAMP reactions to verify the identity of the LAMP amplification product with the target sequence (Kong et al., 2016; Niessen et al., 2018). However, the process of selecting appropriate restriction sites from the complicated concatemeric structure of a LAMP product is cumbersome and time-consuming.

To demonstrate the usefulness and sensitivity of the new assay, its application to the analysis of artificially contaminated apples, grapes, apple juice, apple puree, and grape juice was assessed. For apples and grapes, simple sample washing steps were necessary to process

materials for testing in the LAMP assay with positive results for apples at day 2 after inoculation and for grapes even on the day of inoculation confirming the high sensitivity of the assay. Moreover, also apple juice and grape juice showed positive results right after inoculation. Apple puree gave positive results in the LAMP assay only from day 5 after inoculation. This delay in detection can be explained by the high complexity of the apple puree matrix in which generally higher concentration of LAMP inhibiting polyphenolic compounds are present than in apple juice (Oszmiański et al., 2008; van der Sluis et al., 2002). A higher dilution of the puree might therefore reduce this effect. All negative controls were tested negative over the entire test period indicating that the assay can be used in the different tested food matrices without false-positive results due to matrix compounds. The occurrence of positive results before the appearance of visually detectable mold symptoms shows that the new assay facilitates rapid monitoring of *P. expansum* in agricultural and food production.

The results show the great potential of the developed LAMP assay for the detection of *P. expansum*. Simplification of sample preparation and in-tube detection facilitate economically affordable on-site detection and quality control applications of different matrices in the food and beverage industry, including winemakers.

4.4.2 Detection of gushing-relevant fungi on grape, soil, and must samples from European vineyards

P. expansum, *P. oxalicum*, and *B. cinerea* were demonstrated to be involved in the occurrence of gushing in sparkling wine due to the results of the current study as well as of previous studies (Frisch, 2018; Kupfer et al., 2017b; Vogt et al., 2017b). The presence of these fungi in vineyards can therefore be used as an indicator for an increased risk of gushing in sparkling wines that are produced from base wines made from infected grapes. Detection of these fungi on sample material from the vineyard can help to determine the risk of a future gushing problem early on during the production process. Therefore, grape, soil, and must samples that were collected from different European vineyards were tested for the occurrence of the gushing-relevant fungal species.

Besides the *P. expansum* specific LAMP assay developed during the current study, LAMP assays were used that were previously developed in other studies (Frisch and Niessen, 2019; Tomlinson et al., 2010; Vogt et al., 2017a). Some of the adopted assays needed further optimization in regard to master mix composition and visualization of LAMP products: The LAMP assays for the detection of *P. oxalicum* (Vogt et al., 2017a) and *B. cinerea* (Tomlinson et al., 2010) were optimized for the use of ammonium sulfate buffer and neutral red, so that these assays can be run under the same conditions as the assays for the detection of *P. expansum* and patulin-producing *Penicillium* species (Frisch and Niessen, 2019). Ammonium sulfate buffered LAMP master mixes have a lower buffering capacity than those

buffered with MOPS that was used in the assay of Vogt et al. (2017a). They are therefore well suited for visual signal detection under day light conditions with neutral red as pH-sensitive indicator due to a well distinguishable color change from orange to pink in positive reactions (Niessen et al., 2018; Tanner et al., 2015). Visual indicators such as neutral red have an advantage over agarose gel electrophoresis for evaluation of LAMP products: They facilitate in-tube detection and therefore save time and do not pose the risk of cross-contamination due to opening of reaction vessels after the LAMP reaction is terminated (Parida et al., 2008; Tomita et al., 2008). The optimizations necessitated the adjustment of the incubation temperature in the *P. oxalicum* specific LAMP assay (Vogt et al., 2017a) from 63 °C to 65 °C. After optimization of the *B. cinerea* specific LAMP assay (Tomlinson et al., 2010), the minimum detected genomic DNA was 0.1 pg/rxn as compared to 65 pg/rxn in the original assay before the optimization. Therefore, the optimization steps taken increased the sensitivity of the assay by a factor of 650, while the specificity remained unchanged. A possible explanation for the detected increase in sensitivity can be differences in the LAMP master mixes used in the two studies: Tomlinson et al. (2010) used a higher concentration of dNTPs, a lower concentration of magnesium ions that were added as magnesium sulfate, a Tris-HCl based buffer system, and betaine compared to the LAMP master mix of the current study. In PCR applications, too high concentrations of dNTPs were shown to inhibit the amplification as excessive dNTPs can bind free magnesium ions that are needed for polymerase activity (Markoulatos et al., 2002; Mülhardt, 2009). Therefore, too low concentrations of magnesium ions can have negative effects and the optimization of its concentration is crucial during LAMP assay development. In addition, Niessen (2013) showed that the addition of magnesium ions as magnesium sulfate instead of magnesium chloride resulted in lower LAMP assay sensitivity. Moreover, the same author found LAMP assays that use Tris-HCl based buffers to be considerably less sensitive as compared to assays which use other buffers such as MOPS. Ma et al. (2017) reported that betaine could inhibit the reaction efficiency of LAMP reactions, even though the compound was found to have a positive effect on assay specificity (Notomi, 2000).

Furthermore, optimizations of the sample preparation protocols were necessary: The sample preparation of grapes was performed according to Vogt et al. (2017a). However, dilution of the extracts prior to addition to the LAMP reaction was shown to improve the results by reducing concentrations of inhibitory compounds at maintained sensitivity. Also, Si Ammour et al. (2020) diluted crude berry extracts for the use as template in their *B. cinerea* specific LAMP assay and Luo et al. (2012) showed better detectability of aflatoxigenic fungi in their LAMP assay after dilution of crude extracted DNA, too. Simple washing and cell disruption steps were applied during the current study. This rapid and easy-to-handle sample preparation was shown to be a suitable method to enable on-site application of the LAMP assays that require a minimum of equipment because no DNA extraction was necessary. In contrast, DNA needed

to be extracted from soil and must samples in order to obtain amplifiable nucleic acid and to avoid inhibition of LAMP assays. The need of DNA extraction from soil samples for LAMP was also demonstrated in other studies (Feng et al., 2019; Niu et al., 2011). Due to the low pH of musts, the LAMP buffer used for analyzing the DNA extracted from must samples contained 200 mM ammonium sulfate instead of 100 mM as well as potassium chloride, respectively, to achieve a stronger buffering capacity of the master mix. The optimized sample preparation protocols enabled the analysis of grape, soil, and must samples from vineyards with the optimized LAMP assays.

Besides the assays for the detection of *P. expansum*, *P. oxalicum*, and *B. cinerea*, a LAMP assay for the detection of patulin-producing *Penicillium* species (Frisch and Niessen, 2019) was used in the current study. Since the assay for the specific detection of *P. expansum* was not yet developed and applicable for the screening of samples in the years 2018 and 2019, this group-specific assay was applied for the detection of *P. expansum* as this fungus is the only patulin-producing *Penicillium* species occurring frequently on grapes. In the 2020 screening, the newly developed *P. expansum* specific assay was applied and the group-specific assay was used as a reference to compare results.

In the 2018 screening, only samples from vineyards in Germany were analyzed. The LAMP assay for the detection of patulin-producing *Penicillium* species revealed no positive grape and soil samples for that year. A possible explanation can be the generally hot and dry summer of 2018 throughout Germany with an average precipitation of only 130 L/m^2 in this period that was 46 % less compared to the long-term summer average (Deutscher Wetterdienst, 2018). The analyzed samples originated from wine-growing regions in Hesse (90 L/m^2), Rhineland-Palatinate (125 L/m^2), and Baden-Württemberg (160 L/m^2) which were among the particularly dry German federal states (Deutscher Wetterdienst, 2018). In dry summers, mold infestation on grapes is generally lower than in average years or years with high precipitation due to suboptimal growing conditions. Particularly *P. expansum* prefers humid conditions with frequent precipitation during grape berry ripening (König et al., 2009). These climatic conditions with low fungal pressure may be responsible for the negative results in this LAMP assay. The LAMP assay for the detection of *P. oxalicum* was positive for 2 % of grape samples and for none of the soil samples. This fungus is known to prefer warm Mediterranean climate due to its temperature optimum of 30 °C (Mislivec and Tuite, 1970b). The summer of 2018 in Germany had an average temperature of 19.3 °C (Deutscher Wetterdienst, 2018) which may explain the low presence of the fungus. Nevertheless, the fungus was detected on grapes, and this is to the best of the authors' knowledge the first description of *P. oxalicum* on grapes in Germany. *B. cinerea* was found in 36 % of grape and 59 % of soil samples and was therefore widespread. The pool of samples provided for LAMP testing in 2018 contained, among others,

samples taken from an experimental set-up to analyze the influence of different agronomical factors on *B. cinerea* infestation of grapes and vines. Testing of vines of different ages from the same variety revealed no difference in susceptibility to *B. cinerea* infestation on grapes, while the fungus was not detected in the soil of the youngest vine. Testing of different grape varieties revealed differences in the resistance to *B. cinerea*: The variety “Chardonnay pink” has been undergoing breeding at Hochschule Geisenheim University and is characterized by an increased resistance to *B. cinerea* and the associated bunch rot than Chardonnay white (Lindner, 2015). Higher resistance of the vine was confirmed in the current study by the lower occurrence of *B. cinerea* on the grapes of that variety as compared to the reference Chardonnay variety. Due to climatic changes in the future which may promote the growth of certain fungi and therefore the risk of fungal diseases in wine regions as well as the increase in organic wine production which uses varieties highly susceptible to fungal infestation, the breeding of pest-resistant varieties is becoming increasingly important (Pedneault and Provost, 2016; Santos et al., 2020). Moreover, varieties with a loose bunch structure that can be influenced by partial defoliation of the vine are supposed to be less susceptible to *Botrytis* infection (Walg, 2015). The results regarding LAMP testing of samples that were grown in different cultivation systems will be discussed in a following chapter in more detail. Testing of samples that were treated with different pest control systems revealed the best results for application of Kumar® to prevent infestation. Kumar® is a fungicide that contains potassium hydrogen carbonate as the active agent. The fungicide that is used in organic viticulture is believed to alter the pH and the osmotic pressure resulting in dehydration and killing of spores and mycelium present on the vine (Certis Europe B.V., 2021). VitiSan® is also a potassium hydrogen carbonate based product and is applied as fungicide against *Oidium tuckeri* (powdery mildew of grapes) with side effects on *B. cinerea* (Biofa AG, 2021a). The additional use of the wetting agent WETCIT™ improves the efficacy and saves time by increasing the wetting and distribution of the sprayed fungicide (Biofa AG, 2021b; Fader, 2018). However, application of VitiSan® and WETCIT® according to organic standards did not prevent the detection of *B. cinerea* on grape samples. It is to be considered that organic agents mostly have a low permanent effect and only a preventive contact effect as well as being easily washed off by rainfall which complicates the success of their application (Fader, 2018). The pest control according to integrated standards with the botryticides Teldor® and SWITCH® did also not prevent the detection of *B. cinerea*. It must be mentioned that information on the management measures required for the success of the treatment, such as early defoliation of the grape zone and thinning (Staatliches Weinbauinstitut Freiburg im Breisgau, 2021), was not available to the current study.

In the 2019 screening, *P. expansum* was found in grape, soil, and must samples from Germany as samples that were positive with the LAMP assay for patulin-producing *Penicillium* species

were confirmed by microscopic identification as *P. expansum*. According to an updated Köppen-Geiger climate classification by Kottek et al. (2006), Italy and Portugal have a predominantly temperate climate with hot and dry summers and therefore do not provide the optimal growing conditions of warm and humid climate for *P. expansum* (König et al., 2009). Like Germany, also Luxembourg, the Netherlands, and France have a predominantly temperate climate with warm and humid summers (Kottek et al., 2006). But other than from Germany, few samples were examined from countries in this climatological category which may explain the negative results for *P. expansum* for those countries. The LAMP assay for the detection of *P. oxalicum* revealed the presence of this fungus in samples from all vineyards in 2019. On grapes, the fungus was most frequently detected on samples from Portugal, presumably due to optimal growth conditions under the prevailing climate of the region. Moreover, *P. oxalicum* was detected in must samples from Italy, Germany, Luxembourg, and France. The LAMP assay for the detection of *B. cinerea* revealed a highly frequent presence of this fungus in all tested vineyards. It has been detected more frequently in soil than in grape samples which might be explained by its ability to over-winter in soil as sclerotia (König et al., 2009). It is noteworthy that most of the positive results for all three LAMP assays occurred in the must samples. One reason for this may be the difference in sampling: grape and soil samples were taken selectively at certain vineyard locations. Therefore, these samples reflect only a small proportion of the given vineyard. The must samples, in contrast, represent the average of grapes of an entire vineyard and therefore provide a highly representative cross-section of the fungal population. It is therefore suggested to use must as the preferred sample material for the screening of fungal contamination in vineyards.

In the 2020 screening, both the LAMP assays for the detection of patulin-producing *Penicillium* species and of *P. expansum* revealed positive results only in the samples collected in German vineyards, similar to the results obtained in 2019. The newly developed *P. expansum* assay had slightly lower numbers of positive samples which were microbiologically confirmed as *P. expansum*. Assumingly, the LAMP assay for the detection of patulin-producing *Penicillium* species did also determine the occurrence of patulin-producers other than *P. expansum*. Among them, *P. griseofulvum* may be a possible candidate that was rarely found on grapes (Bau et al., 2005; Bragulat et al., 2008; Felšöciová et al., 2015; Serra et al., 2005; Tančinová et al., 2015). Positive samples for *P. oxalicum* were only found on grapes from Italy indicating the preference of this fungus for the Mediterranean climate. The LAMP assay for the detection of *B. cinerea* confirmed the widespread occurrence of this fungus in vineyards (see section 1.4.3.1).

During the three-year screening, the largest number of samples was obtained from Germany. Therefore, the LAMP results of German samples were compared over the observed years.

P. oxalicum was detected mostly on sample material of the 2019 harvest and not in samples from 2020. 2020 was the year with the lowest average summer temperature in comparison to the other two years (2018: 19,3 °C, 2019: 19,2 °C, 2020: 18,2 °C) which may have resulted in suboptimal growth conditions for *P. oxalicum* with a temperature optimum of 30 °C (Deutscher Wetterdienst, 2018, 2019, 2020; Mislivec and Tuite, 1970b). In contrast, 2018 was the year with the lowest precipitation (2018: 130 L/m^2, 2019: 175 L/m^2, 2020: 230 L/m^2) which could be a supporting factor for the non-occurrence of *P. expansum* in this year since it prefers humid weather conditions (König et al., 2009). *B. cinerea* was detected over all three years.

The European Union (2008) has categorized the wine growing areas in Europe according to climatic conditions in three different zones depending on which the most important vinification methods like enrichment, acidification, and de-acidification are defined. Wine growing zone A comprises, among others, Germany without Baden, the Netherlands, and Luxembourg. Wine growing zone B comprises, among others, Baden in southwestern Germany. Wine growing zone C which is divided into subcategories comprises, among others, Portugal, Italy, France, and Greece. The presence of *P. oxalicum*, *B. cinerea*, and *P. expansum* on grape, soil, and must samples was compared between samples from the 2018, 2019, and 2020 harvest from wine growing zones A and B collectively and samples from wine growing zone C. It was noticeable that the LAMP assay for the detection of patulin-producing *Penicillium* species (primarily *P. expansum*) revealed positive results only in samples from wine growing zones A and B and not from zone C. The fungus prefers humid weather conditions with temperate climate (König et al., 2009) such as in northern European wine growing regions (Kottek et al., 2006). Other studies detected *P. expansum* on samples from wine growing zone C but with differences regarding different regions within that zone: Abrunhosa et al. (2001) isolated the fungus from grapes from the Douro region in Portugal but not from grapes from the Vinho Verde region. The authors made poor weather conditions in the analyzed season and the high acidity of grapes from that region responsible for this observation. In contrast, Serra et al. (2006) identified *P. expansum* especially on grapes from the Vinho Verde region and not from the Douro region. According to the authors, Vinho Verde has a cooler and more humid climate. These conditions are well suited for infestation with *P. expansum* and indicate the strong differences in fungal occurrence due to weather conditions in several years (Serra et al., 2006). Moreover, in the study of Serra et al. (2006), *B. cinerea* dominated the mycobiota of grapes from the Vinho Verde region. It is known that *B. cinerea* is the portal of entry on grapes for secondary infections (Fillinger and Elad, 2016), e.g., by *P. expansum*, and therefore could be one reason for the identification of *P. expansum* on these grapes. In studies about the mycobiota on grapes from countries belonging to wine growing zone C, *P. expansum* did only occur in minor quantities, while *P. brevicompactum*, *P. spinulosum*, *P. citrinum*, *P. glabrum*, and *P. thomii* were the predominant *Penicillium* spp. (Diguta et al., 2011; Serra, 2003; Serra

et al., 2005; Serra et al., 2006). In contrast to *P. expansum*, *P. oxalicum* was detected in the current study mostly on samples from wine growing zone C, especially on grapes and in must. These results are in accordance with the literature that reported the fungus on samples from Spain (Bau et al., 2005), France (Bejaoui et al., 2006; Diguta et al., 2011; Sage et al., 2002), Italy (Lorenzini et al., 2016; Vogt et al., 2017a), and Portugal (Serra et al., 2005; Serra et al., 2006; Vogt et al., 2017a). Due to the climate change with higher temperatures in northern regions, the fungus may become more frequent in non-Mediterranean regions in the future. During the current study, *P. oxalicum* was particularly often found in must samples emphasizing the need of a representative sampling technique which gives an overview of whole vineyards to determine fungal occurrence rather than analyzing individual bunches or berries. The ubiquitous occurrence of *B. cinerea* as described in the literature (see section 1.4.3.1) was confirmed in the current study since it was detected both in samples from wine growing zones A and B as well as from zone C.

In the current study, the viticultural techniques applied, including application of pest control, were found to influence the mycobiota in the vineyards. The results of those vineyards, for which information about the applied viticultural techniques were given, were compared regarding the cumulated presence of the gushing-relevant fungi. On grapes from 2019 and 2020, the highest number of positively tested samples for these fungi were found when no pest control was applied, as expected, followed by samples from organic, integrated, and conventional viticulture. In organic viticulture, synthetic herbicides, insecticides, and fertilizers are completely avoided (Finn, 2013; Schmid and Schmid, 2018). Here, the focus is on measures to promote soil fertility and biodiversity with organic agents. Biodynamic viticulture as a sub-technique of organic viticulture includes the consideration of cosmic forces and follows the spiritual approach of Rudolf Steiner (Finn and Oos, 2017). Here, nature and natural cycles play a major role, while everything needed for the vineyard is not only self-produced but also recycled. Special biodynamic agents are used involving the lunar phases. Integrated viticulture seeks to integrate plant protection into the ecosystem of the vineyard (Glebe, 2002). Here, fertilization or the use of plant protection products should only take place when necessary, so that the protection of resources and the environment is considered. Greening to improve soil fertility is recommended. In conventional viticulture, chemical plant protection products, fungicides, and pesticides are used together with fertilizers (Finn and Oos, 2017). Hereby, the amount of agent applied, the spraying times as well as an alternation of the different active ingredients are prescribed. The results of the current study show that the application of pest control according to the concept of conventional viticulture revealed the lowest number of positively tested grape samples. Also, Schmid et al. (2011) found higher numbers of filamentous fungi in grape, leave, and shoot samples from organically managed plants compared to conventional ones. Döring et al. (2015) observed a significantly increased

frequency of *B. cinerea* in grape bunches after biodynamic treatment compared to integrated treatment. The authors moreover found an interaction between viticultural technique and year of sampling that might be due to the respective weather conditions. Fungal pest control agents are mostly applied on plant surfaces above the ground. Long-term application and wash-off from treated plant surfaces bring the agents into the soil (Komárek et al., 2010). Also, for soil, conventional practices led to the lowest detection of the soil-borne and gushing-relevant fungi during the current study. The effect of other viticultural techniques differed between the tested years, so that a clear correlation cannot be drawn from the available data. Likar et al. (2017) supposed that the fungal community in soil is formed by dispersed propagules and is furthermore influenced by biogeographic factors and environmental conditions. Hartmann et al. (2015) found that fertilizer application and quality are the most important factors affecting soil microbiota. As further information about the pest control agents used in the respective techniques and management measures necessary for successful treatment was not available to the current study, the systems cannot be assessed conclusively here.

The current study has shown that LAMP assays are rapid diagnostic tools that can be applied for on-site investigations in the vineyard. In contrast to common screening studies identifying filamentous fungi on grapes from European countries morphologically (Abrunhosa et al., 2001; Bellí et al., 2006; Felšöciová et al., 2021; Lorenzini et al., 2016; Ostrý et al., 2007; Serra et al., 2005), LAMP facilitates a reliable molecular identification that can be applied and operated by untrained personnel. The testing of grape, soil, and must samples requires minimal DNA preparations and equipment getting a result in short time. *B. cinerea* occurred ubiquitously in the tested vineyards and may serve as portal of entry for other fungal infections. According to Si Ammour et al. (2020), the risk of infection with *B. cinerea* depends on agronomic features such as grape variety or wounding of the berry skin, weather conditions, and the current level of the fungus' establishment in the vineyard. In comparison, the presence of *P. expansum* and *P. oxalicum* showed annual and regional variations. Rousseaux et al. (2014) also found differences in the distribution of *Penicillium* spp. on grapes depending on vineyard, vintage, variety, or environmental factors such as weather. Moreover, Bokulich et al. (2014) associated microbial biogeography of grapes with cultivar, vintage, and climate and proposed *microbial terroir* as determining factor for regional grape variations. Therefore, a general management of fungal control is difficult, but prophylactic treatments like trellising or leaf thinning can repress factors that promote the development of green mold fungi (Rousseaux et al., 2014).

All in all, the screening of the current study gave an insight into the occurrence of the gushing-relevant fungi *P. expansum*, *P. oxalicum*, and *B. cinerea* in tested vineyards across Europe. Due to the limited number of analyzed samples, the general occurrence of these fungi in European vineyards could of course not be fully assessed. However, the results show that an

infection with these fungi can generally occur and therefore the gushing risk of resulting sparkling wines may be increased. According to the results, only healthy grapes should be used to produce base wines intended for sparkling wine production. Hereby, LAMP assays can help wine growers and producers to assess infection with gushing-relevant fungi early on sample materials enabling decisions about viticultural measures and further processing of the material.

4.5 Quantification of ns-LTP1 *Vv* in sparkling wines and evaluation of its influence on the occurrence of gushing

The protein ns-LTP1 from *H. vulgare* was shown to have a gushing-reducing effect in beer (Lutterschmid et al., 2011) raising the question of a similar effect of ns-LTP1 from *V. vinifera* in sparkling wine. As the role of the protein in the gushing mechanism is not yet clear, ns-LTP1 *Vv* should be quantified and its influence on the occurrence of gushing assessed in the current study.

A competitive ELISA against ns-LTP1 from *H. vulgare* was used for the relative quantification of ns-LTP1 *Vv*. Specker (2014) developed this assay for the detection of barley ns-LTP1 and used it to determine relative amounts of the protein in samples of beer. This assay was optimized by Kupfer (2018) to detect the relative intensity of ns-LTP1 *Vv* in sparkling wines due to almost 70 % sequence similarity between the amino acid sequences of ns-LTP1 from *H. vulgare* and *V. vinifera*. In the current study, this assay was further optimized and a larger number of sparkling wine samples were analyzed compared to the study of Kupfer (2018). In contrast to the study of Kupfer (2018), the obtained results were normalized to the total protein content in order to exclude variations in protein content of samples. Protein quantification is commonly conducted by using Bradford or BCA assays. The Bradford assay is based on binding of the Coomassie Brilliant Blue G-250 dye to protein which causes an absorbance shift from 465 nm to 595 nm (Bradford, 1976). However, its response to different proteins varies due to amino acid composition (Compton and Jones, 1985). Moreover, Gazzola et al. (2015) showed that interfering substances present in wine such as ethanol or polyphenols as well as matrix composition affect the protein response in the Bradford assay. From these findings, the authors concluded that this assay is unsuitable for the protein quantification in wine. However, other authors successfully used Bradford or a modified Bradford method (Marchal et al., 1997) in their studies to quantify the protein content in wine samples (Girbau-Solà et al., 2002; Liu et al., 2018). The BCA assay uses the chemical reduction of copper which forms a complex with peptide bonds of proteins and its reaction with bicinchoninic acid which can be detected at 562 nm (Smith et al., 1985). This assay is less sensitive to interferences (Conde et al., 2017) and was applied in studies for protein quantification in wine (Conde et al., 2017; Fusi et al., 2010; Gazzola et al., 2015). In the current study, both quantification methods were applied in

parallel experiments (results not shown). Hereby, an exact determination of the protein content in samples was not necessary, but the comparability of normalized values was important. The results of the ELISA normalized to the total protein content via BCA assay were depicted as normalized relative intensity and showed no significant difference between gushing-positive and -negative sparkling wines. Since ns-LTP1 *Vv* was speculated to have a gushing-reducing effect, a higher concentration of this protein was expected in gushing-negative wines. This expectation was also not met in the analysis of red and white sparkling wines, respectively. Moreover, the relative intensities normalized via Bradford assay showed no significant difference, indicating that both quantification assays in this experimental approach could be used alternatively. In comparison, the ELISA described by Kupfer (2018) revealed the highest amount of ns-LTP1 in a gushing-negative sparkling wine and the lowest amount in a gushing-positive one, even though the authors could not find a significant difference between groups of samples.

In further experiments, the asserted cross-reaction of the antibody against ns-LTP1 from *H. vulgare* used in the ELISA with ns-LTP1 *Vv* was checked via Western blot analysis. Sparkling wines with the highest relative intensity in the ELISA revealed no or only slight protein bands in the Western blot with the Anti-nsLtp1-P2-IgG antibody. To further exclude non-specific binding of the antibody and to obtain a highly specific detection system for ns-LTP1 *Vv*, a peptide antibody against ns-LTP1 *Vv* was generated in the current study.

The newly generated Anti-ns-LTP1 *Vv* antibody was based on a peptide sequence that was calculated by Davids Biotechnologie GmbH (Regensburg, Germany) from the ns-LTP1 *Vv* protein sequence. Several ns-LTP1 *Vv* protein sequences with slight amino acid differences were available on NCBI and the sequence with GenBank accession number ABA29446.1 was chosen which was also used by Kupfer (2018) for the generation of the peptide antibody Anti-nsLtp1-P2-IgG. According to the calculation by Davids Biotechnologie GmbH, the chosen peptide in the current study was predicted to have good antigenicity and good epitope prediction. In contrast to the Anti-nsLtp1-P2-IgG antibody, the newly generated Anti-ns-LTP1 *Vv* antibody detected ns-LTP1 *Vv* in sparkling wines specifically together with a dimer but also in mashed grapes. These results revealed improper binding of the Anti-nsLtp1-P2-IgG antibody to ns-LTP1 *Vv* and thus cast doubt on the ELISA results obtained in the current study as it was unclear what the antibody detected, and demonstrated the need of a specific ELISA for ns-LTP1 *Vv*. A cross-reaction of the new antibody with *Pichia pastoris* culture supernatant overexpressing recombinant ns-LTP1 from *H. vulgare* was found so that barley ns-LTP1 could still be used as positive control in further analyses. Protein sequencing by Edman degradation confirmed the identity of the protein in the Western blot analysis with the Anti-ns-LTP1 *Vv* antibody as ns-LTP1 *Vv* (GenBank accession number RVW40993.1) with a sequence that

differed slightly from the sequence that was used for the Anti-ns-LTP1 *Vv* antibody generation (ABA29446.1). Different isoforms could be the reason for the existence of several slightly different sequences of the protein (Coutos-Thevenot et al., 1993; Gomès et al., 2003; Jégou et al., 2000). The peptide used for antibody production based on the ABA29446.1 sequence differed in one amino acid from the respective sequence of RVW40993.1. A newly generated peptide with the changed amino acid was detected by the antibody just as well as the original peptide. Therefore, the generated antibody was considered suitable for the development of a new ELISA for ns-LTP1 *Vv*. A clear correlation between applied concentrations of the positive control with extinction values was obtained in an optimized ELISA so that the assay could be used to analyze samples.

Similar to the previously used ELISA with the antibody against barley ns-LTP1, analysis of gushing-positive and gushing-negative sparkling wines with the new developed ns-LTP1 *Vv* ELISA revealed no significant difference between the normalized relative intensities of the two groups. Also, when samples of red and white sparkling wines were analyzed, respectively, no significant differences were found between gushing-positive and gushing-negative samples. In white sparkling wines, the normalized relative intensity was higher than in red ones. According to Wigand et al. (2009), LTPs are mainly to be found in the skin of grapes and due to the fermentation of red wines on the mash, a higher concentration in Dornfelder and Portugieser red wines than in rosé and white wines has been observed. The authors explained this result by the fermentation of rosé and white wine on the must so that proteins from the grape skin are present to a much lower extent due to exclusion by the vinification process. However, the same authors did not find the proteins in tested red wines that were not Dornfelder or Portugieser and thus suggested an influence of variety, conditions during grape growth, and vinification as well as the use of fining agents on the presence of LTPs. Moreover, Wigand et al. (2009) emphasized the importance of the time of skin contact during vinification. In the current study, no information about grape varieties used for the tested sparkling wines were available as well as about vinification processes including mash fermentation, coagulation, or fining. Therefore, it is difficult to transfer the results of Wigand et al. (2009) to the current study. Moreover, there are differences in the production of red sparkling wine compared to red wine as e.g., grapes for sparkling wine production are harvested in an earlier ripening stage than grapes for general winemaking which might have an influence and make a comparison difficult.

The results of the current study showed no correlation between the ns-LTP1 *Vv* content and the occurrence of gushing. Lutterschmid (2011) suggested that beer with low levels of barley ns-LTP1 is more susceptible to gushing than beer with normal levels. As in the current study no differences in the ns-LTP1 *Vv* intensity between gushing-positive and -negative sparkling wines were observed, an influence of the protein on gushing occurrence cannot be declared.

In beer gushing, it was found that a modified glycated and unfolded form of ns-LTP1 from *H. vulgare* that is developed during heat treatments in malting and brewing is responsible for foam formation due to improved surface-active properties of the modified protein (Jégou et al., 2000; Perrocheau et al., 2006; Sørensen et al., 1993). In contrast to the production of beer, there is no boiling step in sparkling wine production. Even heating to high temperatures as in mash heating of red grapes is uncommon. It is therefore assumed that no glycation of the ns-LTP1 *Vv* protein will occur by Maillard reactions. Hence, it can be speculated that ns-LTP1 *Vv* is present in sparkling wines in a form that has no influence on foam formation due to minor surface-active properties. Therefore, a gushing-reducing effect of this protein in sparkling wine seems to be unlikely. The experimental data generated during the current study support this assumption. Experiments with mixtures of purified ns-LTP1 *Vv* and gushing-inducing proteins added to sparkling wines could confirm this hypothesis conclusively in future studies.

4.6 Conclusion

In the current study, the direct gushing-inducing effect of recombinant surface-active proteins from *P. expansum* and *P. oxalicum* in sparkling wine was demonstrated. Assays for detection and relative quantification of these proteins in base wines and of the producing fungi on sample materials from vineyards were developed and applied for monitoring these gushing-relevant factors. These assays provide new opportunities in analyzing the phenomenon of gushing in sparkling wines. Analyses via Western blot, dot blot, ELISA, or LAMP facilitate the early detection of relevant markers and can help winemakers and sparkling wine producers to assess the gushing risk associated with their raw materials and products. Moreover, ns-LTP1 *Vv* was shown to be no useful marker for the determination of the gushing potential of sparkling wine. Nonetheless, it must never be forgotten that gushing is a multifactorial phenomenon that is influenced by many different factors. Hence, not only the presence or absence of gushing-inducing and gushing-reducing agents are decisive. The interaction between compounds and the influence of environmental and technological factors must be also considered. Therefore, the current study provides valuable tools that are necessary for further in-depth studies of a phenomenon that is as intriguing for the scientist as it is challenging for the practitioner.

5 Summary

The phenomenon of gushing is described as the spontaneous excessive over-foaming of carbonated beverages that leads to considerable economic losses and reputational damages in the beverage industry. Its causes have been extensively investigated in beer, but recent studies are also dealing with sparkling wine.

Previous studies have shown an involvement of fungal proteins in gushing induction of sparkling wine, but their exact contribution has not been clarified yet. To obtain high quantities of these proteins for experiments, the proteins PEX2_044840 from *Penicillium expansum* and PDE_07106 and PDE_04519 from *P. oxalicum* were successfully heterologously expressed in *Pichia pastoris* and purified. Addition of purified proteins to sparkling wines induced gushing when at least 15 µg (PDE_07106) or 30 µg (PEX2_044840) protein, respectively, were added indicating a direct gushing-inducing effect of these proteins.

For the detection and quantification of gushing-inducing proteins, immunochemical detection assays were developed. Peptide antibodies against PEX2_044840 and PDE_07106 were generated by a third-party and were found to detect their target with high specificity. An ELISA was developed and applied to compare wine samples relatively to each other in regard to their PEX2_044840 protein content. Analysis of sparkling wines revealed a significantly higher concentration of PEX2_044840 in gushing-positive than in gushing-negative sparkling wines indicating an involvement of the protein in gushing induction and its suitability as analytical marker. Both *P. oxalicum* proteins were detected by Western blot and dot blot analyses enabling a qualitative and semi-quantitative determination of their concentrations in base wines.

LAMP assays were shown to be highly suitable as rapid and easy-to-handle detection tool for gushing-relevant filamentous fungi present on sample materials from vineyards. A LAMP assay for the detection of *P. expansum* was developed and revealed high specificity and sensitivity with a detection limit of 25 pg genomic DNA of *P. expansum* per reaction. This assay as well as LAMP assays for the detection of *P. oxalicum*, patulin-producing *Penicillium* species, and *Botrytis cinerea* were optimized and applied for the analysis of sample materials. A screening of grape, soil, and must samples from the harvests 2018, 2019, and 2020 from European vineyards revealed a ubiquitous occurrence of *B. cinerea* and annual and regional variations of *P. expansum* and *P. oxalicum*: *P. expansum* was mostly detected on samples from wine growing zones A and B and *P. oxalicum* on samples from zone C.

The protein ns-LTP1 *Vv* was previously suggested to have a gushing-reducing effect in sparkling wine. To enable the specific detection of the protein, an antibody against ns-LTP1 *Vv* was generated by a third-party and was found to detect its target with high specificity. With this

antibody, an ELISA was developed for the relative quantification of ns-LTP1 *Vv* in wine samples. However, no correlation between the ns-LTP1 *Vv* content in sparkling wine and gushing was found suggesting that the protein has no influence on the gushing mechanism and is therefore no useful marker for the determination of gushing occurrence or absence in sparkling wines.

The current study provides new insights into the phenomenon of gushing of sparkling wine as well as analytical means for its analysis. Based on the results, purified recombinant fungal proteins can be further investigated in sample materials and the occurrence of the respective producing fungi can be monitored. This enables an early detection of gushing-relevant factors and can help winemakers and sparkling wine producers to assess the possible risk of gushing in sparkling wine.

6 Zusammenfassung

Das Phänomen „Gushing“ wird als spontanes, extremes Überschäumen bei kohlensäurehaltigen Getränken beschrieben, das der Getränkeindustrie erhebliche wirtschaftliche Verluste und Imageschäden zufügt. Seine Ursachen wurden ausführlich bei Bier untersucht, aber neuere Studien befassen sich auch mit Sekt.

Frühere Studien haben gezeigt, dass pilzliche Proteine bei der Gushing-Induktion in Sekt involviert sind, jedoch ist ihr genauer Beitrag noch nicht geklärt. Um große Mengen dieser Proteine für Experimente zu erhalten, wurden die Proteine PEX2_044840 von *Penicillium expansum* sowie PDE_07106 und PDE_04519 von *P. oxalicum* erfolgreich heterolog in *Pichia pastoris* exprimiert und aufgereinigt. Die Zugabe der gereinigten Proteine zu Sekt löste Gushing aus, wenn mindestens 15 µg (PDE_07106) beziehungsweise 30 µg (PEX2_044840) Protein zugegeben wurden, was eine direkte Gushing-induzierende Wirkung dieser Proteine zeigt.

Für den Nachweis und die Quantifizierung der Gushing-induzierenden Proteine wurden immunchemische Nachweissysteme entwickelt. Peptidantikörper gegen PEX2_044840 und PDE_07106 wurden als Leistung Dritter hergestellt und detektierten die Zielproteine mit hoher Spezifität. Ein ELISA wurde entwickelt und angewandt, um Weinproben hinsichtlich ihres PEX2_044840-Proteingehalts relativ miteinander zu vergleichen. Die Analyse von Sekt ergab eine signifikant höhere Konzentration von PEX2_044840 in Gushing-positiven als in Gushing-negativen Sekten, was zeigt, dass das Protein an der Gushing-Induktion beteiligt ist und sich als analytischer Marker eignet. Beide Proteine von *P. oxalicum* wurden mittels Western Blot und Dot Blot nachgewiesen, was eine qualitative und semi-quantitative Bestimmung ihrer Konzentrationen in Grundweinen ermöglichte.

Es hat sich gezeigt, dass LAMP-Assays als schnelle und einfach zu handhabende Nachweismethode für Gushing-relevante filamentöse Pilze auf Probenmaterialien aus Weinbergen sehr gut geeignet sind. Es wurde ein LAMP-Assay für den Nachweis von *P. expansum* entwickelt, der eine hohe Spezifität und Sensitivität mit einer Nachweisgrenze von 25 pg genomischer DNA von *P. expansum* pro Reaktion aufwies. Dieser Assay sowie LAMP-Assays für den Nachweis von *P. oxalicum*, Patulin-produzierenden *Penicillium* Spezies und *Botrytis cinerea* wurden optimiert und zur Analyse von Probenmaterialien eingesetzt. Ein Screening von Trauben-, Boden- und Mostproben aus der Lese von 2018, 2019 und 2020 von europäischen Weinbergen ergab ein ubiquitäres Vorkommen von *B. cinerea* sowie jahresbedingte und regionale Unterschiede bei *P. expansum* und *P. oxalicum*: *P. expansum* wurde vor allem in Proben aus den Weinbauzonen A und B und *P. oxalicum* in Proben aus Zone C nachgewiesen.

Bislang wurde vermutet, dass das Protein ns-LTP1 *Vv* einen Gushing-reduzierenden Effekt in Sekt hat. Um einen spezifischen Nachweis des Proteins zu ermöglichen, wurde als Leistung Dritter ein Antikörper gegen ns-LTP1 *Vv* hergestellt, der das Zielprotein mit hoher Spezifität nachwies. Mit diesem Antikörper wurde ein ELISA für die relative Quantifizierung von ns-LTP1 *Vv* in Weinproben entwickelt. Es wurde jedoch keine Korrelation zwischen dem Gehalt an ns-LTP1 *Vv* in Sekt und Gushing festgestellt, was darauf hindeutet, dass dieses Protein keinen Einfluss auf den Gushingmechanismus hat und daher kein geeigneter Marker für die Bestimmung des Auftretens oder Fehlens von Gushing in Sekt ist.

Die aktuelle Studie liefert neue Einblicke in das Phänomen des Gushings von Sekt sowie analytische Mittel zu dessen Untersuchung. Basierend auf den Ergebnissen können aufgereinigte rekombinante Pilzproteine in Probenmaterialien weiter untersucht und das Vorkommen der jeweiligen produzierenden Pilze kontrolliert werden. Dies ermöglicht eine frühzeitige Erkennung von Gushing-relevanten Faktoren und kann Winzern und Sektproduzenten helfen, das mögliche Risiko von Gushing in Sekt einzuschätzen.

7 List of Abbreviations

%	Percentage
Ø	Diameter
(GC)	Content defined by gas chromatography
®	Registered trademark
°C	Degree Celsius
°Oe	Degree Oechsle
µ	Micro
A	Ampere
ACS	American Chemical Society
AfpA	Alkaline foam protein A
AG	*Aktiengesellschaft*
AOX1	Alcohol oxidase 1
AP	Alkaline phosphatase
APS	Ammonium persulfate
AT	Ambient temperature
AU	Absorbance unit
a_w	Water activity
B.	*Botrytis*
B.V.	*Besloten vennootschap*
B3	Backward Outer Primer
BayBioMS	Bavarian Center for Biomolecular Mass Spectrometry
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIP	Backward Inner Primer
BLAST	Basic local alignment search tool
BMG	Buffered Minimal Glycerol Medium
BMGY	Buffered Complex Glycerol Medium
BMM	Buffered Minimal Methanol Medium
BMMY	Buffered Complex Methanol Medium
bp	Base pair
BSA	Bovine serum albumin
c	Centi
CA	California
CBS	Centraalbureau voor Schimmelcultures
Cl	Chlorine
Co.	Corporation
Co. KG	*Compagnie Kommanditgesellschaft*
CO_2	Carbon dioxide
CV	Column volumes
d	Day
Da	Dalton
DFG	*Deutsche Forschungsgemeinschaft*
$_dH_2O$	Sterile deionized water
DMF	N,N-Dimethylformamide

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
€	Euro
E.	*Escherichia*
e.g.	*Exempli gratia*
e.V.	*Eingetragener Verein*
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
et al.	*Et alii*
f	Femto or forward
F	Farad
F.	*Fusarium*
F3	Forward Outer Primer
FIP	Forward Inner Primer
FPLC	Fast protein liquid chromatography
g	Gram or gravitational constant
gDNA	Genomic DNA
GFP	Green fluorescent protein
GmbH	*Gesellschaft mit beschränkter Haftung*
h	Hour
H.	*Hordeum*
HCl	Hydrochloric acid
HGU	Hochschule Geisenheim University
His	Histidine
IgG/Y	Immunoglobulin G/Y
IGS	Intergenic spacer
IMAC	Immobilized metal affinity chromatography
Inc.	Incorporation
ISO	International Standard Organisation
ITS	Internal transcribed spacer
k	Kilo
KgaA	*Kommanditgesellschaft auf Aktien*
KNAW	Royal Netherlands Academy of Arts and Sciences
L	Liter
LAMP	Loop-mediated isothermal amplification
LB	Loop primer backward or lysogeny broth
LC–MS/MS	Liquid chromatography-tandem mass spectrometry
LF	Loop primer forward
LiAc	Lithium acetate
LLC	Limited liability company
Ltd.	Limited
m	Milli or meter
M	Molarity
MA	Massachusetts

max.	Maximal
MC	Multi-copy
ME	Malt extract
$MgCl_2$	Magnesium chloride
min	Minute
MO	Missouri
MOPS	3-(N-morpholino)-propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
Mut	Methanol utilization
MWCO	Molecular weight cut-off
N	Nitrogen
n	Nano
N.V.	*Naamloze vennootschap*
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information
NFC	Not from concentrate
Ni-NTA	Nickel-nitrilotriacetic acid
NJ	New Jersey
no.	Number
ns-LTP	Non-specific lipid transfer protein
NTC	No template control
NY	New York
OD	Optical density
p	Pico
P.	*Penicillium*
p. a.	Pro analysis
PA	Pennsylvania
Pau5p	Protein seripauperin 5
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween
PCR	Polymerase chain reaction
pH	Potential of hydrogen
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
pNPP	para-Nitrophenylphosphate
Pol	Polymerase
PR	Pathogenesis-related
Puriss.	*Purissimum*
PVDF	Polyvinylidene fluoride
r	Reverse
R^2	Coefficient of determination
Reag. Ph. Eur.	Reagent *Pharmacopoea Europaea*
rpm	Revolutions per minute
rxn	Reaction

S.	*Saccharomyces*
S.A.	*Société anonyme*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
Seq	Sequencing
spec. nov.	*Nova species*
spp.	*Species pluralis*
T_a	Annealing temperature
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
™	Trademark
T_m	Melting temperature
TMW	*Technische Mikrobiologie Weihenstephan*
Tris	Tris-(hydroxymethyl)-aminomethane
TUM	Technical University of Munich
U	Enzyme unit
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volt
V.	*Vitis*
v/v	Volume per volume
var.	*Varietas*
Vv	*Vitis vinifera*
w/o	Without
w/v	Weight per volume
WI	Wisconsin
WT	Wild type
YCB	Yeast carbon base
YI	Yeast invertase
YPD	Yeast extract-Peptone-Dextrose
YPDS	Yeast extract-Peptone-Dextrose-Sorbitol
ZfP	Protein Analysis Unit

8 List of Figures

Figure 1: Gushing-positive sparkling wine 2
Figure 2: *P. expansum*, *P. oxalicum*, and *B. cinerea* 9
Figure 3: LAMP primer binding sites 15
Figure 4: LAMP mechanism 16
Figure 5: Competitive ELISA scheme 18
Figure 6: Map of pPICZα vector 19
Figure 7: Alignment of nucleotide sequences of native and modified *PEX2_044840* gene 67
Figure 8: Digested vector and inserts 68
Figure 9: Vector map of pPICZαA_PEX2_044840 69
Figure 10: *E. coli* transformants 70
Figure 11: Linearization of plasmids 71
Figure 12: *Pichia pastoris* transformants 71
Figure 13: *Pichia pastoris* transformants screening for Mut phenotype 72
Figure 14: Screening for multi-copy *Pichia pastoris* transformants 73
Figure 15: Expression experiments with *Pichia pastoris*_PEX2_044840 transformants 74
Figure 16: Expression experiments with *Pichia pastoris*_PDE_07106 transformants 75
Figure 17: Expression experiments with *Pichia pastoris*_PDE_04519 transformants 76
Figure 18: Foams of expression cultures 77
Figure 19: Protein analyses of *Pichia pastoris*_PEX2_044840 transformant and wild type 78
Figure 20: Protein analyses of *Pichia pastoris*_PDE_07106 transformant and wild type 79
Figure 21: Protein analyses of *Pichia pastoris*_PDE_04519 transformant and wild type 80
Figure 22: Gushing tests in sparkling wine with recombinant proteins 82
Figure 23: Testing of antibodies by Western blot analysis 84
Figure 24: ELISA standard curve by coating with peptide 86
Figure 25: ELISA standard curve by coating with PEX2_044840 protein 87
Figure 26: Dot blot analysis of PEX2_044840 protein untreated and denatured 88
Figure 27: ELISA standard curve with denatured protein for solid phase coating and as standard 89
Figure 28: ELISA reproducibility 90
Figure 29: ELISA recovery 91
Figure 30: Dot blot with purified PEX2_044840 protein in base wine 92
Figure 31: ELISA results by testing gushing-negative and gushing-positive sparkling wines 93
Figure 32: Dot blot with purified PDE_07106 protein in base wine 94
Figure 33: Position and orientation of LAMP primers for detection of *P. expansum* 96

Figure 34: Analysis of *P. expansum* LAMP amplification products by agarose gel electrophoresis 97
Figure 35: Sequence alignment of *P. expansum* LAMP product after PCR 98
Figure 36: Determination of *P. expansum* LAMP assay characteristics 98
Figure 37: Optimization of LAMP assays for the detection of *P. oxalicum* and *B. cinerea* 105
Figure 38: LAMP results of screening 2018 111
Figure 39: LAMP results of screening 2019 112
Figure 40: LAMP results of screening 2020 113
Figure 41: Comparison of LAMP results of screenings 2018, 2019, and 2020 in German vineyard samples 114
Figure 42: Comparison of detected fungi in different wine growing zones 115
Figure 43: Comparison of fungal infestation on grapes and soil with different viticultural techniques and pest control applications 116
Figure 44: Alignment of the amino acid sequence of ns-LTP1 proteins from *H. vulgare* and *V. vinifera* 117
Figure 45: ELISA results for testing sparkling wines with antibody against ns-LTP1 from *H. vulgare* 118
Figure 46: Comparison of Anti-ns-LTP1 antibodies 120
Figure 47: Alignment of two amino acid sequences of ns-LTP1 *Vv* 121
Figure 48: ELISA with Anti-ns-LTP1 *Vv* antibody 122
Figure 49: ELISA results for testing sparkling wines with antibody against ns-LTP1 *Vv* 123
Figure 50: Nucleotide sequence of PEX2_044840 from *P. expansum* 185
Figure 51: Codon-optimized nucleotide sequence of PEX2_044840 from *P. expansum* 186
Figure 52: Amino acid sequence of PEX2_044840 from *P. expansum* 186
Figure 53: Amino acid sequence of modified PEX2_044840 187
Figure 54: Nucleotide sequence of PDE_07106 from *P. oxalicum* 187
Figure 55: Amino acid sequence of PDE_07106 from *P. oxalicum* 188
Figure 56: Amino acid sequence of modified PDE_07106 188
Figure 57: Nucleotide sequence of PDE_04519 from *P. oxalicum* 189
Figure 58: Amino acid sequence of PDE_04519 from *P. oxalicum* 189
Figure 59: Amino acid sequence of modified PDE_04519 190
Figure 60: Amino acid sequence of ns-LTP1 from *V. vinifera* 190
Figure 61: Amino acid sequence of ns-LTP1 from *H. vulgare* 190
Figure 62: Vector maps 193

9 List of Tables

Table 1: Equipment 22
Table 2: Consumables 25
Table 3: Chemicals 27
Table 4: Media 31
Table 5: Tripotassium phosphate buffer for BMG, BMM, BMGY, and BMMY medium 33
Table 6: Breaking buffer for preparation of cell lysates 33
Table 7: Buffers for SDS-PAGE 33
Table 8: Solutions for silver staining 34
Table 9: Solutions for Coomassie staining 34
Table 10: Buffers for Western blot and dot blot analysis 35
Table 11: Buffers for ELISA 36
Table 12: Buffer for agarose gel electrophoresis 36
Table 13: Buffer for gDNA isolation of filamentous fungi 36
Table 14: Solutions for cloning procedures 37
Table 15: Buffer for LAMP 37
Table 16: Buffers for protein purification 37
Table 17: Buffers for protein purification by FPLC 38
Table 18: LAMP primers for the detection of *P. oxalicum* 38
Table 19: LAMP primers for the detection of *P. expansum* 39
Table 20: LAMP primers for the detection of *B. cinerea* 39
Table 21: LAMP primers for the detection of patulin-producing *Penicillium* species 40
Table 22: Primers for identification and sequencing of *Penicillium* species 40
Table 23: Primers for cloning procedures with PDE_04519 and PDE_07106 from *P. oxalicum* and PEX2_044840 from *P. expansum* 40
Table 24: Antibodies 41
Table 25: Kits 42
Table 26: Enzymes 42
Table 27: Plasmids 43
Table 28: Software and databases 43
Table 29: Sparkling wines and base wines 44
Table 30: Musts 46
Table 31: Organisms 46
Table 32: Silver staining of SDS-PAGE gels 51
Table 33: Coomassie staining of SDS-PAGE gels 51
Table 34: PCR reagents for amplification of *Penicillium* DNA 57
Table 35: PCR program for amplification of *Penicillium* DNA 58

Table 36: PCR program for amplification of LAMP product....58
Table 37: PCR reagents for amplification of gene of interest and sequencing of *E. coli* and *Pichia pastoris* transformants....59
Table 38: Two-step PCR program for amplification of gene of interest....59
Table 39: PCR program for sequencing of *E. coli* transformants....59
Table 40: PCR program for sequencing of *Pichia pastoris* transformants....60
Table 41: PCR reagents for colony PCR, multi-copy PCR, and determination of Mut phenotype....61
Table 42: PCR program for colony PCR....61
Table 43: PCR program for multi-copy PCR and determination of Mut phenotype....61
Table 44: LAMP master mix....65
Table 45: LAMP primer mix....65
Table 46: Characteristics of the peptides used for antibody production....83
Table 47: Determination of *P. expansum* LAMP assay specificity....99
Table 48: Determination of *B. cinerea* LAMP assay specificity....106
Table 49: Characteristics of the peptide used for Anti-ns-LTP1 *Vv* antibody production....120
Table 50: LAMP results of the screening 2018....194
Table 51: LAMP results of the screening 2019....197
Table 52: LAMP results of the screening 2020....207

10 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.

Abrunhosa, L., Paterson, R.R.M., Kozakiewicz, Z., Lima, N., Venancio, A., 2001. Mycotoxin production from fungi isolated from grapes. *Letters in Applied Microbiology* 32(4), 240–242.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215(3), 403–410.

Amaha, M., Kitabatake, K., Nakagava, A., Yoshida, J., Harada, T., 1973. Gushing inducers produced by some moulds strains, Proceedings of the 14th European Brewery Convention Congress, Salzburg, Elsevier: Amsterdam, pp. 381–398.

Aziz, N.H., Mattar, Z.A., Mahrous, S.R., 2006. Contamination of grains by mycotoxin-producing molds and mycotoxins and control by gamma irradiation. *Journal of Food Safety* 26(3), 184–201.

Bach, H.P., 2001. Das Wildwerden oder Überschäumen von Sekt. Teil 1: Grundlagen und Ursachen. *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. Teil 2: Messmethode und Möglichkeiten der Vermeidung. *Der Deutsche Weinbau* 16-17, 36–41.

Barrero, J.J., Casler, J.C., Valero, F., Ferrer, P., Glick, B.S., 2018. An improved secretion signal enhances the secretion of model proteins from *Pichia pastoris*. *Microbial Cell Factories* 17(1).

Bau, M., Bragulat, M.R., Abarca, M.L., Minguez, S., Cabañes, F.J., 2005. Ochratoxigenic species from Spanish wine grapes. *International Journal of Food Microbiology* 98(2), 125–130.

Bayly, F.C., Berg, H., 1967. Grape and wine proteins of white wine varietals. *American Journal of Enology and Viticulture* 18(1), 18-32.

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

Becker, T., Walter, R., Kortekamp, A., Grimm, E., Knoche, M., 2011. Das Platzen von Weinbeeren (*Vitis vinifera*) bei Befall mit Grauschimmel (*Botrytis cinerea*). *Erwerbs-Obstbau* 53(3), 85–92.

Bejaoui, H., Mathieu, F., Taillandier, P., Lebrihi, A., 2006. Fungal flora and ochratoxin A production during wine grape maturation in French vineyards during three years. *International Journal of Food Microbiology* 111, 46-52.

Bellí, N., Bau, M., Marín, S., Abarca, M.L., Ramos, A.J., Bragulat, M.R., 2006. Mycobiota and ochratoxin A producing fungi from Spanish wine grapes. *International Journal of Food Microbiology* 111 S40–S45.

Biofa AG, 2021a. VitiSan®, Product sheet, Münsingen.

Biofa AG, 2021b. WETCIT®, Product sheet, Münsingen.

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.

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.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences of the United States of America* 111(1), E139-E148.

Bornhorst, J.A., Falke, J.J., 2000. Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology* 326, 245–254.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.

Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2008. Low occurrence of patulin- and citrinin-producing species isolated from grapes. *Letters in Applied Microbiology* 47(4), 286–289.

Brissonnet, F., Maujean, A., 1993. Characterization of foaming proteins in a champagne base wine. *American Journal of Enology and Viticulture* 44(3), 297–301.

Bundesverband der Deutschen Spirituosen-Industrie und -Importeure e. V., 2021. Daten aus der Alkoholwirtschaft 2021. Angelika Wiesgen-Pick, Bonn.

Cenis, J.L., 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* 20(9), 2380.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology* 13(4), 329–332.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* 24, 45–66.

Certis Europe B.V., 2021. Kumar®, User manual, Utrecht.

Chang, S.-W., Lee, G.-C., Shaw, J.-F., 2006. Codon optimization of *Candida rugosa* lip1 gene for improving expression in *Pichia pastoris* and biochemical characterization of the purified recombinant LIP1 lipase. *Journal of Agricultural and Food Chemistry* 54(3), 815–822.

Ciegler, A., Hayes, A.W., Vesonder, R.F., 1980. Production and biological activity of secalonic acid D. *Applied and Environmental Microbiology* 39(2), 285–287.

Ciegler, A., Vesonder, R.F., Jackson, L.K., 1977. Production and biological activity of patulin and citrinin from *Penicillium expansum*. *Applied and Environmental Microbiology* 33(4), 1004–1006.

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.

Cilindre, C., Fasoli, E., D'Amato, A., Liger-Belair, G., Righetti, P.G., 2014. It's time to pop a cork on champagne's proteome! *Journal of Proteomics* 105, 351-362.

Cilindre, C., Jégou, S., Hovasse, A., Schaeffer, C., Castro, A.J., Clément, C., van Dorsselaer, A., Jeandet, P., Marchal, R., 2008. Proteomic approach to identify champagne wine proteins as modified by *Botrytis cinerea* infection. *Journal of Proteome Research* 7(3), 1199–1208.

Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K., Henwood, C.A., 1991. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene* 105, 205-212.

Compton, S.J., Jones, C.G., 1985. Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry* 151, 369–374.

Conde, B.C., Bouchard, E., Culbert, J.A., Wilkinson, K.L., Fuentes, S., Howell, K.S., 2017. Soluble protein and amino acid content affects the foam quality of sparkling wine. *Journal of Agricultural and Food Chemistry* 65(41), 9110-9119.

Coutos-Thevenot, P., Jouenne, T., Maes, O., Guerbette, F., Grosbois, M., Caer, J.P., Boulay, M., Deloire, A., Kader, J.C., Guern, J., 1993. Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid-transfer proteins. *European Journal of Biochemistry* 217(3), 885-889.

Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* 26(12), 1367–1372.

Cregg, J.M., Barringer, K.J., Hessler, A.Y., Madden, K.R., 1985. *Pichia pastoris* as a host system for transformations. *Molecular and Cellular Biology* 5(12), 3376–3385.

Cregg, J.M., Cereghino, J.L., Shi, J., Higgins, D.R., 2000. Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology* 16, 23–52.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P., Stillman, C.A., 1989. Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Molecular and Cellular Biology* 9(3), 1316–1323.

Currie, J.N., Thom, C., 1915. An oxalic acid producing *Penicillium*. *Journal of Biological Chemistry* 22, 287-293.

Dachs, E., Nitschke, R., 1977. Fallstudie zum Problem Gushing. *Brauwelt* 117, 129–131.

Dambrouck, T., Marchal, R., Cilindre, C., Parmentier, M., Jeandet, P., 2005. Determination of the grape invertase content (using PTA-ELISA) following various fining treatments versus changes in the total protein content of wine. Relationships with wine foamability. *Journal of Agricultural and Food Chemistry* 53(22), 8782–8789.

Dambrouck, T., Marchal, R., Marchal-Delahaut, L., Parmentier, M., Maujean, A., Jeandet, P., 2003. Immunodetection of proteins from grapes and yeast in a white wine. *Journal of Agricultural and Food Chemistry* 51(9), 2727–2732.

De Schutter, K., Callewaert, N., 2012. *Pichia* surface display: A tool for screening single domain antibodies. *Methods in Molecular Biology* 911, 125-134.

Deckers, S.M., Gebruers, K., Baggerman, G., Lorgouilloux, Y., Delcour, J.A., Michiels, C., Derdelinckx, G., Martens, J., Neven, H., 2010. CO_2-hydrophobin structures acting as nanobombs in beer. *BrewingScience* 63, 54-61.

Deckers, S.M., Venken, T., Khalesi, M., Gebruers, K., Baggerman, G., Lorgouilloux, Y., Shokribousjein, Z., Ilberg, V., Schoenberger, C., Titze, J., Verachtert, H., Michiels, C., Neven, H., Delcour, J., Martens, J., Derdelinckx, G., De Maeyer, M., 2012. Combined modeling and biophysical characterisation of CO2 interaction with class II hydrophobins: New insight into the mechanism underpinning primary gushing. *Journal of the American Society of Brewing Chemists* 70(4), 249–256.

Denschlag, C., Vogel, R.F., Niessen, L., 2012. Hyd5 gene-based detection of the major gushing-inducing *Fusarium* spp. in a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology* 156(3), 189–196.

Denschlag, C., Vogel, R.F., Niessen, L., 2013. Hyd5 gene based analysis of cereals and malt for gushing-inducing *Fusarium* spp. by real-time LAMP using fluorescence and turbidity measurements. *International Journal of Food Microbiology* 162(3), 245–251.

Deutscher Wetterdienst, 2018. Deutschlandwetter im Sommer 2018, Offenbach. https://www.dwd.de/DE/presse/pressemitteilungen/DE/2018/20180830_deutschlandwetter_sommer_news.html. Retrieved 18.09.2021.

Deutscher Wetterdienst, 2019. Deutschlandwetter im Sommer 2019, Offenbach. https://www.dwd.de/DE/presse/pressemitteilungen/DE/2019/20190830_deutschlandwetter_sommer2019_news.html. Retrieved 18.09.2021.

Deutscher Wetterdienst, 2020. Deutschlandwetter im Sommer 2020, Offenbach. https://www.dwd.de/DE/presse/pressemitteilungen/DE/2020/20200831_deutschlandwetter_sommer2020_news.html. Retrieved 18.09.2021.

Diguta, C.F., Vincent, B., Guilloux-Benatier, M., Alexandre, H., Rousseaux, S., 2011. PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. *Food Microbiology* 28(6), 1145–1154.

Dizy, M., Bisson, L.F., 1999. White wine protein analysis by capillary zone electrophoresis. *American Journal of Enology and Viticulture* 50(1), 120-127.

Döring, J., Frisch, M., Tittmann, S., Stoll, M., Kauer, R., 2015. Growth, yield and fruit quality of grapevines under organic and biodynamic management. *PloS One* 10(10).

Draeger, M., 1996. Physical observations on the subject of gushing. *Brauwelt International* 4, 363–367.

Drusch, S., Ragab, W., 2003. Mycotoxins in fruits, fruit juices, and dried fruits. *Journal of Food Protection* 66(8), 1514-1527.

Duan, Y.-B., Ge, C.-Y., Zhang, X.-K., Wang, J.-X., Zhou, M.-G., 2014. Development and evaluation of a novel and rapid detection assay for *Botrytis cinerea* based on loop-mediated isothermal amplification. *PloS One* 9(10), e111094.

Duan, Y.B., Yang, Y., Li, M.X., Li, T., Fraaije, B.A., Zhou, M.G., 2018a. Development and application of a simple, rapid and sensitive method for detecting moderately carbendazim-resistant isolates in *Botrytis cinerea*. *Annals of Applied Biology* 172(3), 355–365.

Duan, Y.B., Yang, Y., Wang, J.X., Chen, C.J., Steinberg, G., Fraaije, B.A., Zhou, M.G., 2018b. Simultaneous detection of multiple benzimidazole-resistant β-tubulin variants of *Botrytis cinerea* using loop-mediated isothermal amplification. *Plant Disease* 102(10), 2016–2024.

Ehrlich, K.C., Lee, L.S., Ciegler, A., Palmgren, M.S., 1982. Secalonic acid D: Natural contaminant of corn dust. *Applied and Environmental Microbiology* 44(4), 1007–1008.

European Commission, 2006. Commission regulation no 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in food stuff. *Official Journal of the European Union* L 364/5, 5-24.

European Union, 2008. Council regulation (EC) No 479/2008 of 29 April 2008 on the common organisation of the market in wine. *Official Journal of the European Union* L 148/2.

Evans, D.E., Hejgaard, J., 1999. The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *Journal of the Institute of Brewing* 105, 159-169.

Evans, D.E., Sheehan, M.C., Stewart, D.C., 1999. The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. *Journal of the Institute of Brewing* 105(2), 171-177.

Fader, B., 2018. Rebschutz im ökologischen Weinbau- Mit alternativen Mitteln zum Ziel, Dienstleistungszentrum Ländlicher Raum Rheinhessen-Nahe-Hunsrück Oppenheim.

Fan, F., Hahn, M., Li, G.-Q., Lin, Y., Luo, C.-X., 2019. Rapid detection of benzimidazole resistance in *Botrytis cinerea* by loop-mediated isothermal amplification. *Phytopathology Research* 1(1).

Fan, F., Yin, W.X., Li, G.Q., Lin, Y., Luo, C.X., 2018. Development of a LAMP method for detecting SDHI fungicide resistance in *Botrytis cinerea*. *Plant Disease* 102(8), 1612–1618.

Felšöciová, S., Sabo, J., Galovičová, L., Kačániová, M., 2021. The diversity of fungal population from grape harvest to young wine in Small Carpathian wine region. *Potravinarstvo Slovak Journal of Food Sciences* 15, 340-349.

Felšöciová, S., Tančinová, D., Rybárik, Ľ., Mašková, Z., Kačániova, M., 2015. Mycobiota of Slovak wine grapes with emphasis on *Aspergillus* and *Penicillium* species in the small carpathian area. *Potravinarstvo* 9(1), 501-508.

Feng, W., Hieno, A., Kusunoki, M., Suga, H., Kageyama, K., 2019. LAMP detection of four plant-pathogenic Oomycetes and its application in lettuce fields. *Plant Disease* 103(2), 298–307.

Ferrara, M., Perrone, G., Gallo, A., Epifani, F., Visconti, A., Susca, A., 2015. Development of loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Penicillium nordicum* in dry-cured meat products. *International Journal of Food Microbiology* 202, 42–47.

Fillinger, S., Elad, Y., 2016. *Botrytis* – the fungus, the pathogen and its management in agricultural systems. Springer International Publishing, Cham, Switzerland.

Finn, I., 2013. Weinwissen- In 2 Tagen zum Kenner. Hallwag, München.

Finn, I., Oos, A., 2017. Warenkunde Wein. Stiftung Warentest.

Frisch, L.M., 2018. Investigations of the production of surface-active proteins and patulin by *Penicillium expansum*. Technical University of Munich, *Master's Thesis*.

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

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

Frisch, L.M., Niessen, L., 2019. Development and optimization of a group-specific loop-mediated isothermal amplification (LAMP) assay for the detection of patulin-producing *Penicillium* species. *International Journal of Food Microbiology* 298, 20-30.

Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*: A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology* 49, 1–174.

Frisvad, J.C., Smedsgaard, J., Larsen, T.O., Samson, R.A., 2004. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Studies in Mycology* 49, 201–241.

Fusi, M., Mainente, F., Rizzi, C., Zoccatelli, G., Simonato, B., 2010. Wine hazing: A predictive assay based on protein and glycoprotein independent recovery and quantification. *Food Control* 21(6), 830–834.

Gardner, R.J., 1973. The mechanism of gushing-A review. *Journal of the Institute of Brewing* 79(4), 275–283.

Gatignol, A., Durand, H., Tiraby, G., 1988. Bleomycin resistance conferred by a drug-binding protein. *FEBS Letters* 230(1-2), 171–175.

Gazzola, D., van Sluyter, S.C., Curioni, A., Waters, E.J., Marangon, M., 2012. Roles of proteins, polysaccharides, and phenolics in haze formation in white wine via reconstitution experiments. *Journal of Agricultural and Food Chemistry* 60(42), 10666–10673.

Gazzola, D., Vincenzi, S., Pasini, G., Lomolino, G., Curioni, A., 2015. Advantages of the KDS/BCA assay over the Bradford assay for protein quantification in white wine and grape juice. *American Journal of Enology and Viticulture* 66(2), 227-233.

Girbau-Solà, T., López-Tamames, E., Buján, J., Buxaderas, S., 2002. Foam aptitude of trepat and monastrell red varieties in cava elaboration. 1. Base wine characteristics. *Journal of Agricultural and Food Chemistry* 50(20), 5596-5599.

Gjertsen, P., 1967. Gushing in beer: Its nature, cause and prevention. *Brewers Digest* 42(5), 80–84.

Gjertsen, P., Trolle, B., Andersen, K., 1963. Weathered barley as a contributory cause of gushing in beer, Proceedings of the Europen Brewery Convention Congress, Brussels, Elsevier: Amsterdam, pp. 320–341.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* 61(4), 1323–1330.

Glebe, D., 2002. Deutscher Wein für Anfänger - EW Edition Weinwissen. Books on Demand.

Golding, N.S., 1945. The gas requirements of molds. IV. a preliminary interpretation of the growth rates of four common mold cultures on the basis of absorbed gases. *Journal of Dairy Science* 28(10), 737–750.

Goldman, A., Harper, S., Speicher, D.W., 2016. Detection of proteins on blot membranes. *Current Protocols in Protein Science* 86, 10.18.11-10.18.11.

Gomès, E., Sagot, E., Gaillard, C., Laquitaine, L., Poinssot, B., Sanejouand, Y.-H., Delrot, S., Coutos-Thévenot, P., 2003. Nonspecific lipid-transfer protein genes expression in grape (*Vitis* sp.) cells in response to fungal elicitor treatments. *Molecular Plant-Microbe Interactions* 16(5), 456–464.

Gorjanović, S., 2007. Barley grain non-specific lipid-transfer proteins (ns-LTPs) in beer production and quality. *Journal of the Institute of Brewing* 113(3), 310–324.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K.-I., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *BioTechniques* 46(3), 167–172.

Guggenberger, J., Kleber, W., 1963. Über den Mechanismus des Wildwerdens von Bier, Proceedings of the Europen Brewery Convention Congress, Brussels, Elsevier: Amsterdam, pp. 299–363.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., Widmer, F., 2015. Distinct soil microbial diversity under long-term organic and conventional farming. *The ISME Journal* 9(5), 1177–1194.

Harwig, J., Chen, Y.-K., Kennedy, B.P.C., Scott, P.M., 1973. Occurrence of patulin and patulin-producing strains of *Penicillium expansum* in natural rots of apple in Canada. *Canadian Institute of Food Science and Technology Journal* 6(1), 22–25.

Hejgaard, J., Kaersgaard, P., 1983. Purification and properties of the major antigenic beer protein of barley origin. *Journal of the Institute of Brewing* 89(6).

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

Higgins, D.R., Cregg, J., 1998. *Pichia* protocols. Humana Press, New Jersey.

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 C* 57(1-2), 1-8.

Hippeli, S., Hecht, D., 2008. Die Rolle von nsLTP1 und Proteasen bei der Entstehung des primären Gushing. *Brauwelt* 32, 900–904.

Hnasko, R., Lin, A., McGarvey, J.A., Stanker, L.H., 2011. A rapid method to improve protein detection by indirect ELISA. *Biochemical and Biophysical Research Communications* 410(4), 726–731.

Houbraken, J., Kocsube, S., Visagie, C.M., Yilmaz, N., Wang, X.C., Meijer, M., Kraak, B., Hubka, V., Bensch, K., Samson, R.A., Frisvad, J.C., 2020. Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (*Eurotiales*): An overview of families, genera, subgenera, sections, series and species. *Studies in Mycology* 95, 5-169.

Hovasse, A., Alayi, T.D., van Dorsselaer, A., Marchal, R., Jégou, S., Schaeffer-Reiss, C., 2016. In-depth glycoproteomic characterisation of grape berry vacuolar invertase using a combination of mass spectrometry-based approaches. *Food Chemistry* 200, 237–244.

Hsu, J.-C., Heatherbell, D.A., 1987. Isolation and characterization of soluble proteins in grapes, grape juice, and wine. *American Journal of Enology and Viticulture* 38, 6-10.

Hu, X.R., Dai, D.J., Wang, H.D., Zhang, C.Q., 2017. Rapid on-site evaluation of the development of resistance to quinone outside inhibitors in *Botrytis cinerea*. *Scientific Reports* 7(1).

International Organisation of Vine and Wine, 2015. International Code of Oenological Practices, Resolution 18/73 & 6/79.

International Organisation of Vine and Wine, 2020. OIV Focus: The global sparkling wine market.

Invitrogen, 2010a. EasySelect™ *Pichia* Expression Kit, User manual. Thermo Fisher Scientific, Carlsbad, CA, USA.

Invitrogen, 2010b. pPICZα A, B, and C - User manual. Thermo Fisher Scientific, Carlsbad, CA, USA.

Jansohn, M., Rothhämel, S., 2012. Gentechnische Methoden. Spektrum Akademischer Verlag, Heidelberg.

Jégou, S., Douliez, J.-P., Mollé, D., Boivin, P., Marion, D., 2000. Purification and structural characterization of LTP1 polypeptides from beer. *Journal of Agricultural and Food Chemistry* 48(10), 5023–5029.

Jiang, F., Kongsaeree, P., Schilke, K., Lajoie, C., Kelly, C., 2008. Effects of pH and temperature on recombinant manganese peroxidase production and stability. *Applied Biochemistry and Biotechnology* 146(1-3), 15–27.

Kader, J.C., 1996. Lipid-transfer proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 47, 627-654.

Kaersgaard, P., Hejgaard, J., 1979. Antigenic beer macromolecules- an experimental survey of purification methods. *Journal of the Institute of Brewing* 85, 103-111.

Kastner, H., 1909. Das "Wildwerden" des Malzbieres. *Wochenschrift für Brauerei* 26(13), 169–170.

Kemp, B., Condé, B., Jégou, S., Howell, K., Vasserot, Y., Marchal, R., 2018. Chemical compounds and mechanisms involved in the formation and stabilization of foam in sparkling wines. *Critical Reviews in Food Science and Nutrition*, 1–23.

Kershaw, M.J., Talbot, N.J., 1998. Hydrophobins and repellents: Proteins with fundamental roles in fungal morphogenesis. *Fungal Genetics and Biology* 23(1), 18–33.

Kitabatake, K., 1978. A wort component responsible for gushing in beer. *Bulletin of Brewing Science* 24, 21-32.

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

Koestel, C., Simonin, C., Belcher, S., Rosti, J., 2016. Implementation of an enzyme linked immunosorbent assay for the quantification of allergenic egg residues in red wines using commercially available antibodies. *Journal of Food Science* 81(8), T2099-2106.

Komárek, M., Čadková, E., Chrastný, V., Bordas, F., Bollinger, J.-C., 2010. Contamination of vineyard soils with fungicides: a review of environmental and toxicological aspects. *Environment International* 36(1), 138–151.

Kong, X., Qin, W., Huang, X., Kong, F., Schoen, C.D., Feng, J., Wang, Z., Zhang, H., 2016. Development and application of loop-mediated isothermal amplification (LAMP) for detection of *Plasmopara viticola*. *Scientific Reports* 6, 28935.

König, H., Unden, G., Fröhlich, J., 2009. Biology of microorganisms on grapes, in must and in wine. Springer Heidelberg.

Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World map of the Köppen-Geiger climate classification updated. *Meteorologische Zeitschrift* 15(3), 259–263.

Kretschmer, M., Hahn, M., 2008. Fungicide resistance and genetic diversity of *Botrytis cinerea* isolates from a vineyard in Germany. *Journal of Plant Diseases and Protection* 115(5), 214–219.

Kück, U., 2009. Schimmelpilze: Lebensweise, Nutzen, Schaden, Bekämpfung, 3. ed. Springer, Heidelberg.

Kupfer, V.M., 2018. The influence of plant- and yeast-derived proteins on gushing in sparkling wine. Technical University of Munich, *Dissertation*.

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.

Kurtzman, C.P., 2009. Biotechnological strains of *Komagataella* (*Pichia*) *pastoris* are *Komagataella phaffii* as determined from multigene sequence analysis. *Journal of Industrial Microbiology & Biotechnology* 36(11), 1435-1438.

Kwon, J.-H., Shen, S.-S., Jee, H.-J., 2008. Occurence of blue mold on tomato caused by *Penicillium oxalicum* in Korea. *Plant Pathology* 21(1), 87–89.

Kwon, S.W., 2004. Profiling of soluble proteins in wine by nano-high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* 52(24), 7258–7263.

La Guerche, S., Chamont, S., Blancard, D., Dubourdieu, D., Darriet, P., 2005. Origin of (-)-geosmin on grapes: On the complementary action of two fungi, *Botrytis cinerea* and *Penicillium expansum*. *Antonie Van Leeuwenhoek* 88(2), 131–139.

Larsen, T.O., Frisvad, J.C., 1994. A simple method for collection of volatile metabolites from fungi based on diffusive sampling from Petri dishes. *Journal of Microbiological Methods* 19(4), 297-305.

Latt, S.A., Stetten, G., Juergens, L.A., Willard, H.F., Scher, C.D., 1975. Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. *Journal of Histochemistry and Cytochemistry* 23(7), 493–505.

Leroch, M., Kretschmer, M., Hahn, M., 2011. Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in South West Germany. *Journal of Phytopathology* 159(1), 63-65.

Li, P., Anumanthan, A., Gao, X.-G., Ilangovan, K., Suzara, V.V., Düzgüneş, N., Renugopalakrishnan, V., 2007. Expression of recombinant proteins in *Pichia pastoris*. *Applied Biochemistry and Biotechnology* 142(2), 105–124.

Li, Y., Zhang, B., Chen, X., Chen, Y., Cao, Y., 2010. Improvement of *Aspergillus sulphureus* endo-beta-1,4-xylanase expression in *Pichia pastoris* by codon optimization and analysis of the enzymic characterization. *Applied Biochemistry and Biotechnology* 160(5), 1321–1331.

Liger-Belair, G., 2005. The physics and chemistry behind the bubbling properties of champagne and sparkling wines: A state-of-the-art review. *Journal of Agricultural and Food Chemistry* 53(8), 2788–2802.

Likar, M., Stres, B., Rusjan, D., Potisek, M., Regvar, M., 2017. Ecological and conventional viticulture gives rise to distinct fungal and bacterial microbial communities in vineyard soils. *Applied Soil Ecology* 113, 86–95.

Lin, J., Panigraphy, D., Trinh, L.B., Folkman, J., Shiloach, J., 2000. Production process for recombinant human angiostatin in *Pichia pastoris*. *Journal of Industrial Microbiology and Biotechnology* 24(1), 31–35.

Linder, M.B., Szilvay, G.R., Nakari-Setälä, T., Penttilä, M.E., 2005. Hydrophobins: The protein-amphiphiles of filamentous fungi. *FEMS Microbiology Reviews* 29(5), 877–896.

Lindner, B., 2015. Er ist im Keller, der „Rosa Chardonnay“, Institut für Rebenzüchtung, Hochschule Geisenheim University. https://www.hs-geisenheim.de/studieninfotag-digital/forschung/neuigkeiten/n/er-ist-im-keller-der-rosa-chardonnay/. Retrieved 18.09.2021.

Liu, P.H., Vrigneau, C., Salmon, T., Hoang, D.A., Boulet, J.C., Jegou, S., Marchal, R., 2018. Influence of grape berry maturity on juice and base wine composition and foaming properties of sparkling wines from the champagne region. *Molecules* 23(6).

Liu, Y.H., Yuan, S.K., Hu, X.R., Zhang, C.Q., 2019. Shift of sensitivity in *Botrytis cinerea* to benzimidazole fungicides in strawberry greenhouse ascribing to the rising-lowering of E198A subpopulation and its visual, on-site monitoring by loop-mediated isothermal amplification. *Scientific Reports* 9(1).

Looke, M., Kristjuhan, K., Kristjuhan, A., 2011. Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques* 50(5), 325-328.

Lopez Pinar, A., Rauhut, D., Ruehl, E., Buettner, A., 2017. Effects of Bunch Rot (*Botrytis cinerea*) and Powdery Mildew (*Erysiphe necator*) fungal diseases on wine aroma. *Frontiers in Chemistry* 5.

Lorenzini, M., Cappello, M.S., Logrieco, A., Zapparoli, G., 2016. Polymorphism and phylogenetic species delimitation in filamentous fungi from predominant mycobiota in withered grapes. *International Journal of Food Microbiology* 238, 56–62.

Luo, J., Vogel, R.F., Niessen, L., 2012. Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. *International Journal of Food Microbiology* 159(3), 214–224.

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

Lutterschmid, G., 2011. Surface-active proteins from *Fusarium* spp. and their role in gushing in carbonated beverages. Technical University of Munich, *Dissertation*.

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(1), 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.

Luttmann, W., Bratke, K., Küpper, M., Myrtek, D., 2014. Der Experimentator: Immunologie. Springer Berlin, Heidelberg.

Ma, C., Wang, Y., Zhang, P., Shi, C., 2017. Accelerated isothermal nucleic acid amplification in betaine-free reaction. *Analytical Biochemistry* 530, 1-4.

Marangon, M., van Sluyter, S.C., Neilson, K.A., Chan, C., Haynes, P.A., Waters, E.J., Falconer, R.J., 2011. Roles of grape thaumatin-like protein and chitinase in white wine haze formation. *Journal of Agricultural and Food Chemistry* 59(2), 733–740.

Marchal, R., Berthier, L., Legendre, L., Marchal-Delahaut, L., Jeandet, P., Maujean, A., 1998. Effects of *Botrytis cinerea* infection on the must protein electrophoretic characteristics. *Journal of Agricultural and Food Chemistry* 46(12), 4945–4949.

Marchal, R., Bouquelet, S., Maujean, A., 1996. Purification and partial biochemical characterization of glycoproteins in a Champenois Chardonnay wine. *Journal of Agricultural and Food Chemistry* 44(7), 1716–1722.

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).

Marchal, R., Seguin, V., Maujean, A., 1997. Quantification of interferences in the direct measurement of proteins in wines from the Champagne region using the Bradford method. *American Journal of Enology and Viticulture* 48, 303-309.

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.

Marchal, R., Warchol, M., Cilindre, C., Jeandet, P., 2006. Evidence for protein degradation by *Botrytis cinerea* and relationships with alteration of synthetic wine foaming properties. *Journal of Agricultural and Food Chemistry* 54(14), 5157–5165.

Markoulatos, P., Siafakas, N., Moncany, M., 2002. Multiplex polymerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis* 16(1), 47–51.

Martínez-Lapuente, L., Ayestarán, B., Guadalupe, Z., 2018. Influence of wine chemical compounds on the foaming properties of sparkling wines, in: Jordão, A.M., Cosme, F. (Eds.), Grapes and Wines - Advances in production, processing, analysis and valorization. IntechOpen, London.

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.

Mastanjević, K., Mastanjević, K., Krstanović, V., 2017. The gushing experience—A quick overview. *Beverages* 3(4).

Mikusová, P., Ritieni, A., Santini, A., Juhasová, G., Srobárová, A., 2010. Contamination by moulds of grape berries in Slovakia. *Food Additives & Contaminants Part A* 27(5), 738–747.

Mislivec, P.B., Tuite, J., 1970a. Species of *Penicillium* occurring in freshly-harvested and in stored dent corn kernels. *Mycologia* 62(1), 67–74.

Mislivec, P.B., Tuite, J., 1970b. Temperature and relative humidity requirements of species of *Penicillium* isolated from yellow dent corn kernels. *Mycologia* 62(1), 75–88.

Morales, H., Marín, S., Rovira, A., Ramos, A.J., Sanchis, V., 2007. Patulin accumulation in apples by *Penicillium expansum* during postharvest stages. *Letters in Applied Microbiology* 44(1), 30–35.

Mori, Y., Kitao, M., Tomita, N., Notomi, T., 2004. Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods* 59(2), 145–157.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications* 289(1), 150–154.

Mülhardt, C., 2009. Der Experimentator: Molekularbiologie/ Genomics, 6 ed. Spektrum Akademischer Verlag; Springer, Heidelberg.

Munar, M.J., Sebree, B., 1997. Gushing—A maltster's view. *Journal of the American Society of Brewing Chemists* 55(3), 119–122.

Murakami-Yamaguchi, Y., Hirose, J., Fujii, W., Narita, H., 2009. An novel enzyme-linked immunosorbent assay for barley lipid transfer protein as a quality control system of beer. *Journal of the Japanese Society for Food Science and Technology* 56(2), 64–71.

Murakami-Yamaguchi, Y., Hirose, J., Kizu, K., Okazaki, F., Fujii, W., Narita, H., 2012. Quality control system for beer developed with monoclonal antibodies specific to barley lipid transfer protein. *Antibodies* 1(3), 259–272.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes* 16(3), 223–229.

Nakano, R., Nakano, A., Ishii, Y., Ubagai, T., Kikuchi-Ueda, T., Kikuchi, H., Tansho-Nagakawa, S., Kamoshida, G., Mu, X., Ono, Y., 2015. Rapid detection of the *Klebsiella pneumoniae* carbapenemase (KPC) gene by loop-mediated isothermal amplification (LAMP). *Journal of Infection and Chemotherapy* 21(3), 202–206.

Neiers, F., Belloir, C., Poirier, N., Naumer, C., Krohn, M., Briand, L., 2021. Comparison of different signal peptides for the efficient secretion of the sweet-tasting plant protein brazzein in *Pichia pastoris*. *Life* 11(1).

Niessen, L., 2013. Loop-mediated isothermal amplification-based detection of *Fusarium graminearum*, in: O'Connor, L., Glynn, B. (Eds.), Fungal Diagnostics. Humana Press, Totowa, NJ, pp. 177-193.

Niessen, L., 2015. Current state and future perspectives of loop-mediated isothermal amplification (LAMP)-based diagnosis of filamentous fungi and yeasts. *Applied Microbiology and Biotechnology* 99(2), 553–574.

Niessen, L., Bechtner, J., Fodil, S., Taniwaki, M.H., Vogel, R.F., 2018. LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection. *International Journal of Food Microbiology* 266, 241–250.

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* 132, 702–714.

Niessen, L., Vogel, R.F., 2010. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. *International Journal of Food Microbiology* 140(2-3), 183–191.

Niu, B., Wang, D., Yang, Y., Xu, H., Qiao, M., 2012. Heterologous expression and characterization of the hydrophobin HFBI in *Pichia pastoris* and evaluation of its contribution to the food industry. *Amino Acids* 43(2), 763–771.

Niu, J.-h., Guo, Q.-x., Jian, H., Chen, C.-l., Yang, D., Liu, Q., Guo, Y.-d., 2011. Rapid detection of *Meloidogyne* spp. by LAMP assay in soil and roots. *Crop Protection* 30(8), 1063–1069.

Noble, R.T., Fuhrman, J.A., 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology* 14, 113–118.

Notomi, T., 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28(12), 63e-63.

Núñez, Y.P., Carrascosa, A.V., Gonzalez, R., Polo, M.C., Martínez-Rodríguez, A., 2006. Isolation and characterization of a thermally extracted yeast cell wall fraction potentially useful for improving the foaming properties of sparkling wines. *Journal of Agricultural and Food Chemistry* 54(20), 7898–7903.

Núñez, Y.P., Carrascosa, A.V., González, R., Polo, M.C., Martínez-Rodríguez, A.J., 2005. Effect of accelerated autolysis of yeast on the composition and foaming properties of sparkling wines elaborated by a champenoise method. *Journal of Agricultural and Food Chemistry* 53(18), 7232–7237.

O'Neill, T.M., M., B., Ann, D., 1991. Aspects of biology and control of a stem rot of cucumber caused by *Penicillium oxalicum*. *Plant Pathology* 40(1), 78–84.

Okigbo, R.N., Ogbonnaya, U.O., 2006. Antifungal effects of two tropical plant leaf extracts (*Ocimum gratissimum* and *Aframomum melegueta*) on postharvest yam (*Dioscorea* spp.) rot. *African Journal of Biotechnology* 5(9), 727–731.

Okuda, T., Fukui, M., Takayanagi, T., Yokotsuka, K., 2006. Characterization of major stable proteins in Chardonnay wine. *Food Science and Technology Research* 12(2), 131–136.

Ostrý, V., Škarková, J., Procházková, I., Kubátová, A., Malíř, F., Ruprich, J., 2007. Mycobiota of Czech wine grapes and occurrence of ochratoxin A and *Alternaria* mycotoxins in fresh grape juice, must and wine. *Czech Mycology* 59(2), 241–254.

Oszmiański, J., Wolniak, M., Wojdyło, A., Wawer, I., 2008. Influence of apple purée preparation and storage on polyphenol contents and antioxidant activity. *Food Chemistry* 107(4), 1473–1484.

Outchkourov, N.S., Stiekema, W.J., Jongsma, M.A., 2002. Optimization of the expression of equistatin in *Pichia pastoris*. *Protein Expression and Purification* 24(1), 18-24.

Panasenko, V.T., 1967. Ecology of microfungi. *The Botanical Review* 33(3), 189–215.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L., Morita, K., 2008. Loop mediated isothermal amplification (LAMP): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology* 18(6), 407–421.

Pedneault, K., Provost, C., 2016. Fungus resistant grape varieties as a suitable alternative for organic wine production: Benefits, limits, and challenges. *Scientia Horticulturae* 208, 57-77.

Pellaud, J., 2002. Gushing: State of the art. *Cerevisia* 27, 189–205.

Perrocheau, L., Bakan, B., Boivin, P., Marion, D., 2006. Stability of barley and malt lipid transfer protein 1 (LTP1) toward heating and reducing agents: relationships with the brewing process. *Journal of Agricultural and Food Chemistry* 54(8), 3108–3113.

Pitt, J.I., Hocking, A.D., 1997. Fungi and food spoilage, 2. ed. Blackie Academic & Professional, London.

Pitt, J.I., Hocking, A.D., 2009. Fungi and food spoilage, 3. ed. Springer US, Boston, MA.

Rankine, B.C., 1977. Gushing in sparkling wine. *Australian Grapegrower and Winemaker* 160, 26.

Ribéreau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D., 2006. Handbook of Enology, volume 2: The chemistry of wine stabilization and treatments, 2 ed. John Wiley & Sons Ltd, Chichester.

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

Rousseaux, S., Diguta, C.F., Radoï-Matei, F., Alexandre, H., Guilloux-Bénatier, M., 2014. Non-*Botrytis* grape-rotting fungi responsible for earthy and moldy off-flavors and mycotoxins. *Food Microbiology* 38, 104–121.

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

Russo, P.S., Blum, F.D., Ipsen, J.D., Abul-Hajj, Y.J., Miller, W.G., 1982. The surface activity of the phytotoxin cerato-ulmin. *Canadian Journal of Botany* 60(8).

Sage, L., Krivobok, S., Delbos, É., Seigle-Murandi, F., Creppy, E.E., 2002. Fungal flora and ochratoxin A production in grapes and musts from France. *Journal of Agricultural and Food Chemistry* 50(5), 1306–1311.

Salminen, T.A., Blomqvist, K., Edqvist, J., 2016. Lipid transfer proteins: classification, nomenclature, structure, and function. *Planta* 244(5), 971–997.

Samson, R.A., Houbraken, J., Thrane, U., Frisvald, J.C., Andersen, B., 2019. Food and indoor fungi. Westerdijk Fungal Biodiversity Institute, Utrecht.

Santos, J.A., Fraga, H., Malheiro, A.C., Moutinho-Pereira, J., Dinis, L.-T., Correia, C., Moriondo, M., Leolini, L., Dibari, C., Costafreda-Aumedes, S., Kartschall, T., Menz, C., Molitor, D., Junk, J., Beyer, M., Schultz, H.R., 2020. A review of the potential climate change impacts and adaptation options for European viticulture. *Applied Sciences* 10(9), 3092.

Sarlin, T., 2012. Detection and characterisation of *Fusarium* hydrophobins inducing gushing in beer. Aalto University School of Chemical Technology, *Dissertation*.

Sarlin, T., Laitila, A., Pekkarinen, A., Haikara, A., 2005a. Effects of three *Fusarium* species on the quality of barley and malt. *Journal of the American Society of Brewing Chemists* 63(2), 43–49.

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

Sarlin, T., Vilpola, A., Kotaviita, E., Olkku, J., Haikara, A., 2007. Fungal hydrophobins in the barley-to-beer chain. *Journal of the Institute of Brewing* 113(2).

Schägger, H., Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* 166, 368–379.

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

Scheurer, S., Schülke, S., 2018. Interaction of non-specific lipid-transfer proteins with plant-derived lipids and its impact on allergic sensitization. *Frontiers in Immunology* 9.

Schmid, F., Moser, G., Müller, H., Berg, G., 2011. Functional and structural microbial diversity in organic and conventional viticulture: organic farming benefits natural biocontrol agents. *Applied and Environmental Microbiology* 77(6), 2188–2191.

Schmid, S., Schmid, T., 2018. Bio-Wein im eigenen Garten. Löwenzahn Verlag.

Schumacher, T., 2002. Gushing in Fruchtsaftschorlen – Ursachen und Gegenmaßnahmen. *Flüssiges Obst* 69(5), 304–310.

Schuren, F.H.J., Wessels, J.G.H., 1990. Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating-type genes. *Gene* 90(2).

Schwarz, P.B., 1996. Relationship between *Fusarium* infestation of barley and the gushing potential of malt. *Journal of the Institute of Brewing* 102, 93-96.

Serra, R., 2003. Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. *International Journal of Food Microbiology* 88(1), 63–68.

Serra, R., Braga, A., Venâncio, A., 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. *Research in Microbiology* 156(4), 515–521.

Serra, R., Lourenço, A., Alípio, P., Venâncio, A., 2006. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. *Mycological Research* 110(Pt 8), 971–978.

Shah, K., Maghsoudlou, P., 2016. Enzyme-linked immunosorbent assay (ELISA): the basics. *British Journal of Hospital Medicine* 77(7).

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M., 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols* 1(6), 2856–2860.

Si Ammour, M., Castaldo, E., Fedele, G., Rossi, V., 2020. Use of LAMP for assessing *Botrytis cinerea* colonization of bunch trash and latent infection of berries in grapevines. *Plants* 9(11).

Sinclair, G., Choy, F.Y.M., 2002. Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. *Protein Expression and Purification* 26, 96–105.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* 150, 76-85.

Snowdon, A.L., 1990. A colour atlas of post-harvest diseases and disorders of fruits and vegetables. Vol. 1. General introduction and fruits. Wolfe Scientific, London.

Sommer, N., Fortlage, R.J., Edwards, D.C., 2002. Postharvest diseases of selected commodities, in: Kader, A.A. (Ed.), Postharvest technology of horticultural crops, 3. ed. Univ. of California Agriculture and Natural Resources Communication Services, Davis, CA.

Sørensen, S.B., Bech, L.M., Muldbjerg, M., Beenfeldt, T., Breddam, K., 1993. Barley lipid transfer protein 1 is involved in beer foam formation. *Technical Quarterly - Master Brewers Association of the Americas* 30(4), 136-145.

Specker, C., 2014. Analysis of the interaction of gushing inducing hydrophobins with beer foam proteins. Technical University of Munich, *Dissertation*.

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.

Staatliches Weinbauinstitut Freiburg im Breisgau, 2021. Rebschutz 2021. *Der Badische Winzer*.

Statistisches Bundesamt, 2021a. Absatz, Verbrauch und Hersteller von Schaumwein, https://www-genesis.destatis.de/genesis/online?language=de&sequenz=tabelleErgebnis&selectionname=73423-0001#abreadcrumb. Retrieved 18.09.2021.

Statistisches Bundesamt, 2021b. Annähernder Verbrauch alkoholischer Getränke in Deutschland, https://www.destatis.de/DE/Themen/Staat/Steuern/Verbrauchsteuern/Tabellen/alkoholische-getraenke.html. Retrieved 18.09.2021.

Steyn, P.S., 1970. The isolation, structure and absolute configuration of secalonic acid d, the toxic metabolite of *Penicillium oxalicum*. *Tetrahedron* 26, 51–57.

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(1-2), 110–115.

Svendsen, A., Frisvad, J.C., 1994. A chemotaxonomic study of the terverticillate penicillia based on high performance liquid chromatography of secondary metabolities. *Mycological Research* 98(11), 1317-1328.

Tančinová, D., Felšöciová, S., Rybárik, Ľ., Mašková, Z., Císarová, M., 2015. Colonization of grapes berries and cider by potential producers of patulin. *Potravinarstvo* 9(1).

Tanner, N.A., Zhang, Y., Evans, T.C., 2015. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *BioTechniques* 58(2), 59–68.

Tannous, J., Atoui, A., El Khoury, A., Kantar, S., Chdid, N., Oswald, I.P., Puel, O., Lteif, R., 2015. Development of a real-time PCR assay for *Penicillium expansum* quantification and patulin estimation in apples. *Food Microbiology* 50, 28–37.

ten Have, A., Dekkers, E., Kay, J., Phylip, L.H., van Kan, J.A.L., 2004. An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. *Microbiology* 150, 2475–2489.

Thiessen, L.D., Keune, J.A., Neill, T.M., Turechek, W.W., Grove, G.G., Mahaffee, W.F., 2016. Development of a grower-conducted inoculum detection assay for management of grape powdery mildew. *Plant Pathology* 65(2), 238–249.

Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols* 3(5), 877–882.

Tomlinson, J.A., Dickinson, M.J., Boonham, N., 2010. Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. *Letters in Applied Microbiology* 51(6), 650–657.

Tone, K., Fujisaki, R., Yamazaki, T., Makimura, K., 2017. Enhancing melting curve analysis for the discrimination of loop-mediated isothermal amplification products from four pathogenic molds: Use of inorganic pyrophosphatase and its effect in reducing the variance in melting temperature values. *Journal of Microbiological Methods* 132, 41–45.

Trinh, L.B., Phue, J.N., Shiloach, J., 2003. Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from *Pichia pastoris*. *Biotechnology and Bioengineering* 82(4), 438–444.

Troost, G., Bach, H.P., Rhein, O.H., 1995. Sekt, Schaumwein, Perlwein, 2. ed. Ulmer, Stuttgart.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A., Gingeras, T.R., 1987. Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Research* 15(9).

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., Cox, J., 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods* 13(9), 731–740.

Umemoto, S., Odake, Y., Takeuchi, T., Yoshida, S., Tsushima, S., Koitabashi, M., 2009. Blue mold of tomato caused by *Penicillium oxalicum* in Japan. *Journal of General Plant Pathology* 75(5), 399–400.

van der Sluis, A.A., Dekker, M., Skrede, G., Jongen, W.M.F., 2002. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *Journal of Agricultural and Food Chemistry* 50(25), 7211–7219.

van der Vegt, W., van der Mei, H.C., Wösten, H.A.B., Wessels, J.G.H., Busscher, H.J., 1996. A comparison of the surface activity of the fungal hydrophobin SC3p with those of other proteins. *Biophysical Chemistry* 57(2-3).

van Sluyter, S.C., McRae, J.M., Falconer, R.J., Smith, P.A., Bacic, A., Waters, E.J., Marangon, M., 2015. Wine protein haze: mechanisms of formation and advances in prevention. *Journal of Agricultural and Food Chemistry* 63(16), 4020–4030.

Varga, J., Koncz, Z., Kocsubé, S., Mátrai, T., Téren, J., Ostry, V., Skarkova, J., Ruprich, J., Kubatova, A., Kozakiewicz, Z., 2007. Mycobiota of grapes collected in Hungarian and Czech vineyards in 2004. *Acta Alimentaria* 36(3), 329–341.

Vesper, S.J., Wymer, L.J., Meklin, T., Varma, M., Stott, R., Richardson, M., Haugland, R.A., 2005. Comparison of populations of mould species in homes in the UK and USA using mould-specific quantitative PCR. *Letters in Applied Microbiology* 41(4), 367–373.

Vico, I., Duduk, N., Vasic, M., Nikolic, M., 2014. Identification of *Penicillium expansum* causing postharvest blue mold decay of apple fruit. *Pesticidi i Fitomedicina* 29(4), 257–266.

Vincenzi, S., Crapisi, A., Curioni, A., 2014. Foamability of Prosecco wine: Cooperative effects of high molecular weight glycocompounds and wine PR-proteins. *Food Hydrocolloids* 34, 202-207.

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, 149–153.

Vogt-Hrabak, E.I., 2017. Influence of grape-associated filamentous fungi and their exoproteome on the gushing in sparkling wine. Technical University of Munich, *Dissertation*.

Vogt, E.I., Kupfer, V.M., Bechtner, J.D., Frisch, L.M., Vogel, R.F., Niessen, L., 2017a. Detection of *Penicillium oxalicum* in grapes with a loop-mediated isothermal amplification (LAMP) assay. *Journal of Microbiology, Biotechnology and Food Sciences* 7(3), 265–270.

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., 2017b. Evidence of gushing induction by *Penicillium oxalicum* proteins. *Journal of Applied Microbiology* 122(3), 708–718.

Walg, O., 2015. Entblätterung der Traubenzone – Einflüsse auf Traubengesundheit, Ertrag und Qualität, Tagungsband zur 59. Wintertagung für Weinbau und Landwirtschaft, Bad Kreuznach, pp. 36–50.

Walter, R., 2008. Untersuchungen zur Grünfäule (*Penicillium* spec.) an Weintrauben. University of Hohenheim, *Dissertation*.

Wang, W., Chen, K., Xu, C., 2006. DNA quantification using EvaGreen and a real-time PCR instrument. *Analytical Biochemistry* 356(2), 303–305.

Waters, E.J., Pellerin, P., Brillouet, J.-M., 1994. A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydrate Polymers* 23, 185-191.

Weber, P., Steinhart, H., Paschke, A., 2009. Determination of the bovine food allergen casein in white wines by quantitative indirect ELISA, SDS-PAGE, Western blot and immunostaining. *Journal of Agricultural and Food Chemistry* 57(18), 8399–8405.

Wessels, J.G.H., 1994. Developmental regulation of fungal cell wall formation. *Annual Review of Phytopathology* 32(1).

Wessels, J.G.H., 1996. Fungal hydrophobins: Proteins that function at an interface. *Trends in Plant Science* 1(1), 9–15.

Wessels, J.G.H., de Vries, O.M.H., Asgeirsdottir, S.A., Schuren, F.H.J., 1991. Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum*. *The Plant Cell* 3(8).

Wigand, P., Tenzer, S., Schild, H., Decker, H., 2009. Analysis of protein composition of red wine in comparison with rose and white wines by electrophoresis and high-pressure liquid chromatography-mass spectrometry (HPLC-MS). *Journal of Agricultural and Food Chemistry* 57(10), 4328-4333.

Wilson, S.M., 1999. A study of gushing in ciders: its mechanism and causative effects. University of Guelph, *Dissertation*.

Wösten, H.A., Schuren, F.H., Wessels, J.G., 1994. Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. *The EMBO Journal* 13(24).

Wösten, H.A.B., de Vries, O.M.H., Wessels, J.G.H., 1993. Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. *The Plant Cell* 5(11), 1567–1574.

Wösten, H.A.B., Wessels, J.G.H., 1997. Hydrophobins, from molecular structure to multiple functions in fungal development. *Mycoscience* 38(3), 363–374.

Wu, S., Letchworth, G.J., 2004. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *BioTechniques* 36(1), 152-154.

Yaguchi, M., Pusztai-Carey, M., Roy, C., Surewicz, W.K., Carey, P.R., Stevenson, K.J., Richards, W.C., Takai, S., 1993. Amino acid sequence and spectroscopic studies of Dutch elm disease toxin, cerato-ulmin, in: Sticklen, S. (Ed.), Dutch elm disease research. Springer, New York, pp. 152–170.

Yount, D.E., 1982. On the evolution, generation, and regeneration of gas cavitation nuclei. *The Journal of the Acoustical Society of America* 71(6), 1473–1481.

Yu, P., Yan, Y., Gu, Q., Wang, X., 2013. Codon optimisation improves the expression of *Trichoderma viride* sp. endochitinase in *Pichia pastoris*. *Scientific Reports* 3.

Zapf, M., 2006. Charakterisierung oberflächenaktiver Proteine aus *Fusarium* spp. und deren Einfluss auf die Blasenstabilisierung in Bier. Technical University of Munich, *Dissertation*.

Zapf, M.W., Theisen, S., Rohde, S., Rabenstein, F., Vogel, R.F., Niessen, L., 2007. Characterization of AfpA, an alkaline foam protein from cultures of *Fusarium culmorum* and its identification in infected malt. *Journal of Applied Microbiology* 103(1), 36-52.

Zarnkow, M., Back, W., 2001. Neue Erkenntnisse über gushingauslösende Substanzen. *Brauwelt* 141, 363–367.

Zepf, M., Geiger, E., 2000. Gushing problems caused by calcium oxalate (Part 1). *Brauwelt International* 18, 473–475.

Zhang, X., Lowe, S.B., Gooding, J.J., 2014. Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP). *Biosensors & Bioelectronics* 61, 491–499.

11 Appendix

11.1 Sequences

Nucleotide and amino acid sequences that are relevant for the current study are shown in the following figures.

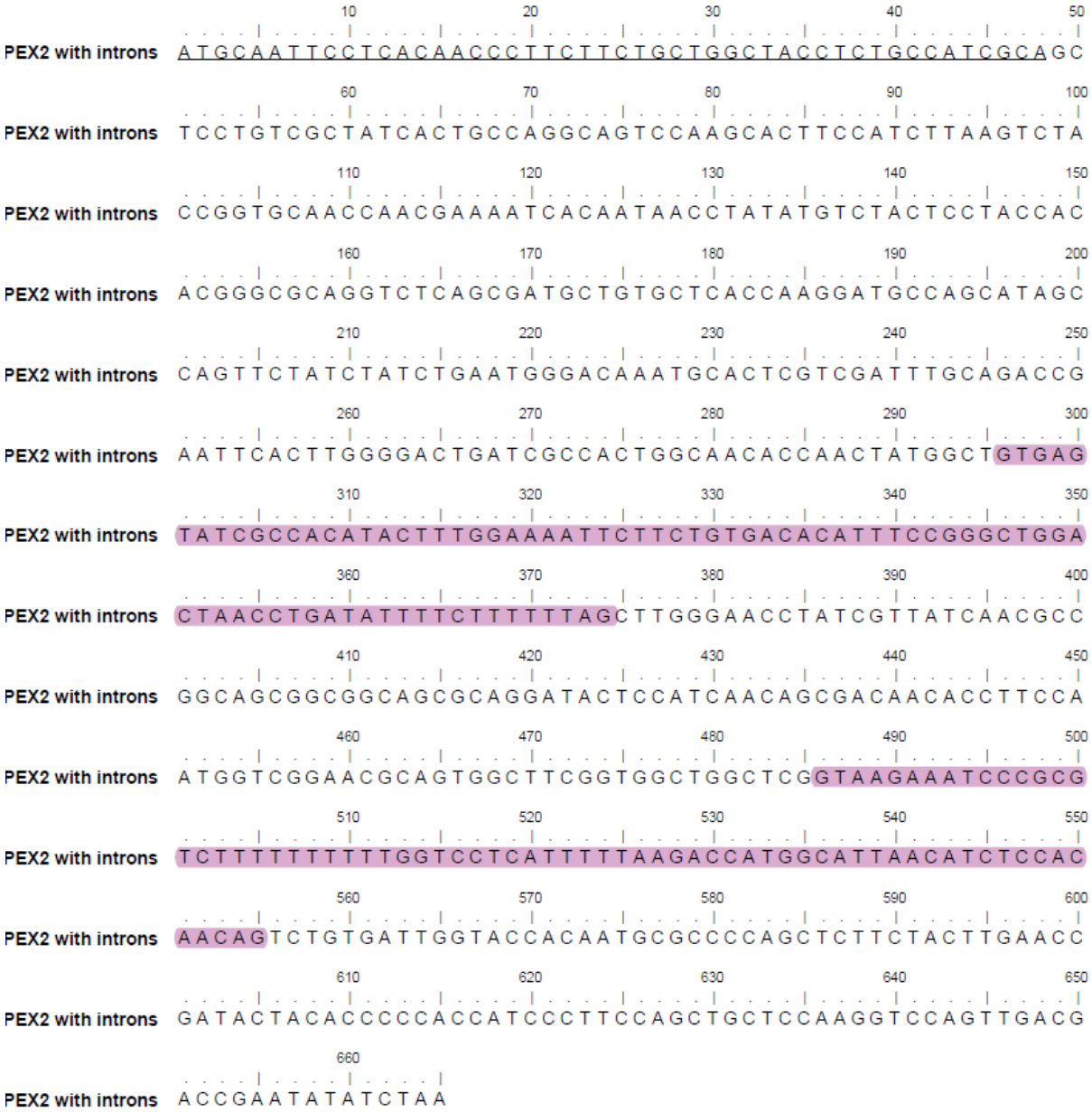


Figure 50: Nucleotide sequence of PEX2_044840 from *P. expansum*

The nucleotide sequence of the gene coding for the protein PEX2_044840 from *P. expansum* (GenBank accession number NW_015971172.1:c457334-456670) is shown. The signal peptide is underlined. Introns are marked in purple.

Figure 51: Codon-optimized nucleotide sequence of PEX2_044840 from *P. expansum*

The intron-free nucleotide sequence of the gene coding for the protein PEX2_044840 (GenBank accession number XM_016741759.1) with codon optimization for optimal expression in *Pichia pastoris* is shown. The signal peptide is underlined.

Figure 52: Amino acid sequence of PEX2_044840 from *P. expansum*

The amino acid sequence of the protein PEX2_044840 from *P. expansum* (GenBank accession number XP_016603461.1) is shown. The signal peptide is underlined and the peptide for the Anti-PEX2_044840 antibody (see Table 24) is marked in orange.

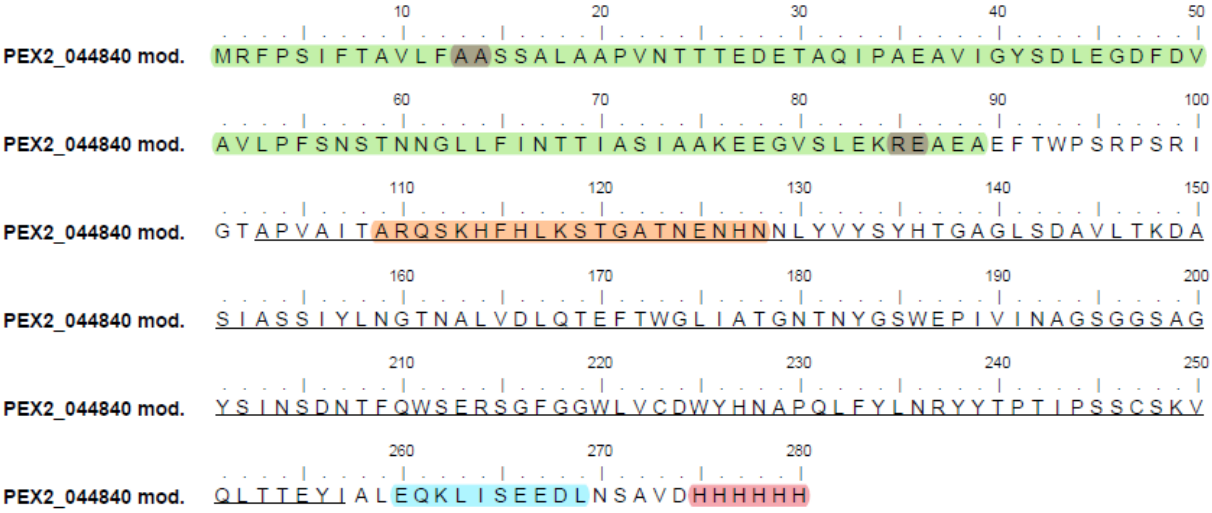


Figure 53: Amino acid sequence of modified PEX2_044840

The amino acid sequence of the modified (mod.) PEX2_044840 protein from *P. expansum* is shown. The α-factor secretion signal is marked in green with cleavage sites marked in grey. The PEX2_044840 protein sequence is underlined and the peptide for the Anti-PEX2_044840 antibody is marked in orange. The c-myc epitope is marked in blue and the 6xHis-tag in pink. Non-marked amino acids were added to be in-frame.

Figure 54: Nucleotide sequence of PDE_07106 from *P. oxalicum*

The nucleotide sequence of the gene coding for the protein PDE_07016 from *P. oxalicum* (GenBank accession number KB644414.1:1493352-1493789) is shown. The signal peptide is underlined.

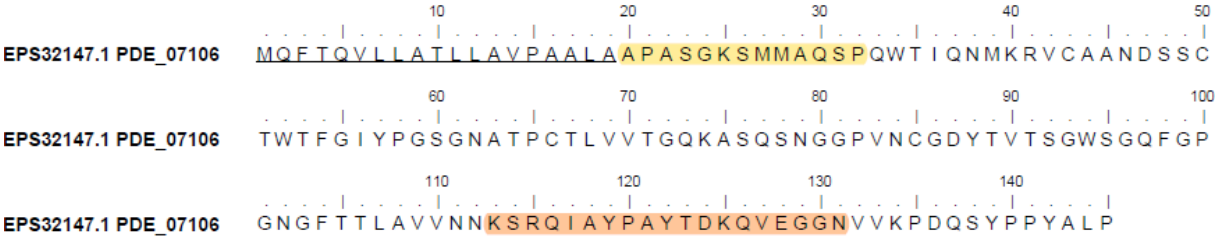


Figure 55: Amino acid sequence of PDE_07106 from *P. oxalicum*

The amino acid sequence of the protein PDE_07016 from *P. oxalicum* (GenBank accession number EPS32147.1) is shown. The signal peptide is underlined, the peptide for the Anti-PDE_07106 antibody (see Table 24) is marked in orange, and the peptide for the Anti-VOG-APA-IgG antibody (see Table 24) in yellow.

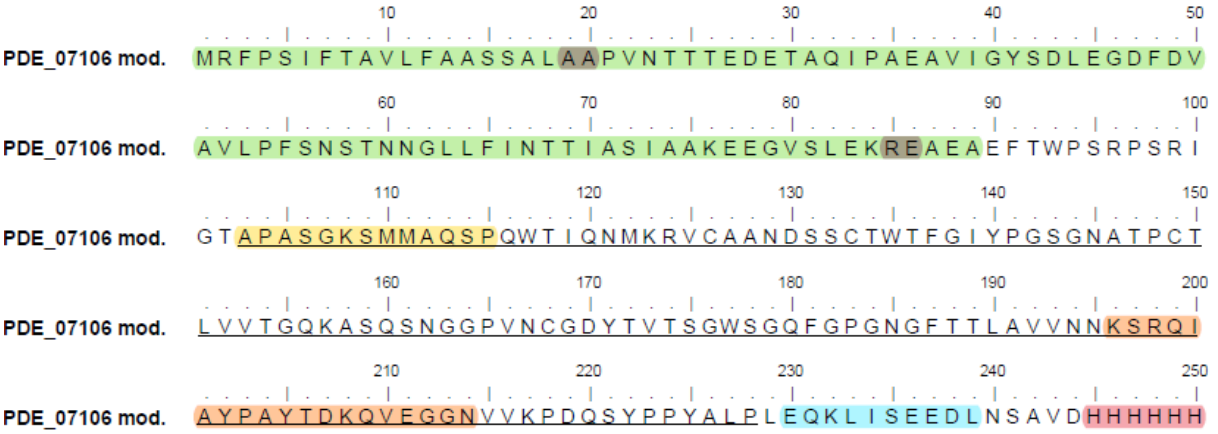


Figure 56: Amino acid sequence of modified PDE_07106

The amino acid sequence of the modified (mod.) PDE_07106 protein from *P. oxalicum* is shown. The α-factor secretion signal is marked in green with cleavage sites marked in grey. The PDE_07106 protein sequence is underlined, the peptide for the Anti-PDE_07106 antibody is marked in orange, and the peptide for the Anti-VOG-APA-IgG antibody in yellow. The c-myc epitope is marked in blue and the 6xHis-tag in pink. Non-marked amino acids were added to be in-frame.

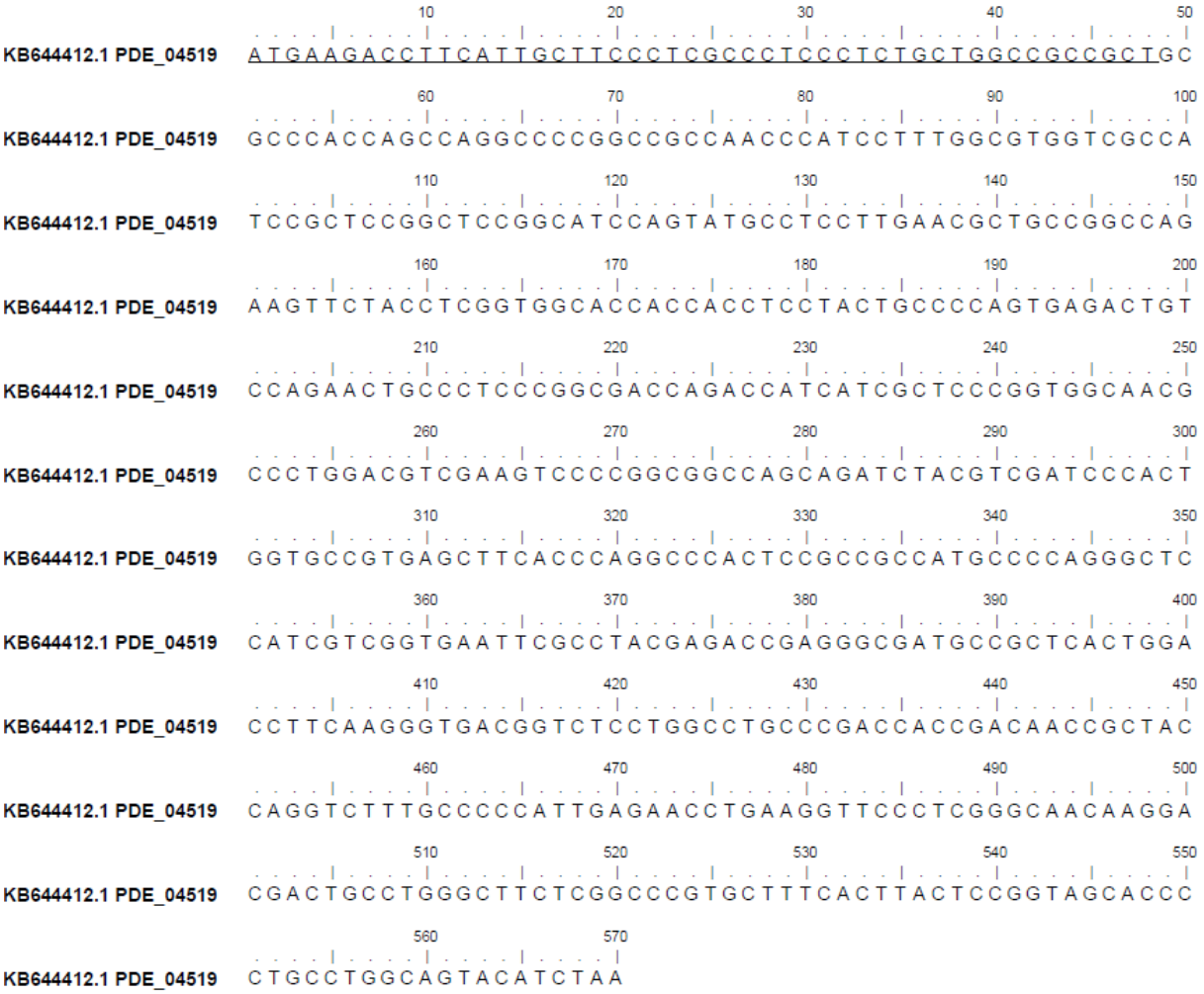


Figure 57: Nucleotide sequence of PDE_04519 from *P. oxalicum*

The nucleotide sequence of the gene coding for the protein PDE_04519 from *P. oxalicum* (GenBank accession number KB644412.1:210536-211105) is shown. The signal peptide is underlined.

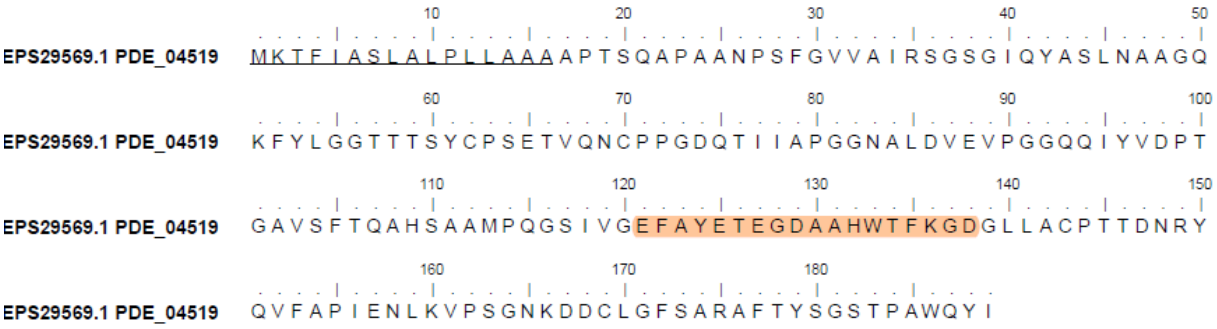


Figure 58: Amino acid sequence of PDE_04519 from *P. oxalicum*

The amino acid sequence of the protein PDE_04519 from *P. oxalicum* (GenBank accession number EPS29569.1) is shown. The signal peptide is underlined and the peptide for the Anti-VOG-EFA-IgG antibody (see Table 24) is marked in orange.

Figure 59: Amino acid sequence of modified PDE_04519

The amino acid sequence of the modified (mod.) PDE_04519 protein from *P. oxalicum* is shown. The α-factor secretion signal is marked in green with cleavage sites marked in grey. The PDE_04519 protein sequence is underlined and the peptide for the Anti-VOG-EFA-IgG antibody is marked in orange. The c-myc epitope is marked in blue and the 6xHis-tag in pink. Non-marked amino acids were added to be in-frame.

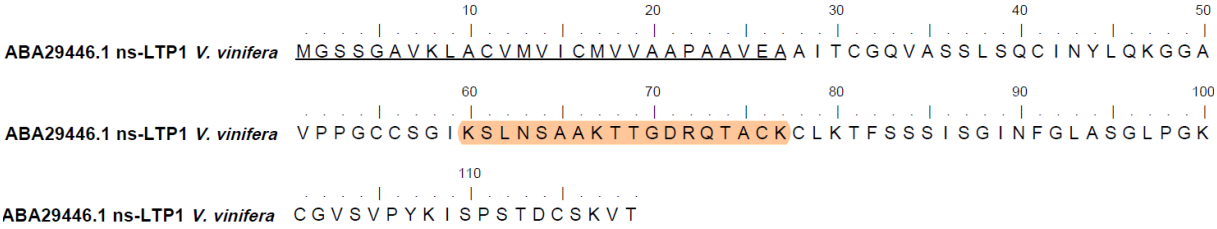


Figure 60: Amino acid sequence of ns-LTP1 from *V. vinifera*

The amino acid sequence of the protein ns-LTP1 from *V. vinifera* (GenBank accession number ABA29446.1) is shown. The signal peptide is underlined and the peptide for the Anti-ns-LTP1-*Vv* antibody (see Table 24) is marked in orange.

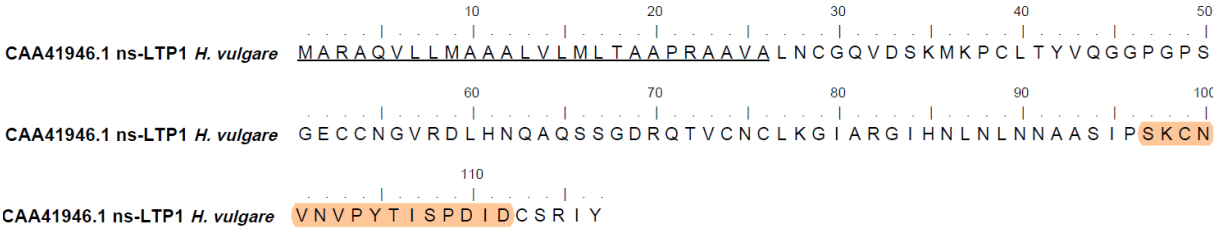


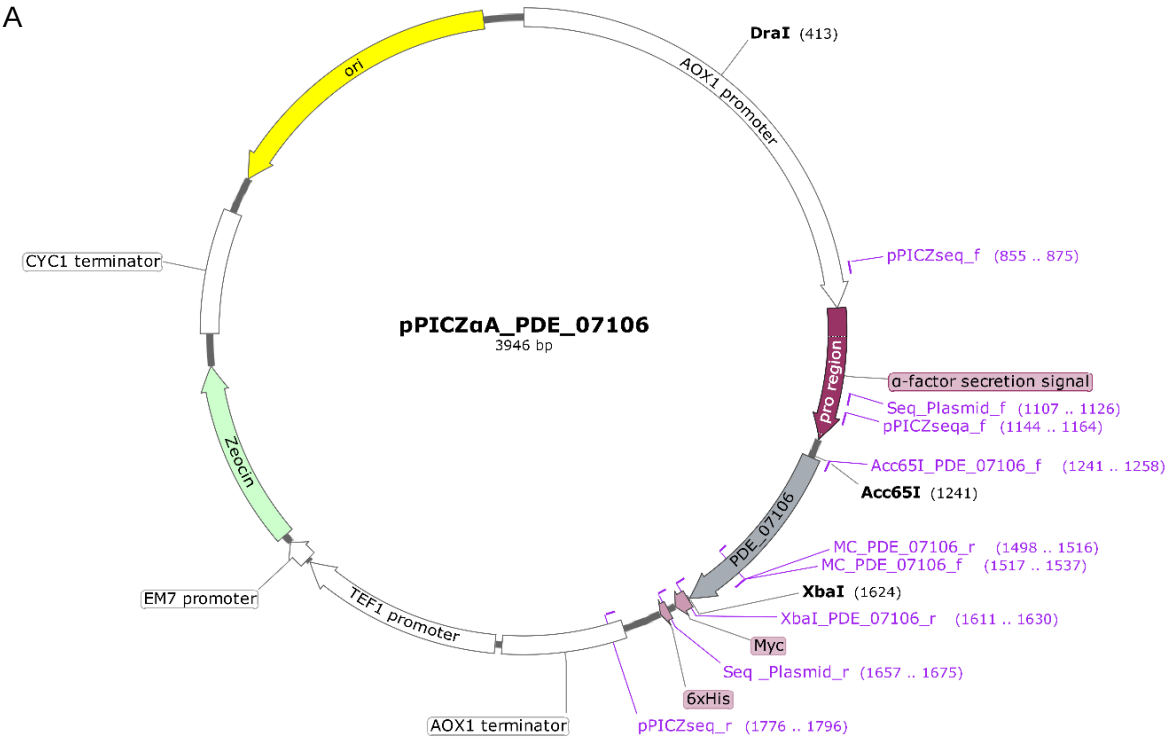
Figure 61: Amino acid sequence of ns-LTP1 from *H. vulgare*

The amino acid sequence of the protein ns-LTP1 from *H. vulgare* (GenBank accession number CAA41946.1) is shown. The signal peptide is underlined and the peptide for the Anti-nsLtp1-P2-IgG antibody (see Table 24) is marked in orange.

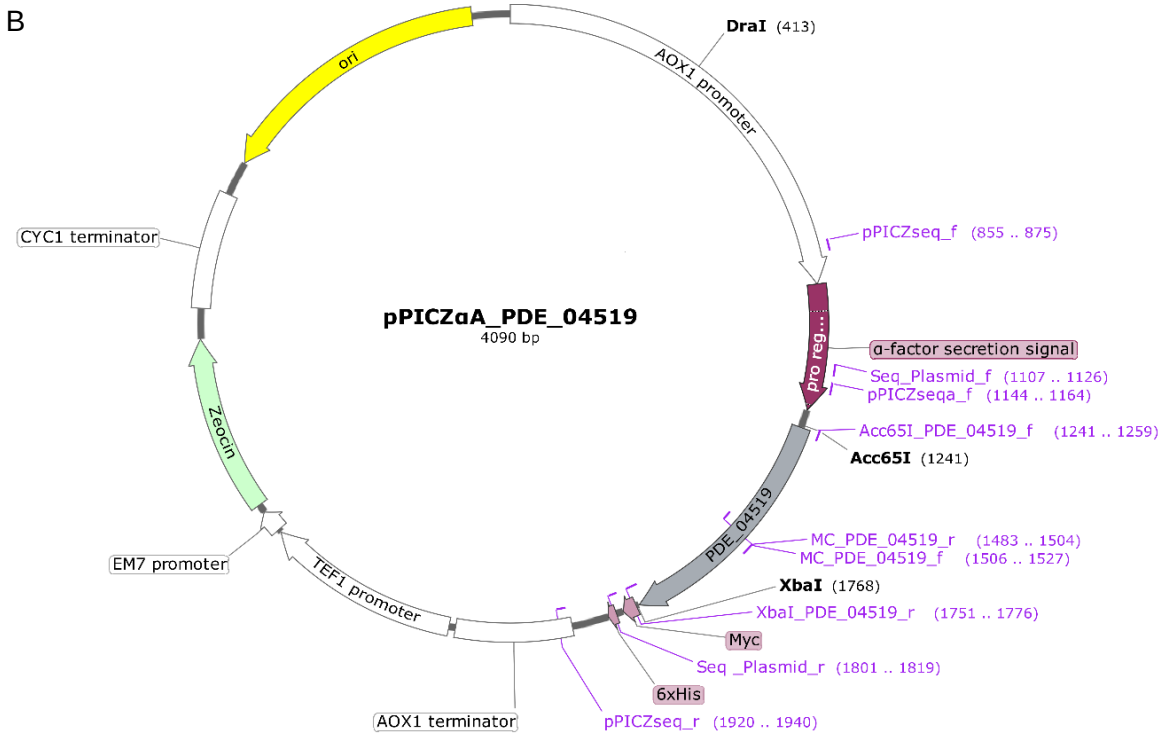
11.2 Vector maps

The vector maps are shown in the following figure.

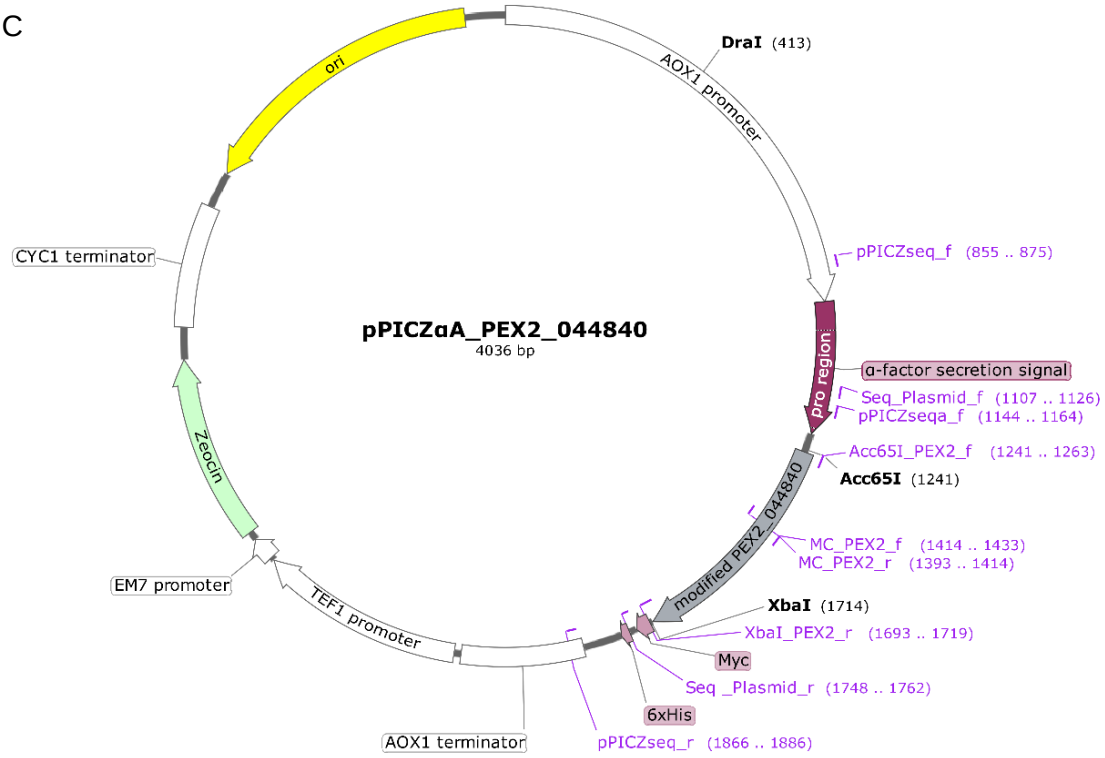
A



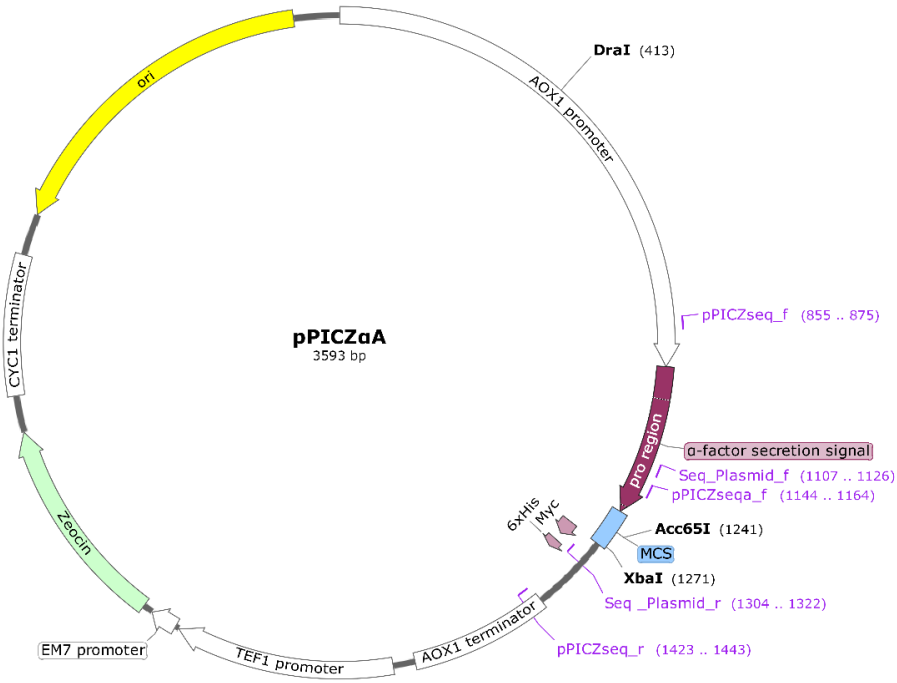
B



C


pPICZαA_PEX2_044840
4036 bp
DraI (413)
AOX1 promoter
pPICZseq_f (855 .. 875)
pro region
α-factor secretion signal
Seq_Plasmid_f (1107 .. 1126)
pPICZseqa_f (1144 .. 1164)
Acc65I_PEX2_f (1241 .. 1263)
Acc65I (1241)
modified PEX2_044840
MC_PEX2_f (1414 .. 1433)
MC_PEX2_r (1393 .. 1414)
XbaI (1714)
XbaI_PEX2_r (1693 .. 1719)
Myc
Seq _Plasmid_r (1748 .. 1762)
6xHis
pPICZseq_r (1866 .. 1886)
AOX1 terminator
TEF1 promoter
EM7 promoter
Zeocin
CYC1 terminator
ori

D


pPICZαA
3593 bp
DraI (413)
AOX1 promoter
pPICZseq_f (855 .. 875)
pro region
α-factor secretion signal
Seq_Plasmid_f (1107 .. 1126)
pPICZseqa_f (1144 .. 1164)
6xHis
Myc
Acc65I (1241)
MCS
XbaI (1271)
Seq _Plasmid_r (1304 .. 1322)
pPICZseq_r (1423 .. 1443)
AOX1 terminator
TEF1 promoter
EM7 promoter
Zeocin
CYC1 terminator
ori

E

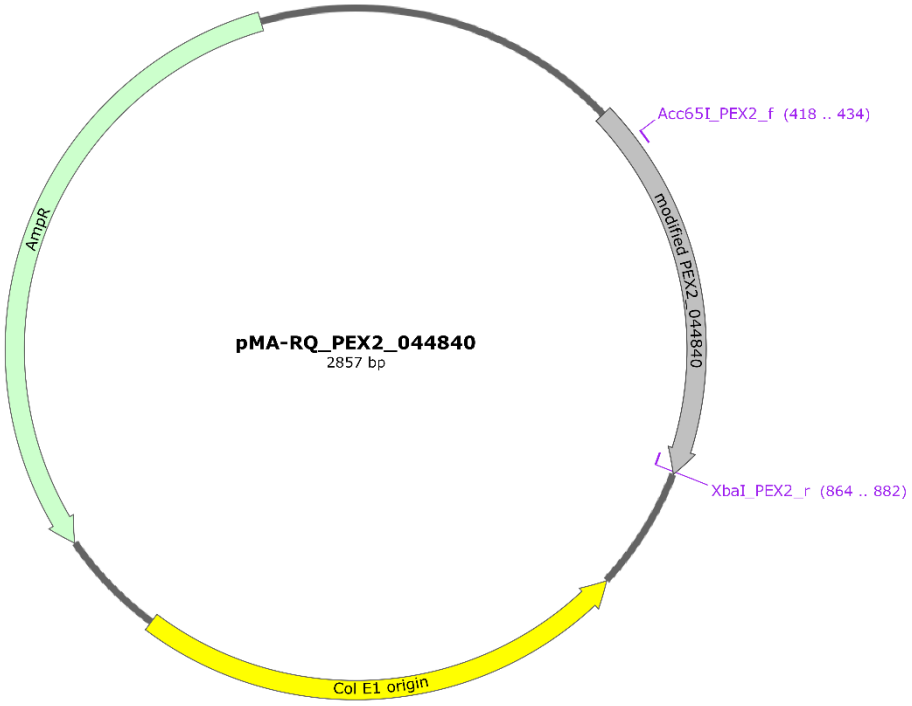


Figure 62: Vector maps

The vector maps of pPICZαA_PDE_07106 (A), pPICZαA_PDE_04519 (B), pPICZαA_PEX2_044840 (C), pPICZαA (D), and pMA-RQ_PEX2_044840 (E) are shown. The *AOX1* promoter is methanol-inducible. The α-factor secretion signal is responsible for the secretion of the expressed protein into the culture medium. The myc epitope and 6xHis-tag enable detection of the protein by specific antibodies and purification via IMAC. A Zeocin™ or ampicillin (Amp) resistance gene and bacterial replication origin are coded. Restriction sites for the enzymes DraI, Acc65I, and XbaI are marked in bold. Primer binding sites are marked in purple. MCS in D = multiple cloning site.

11.3 Screening results

The LAMP assay results (see section 3.4.3) of the screening of samples from the harvests 2018, 2019, and 2020 are listed in the following tables.

Table 50: LAMP results of the screening 2018

The results of the LAMP assays for the detection of patulin-producing *Penicillium* species (Pat. *Penicillium*), *B. cinerea*, and *P. oxalicum* by testing sample material from Germany of the harvest 2018 are listed. + = positive result, - = negative result, ns = not specified.

Source	Labeling of samples	Variety of the grapes	Test variant	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
				Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Old vine, healthy	Riesling	Test vine age: 47 years old	-	-	-	-	-	-
	Old vine, diseased			-	-	**+**	**+**	-	-
	Soil old vine			-		**+**		-	
	Medium old vine, healthy	Riesling	Test vine age: 22 years old	-	-	-	-	-	-
	Medium old vine, diseased			-	-	**+**	-	-	-
	Soil medium old vine			-		**+**		-	
	Young vine, healthy	Riesling	Test vine age: 6 years old	-	-	-	-	-	-
	Young vine, diseased			-	-	**+**	**+**	-	-
	Soil young vine			-		-		-	
	Traminer, healthy	Traminer	Test variety of the grapes	-	-	-	-	-	-
	Traminer, diseased			-	-	-	-	-	-
	Soil Traminer			-		**+**		-	
	Chardonnay white, healthy	Chardonnay white	Test variety of the grapes	-	-	-	-	-	-
	Chardonnay white, diseased			-	-	**+**	**+**	-	**+**
	Soil Chardonnay white			-		**+**		-	

Table 50 (continued)

Source	Labeling of samples	Variety of the grapes	Test variant	LAMP Pat. P*enicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
				Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Chardonnay pink, healthy	Chardonnay pink	Test variety of the grapes	-	-	-	-	-	-
	Chardonnay pink, diseased			-	-	-	-	-	-
	Soil Chardonnay pink			-		+		-	
	Integrated, healthy	Riesling	Test cultivation system: Integrated	-	-	-	-	-	-
	Integrated, diseased			-	-	-	-	-	-
	Soil integrated			-		-		-	
	Organic, healthy	Riesling	Test cultivation system: Organic	-	-	-	-	-	-
	Organic, diseased			-	-	+	+	-	-
	Soil organic			-		-		-	
	Biodynamic, healthy	Riesling	Test cultivation system: Biodynamic	-	-	-	-	-	-
	Biodynamic, diseased			-	-	+	-	-	-
	Soil biodynamic			-		+		-	
	Control, healthy	Riesling	Test pest control *B. cinerea*: No botryticide, product against *Plasmopara viticola* and *Oidium tuckeri*	-	-	-	+	-	-
	Control, diseased			-	-	-	-	-	-
	Soil control			-		+		-	
	Standard, healthy	Riesling	Test pest control *B. cinerea*: according to integrated standards, + Teldor, Switch	-	-	-	-	-	-
	Standard, diseased			-	-	+	+	-	-
	Soil standard			-		+		-	

Table 50 (continued)

Source	Labeling of samples	Variety of the grapes	Test variant	LAMP Pat. P*enicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
				Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Vitisan, healthy	Riesling	Test pest control *B. cinerea*: according to organic standards, + Wetcit, Vitisan	-	-	+	-	-	-
	Vitisan, diseased			-	-	+	+	-	-
	Soil Vitisan			-		-		-	
	Kumar, healthy	Riesling	Test pest control *B. cinerea*: according to organic standards, + Kumar	-	-	-	-	-	-
	Kumar, diseased			-	-	-	-	-	-
	Soil Kumar			-		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Test variant**	**LAMP Pat. P*enicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
				Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Rhineland-Palatinate	White variety 1	Pinot blanc	ns	-	-	+	+	-	-
	Soil 1			-		+		-	
	Soil 2			-		-		-	
	Soil 3			-		-		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Test variant**	**LAMP Pat. P*enicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
				Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Baden-Wuerttem-berg	White variety 1	ns	ns	-	-	+	+	-	-
	Soil 1			-		-		-	

Table 51: LAMP results of the screening 2019

The results of the LAMP assays for the detection of patulin-producing *Penicillium* species (Pat. *Penicillium*), *B. cinerea*, and *P. oxalicum* by testing sample material from Portugal, Italy, Germany, Luxembourg, the Netherlands, and France of the harvest 2019 are listed. + = positive result, - = negative result, ns = not specified, n.a. = not available.

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Portugal, Douro, Beira	White variety 1, sample 1	Bical	Clay	38	ns	-	-	+	+	-	-
	Soil 1, sample 1					-		+		-	
	White variety 1, sample 2	Bical	Clay	38	ns	-	-	+	+	-	+
	Soil 1, sample 2					-		-		-	
	White variety 2, sample 1	Sercial	Clay	38	ns	-	-	+	+	+	+
	Soil 2, sample 1					-		+		-	
	White variety 2, sample 2	Sercial	Clay	38	ns	-	-	+	+	-	-
	Soil 2, sample 2					-		+		-	
	Red variety 1, sample 1	Touriga Nacional	Slate	21	ns	-	-	+	+	+	-
	Soil 1, sample 1					-		+		-	
	Red variety 1, sample 2	Touriga Nacional	Slate	21	ns	-	-	+	+	-	-
	Soil 1, sample 2					-		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Italy, Emilia-Romagna	White variety 1, sample 1	Trebbiano	Clay	18	Yes	-	-	+	-	-	-
	Soil 1, sample 1					-		+		+	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Italy, Emilia-Romagna	White variety 1, sample 2	Trebbiano	Clay	18	Yes	-	-	+	-	-	-
	Soil 1, sample 2					-		+		+	
	Red variety 1, sample 1	Sangiovese	Clay	18	Yes	-	-	+	+	-	-
	Soil 1, sample 1					-		+		-	
	Red variety 1, sample 2	Sangiovese	Clay	18	Yes	-	-	+	+	-	-
	Soil 1, sample 2					-		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Trebbiano	Grape crusher, wine press	100 kg	80 L	-		+		+	
	Must sample 2	Ciliegiolo	Grape crusher, wine press	100 kg	78 L	-		+		+	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Baden-Wuerttem-berg	White variety 1, sample 1	Riesling	ns	ns	ns	-	-	+	+	-	-
	Soil 1, sample 1					+		+		+	
	White variety 1, sample 2	Riesling	ns	ns	ns	-	-	+	+	-	-
	Soil 1, sample 2					+		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Pinot noir	ns	ns	ns	-		+		+	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Luxem-bourg, Remich canton	White variety 1, sample 1	Chardonnay	Keuper	5	ns	-	-	-	-	-	-
	Soil 1, sample 1					-		+		+	
	White variety 1, sample 2	Chardonnay	Keuper	5	ns	-	-	-	-	-	-
	Soil 1, sample 2					-		-		+	
	White variety 2, sample 1	Pinot gris	Keuper	23	ns	-	-	-	+	-	-
	Soil 2, sample 1					-		+		+	
	White variety 2, sample 2	Pinot gris	Keuper	23	ns	-	-	-	-	-	-
	Soil 2, sample 2					-		-		+	
	Red variety 1, sample 1	Cabaret Noir	Keuper	5	ns	-	-	-	+	-	-
	Soil 1, sample 1					-		-		+	
	Red variety 1, sample 2	Cabaret Noir	Keuper	5	ns	-	-	-	-	-	-
	Soil 1, sample 2					-		-		+	
	Red variety 2, sample 1	Pinot Noir Précoce	Keuper	18	ns	-	-	-	-	-	-
	Soil 2, sample 1					-		+		+	
	Red variety 2, sample 2	Pinot Noir Précoce	Keuper	18	ns	-	-	-	-	+	-
	Soil 2, sample 2					-		+		+	

Table 51 (continued)

Source	Labeling of samples	Selection of grapes for must	Processing of must	Input (weight of grapes)	Output (liters of must)	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
Luxem-bourg, Remich canton	Must sample 1	Chardonnay	ns	ns	ns	-		+		+	
	Must sample 2	Pinot gris	ns	ns	ns	-		+		+	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Baden-Wuerttem-berg	Red variety 1, sample 1	Pinot noir	ns	ns	ns	-	-	-	-	-	-
	Soil 1, sample 1					+		-		-	
	Red variety 1, sample 2	Pinot noir	ns	ns	ns	-	-	-	-	-	-
	Soil 1, sample 2					-		-		+	
	White variety 1, sample 1	Pinot gris	ns	ns	ns	-	-	-	-	-	-
	Soil 1, sample 1					-		+		-	
	White variety 1, sample 2	Pinot gris	ns	ns	ns	-	-	-	-	-	-
	Soil 1, sample 2					-		-		+	
	White variety 2, sample 1	Riesling	ns	ns	ns	-	-	-	-	-	-
	Soil 2, sample 1					-		+		+	
	White variety 2, sample 2	Riesling	ns	ns	ns	-	-	-	-	-	-
	Soil 2, sample 2					-		+		-	
	White variety 3, sample 1	Chasselas	ns	ns	ns	-	-	+	-	-	-
	Soil 3, sample 1					-		-		-	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Baden-Wuerttem-berg	White variety 3, sample 2	Chasselas	ns	ns	ns	-	-	-	-	-	-
	Soil 3, sample 2					-		-		+	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Pinot Noir	Without yeast, not filtrated	ns	ns	-		-		+	
	Must sample 2	Pinot Noir	Without yeast, not filtrated	ns	ns	-		+		+	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Baden-Wuerttem-berg, Bavaria	White variety 1, sample 1	Silvaner	Shell limestone	7	Conventional	-	-	-	-	-	-
	Soil 1, sample 1					-		+		-	
	White variety 1, sample 2	Silvaner	Shell limestone	7	Conventional	-	-	-	+	-	-
	Soil 1, sample 2					-		-		+	
	White variety 2, sample 1	Riesling	Shell limestone	6	Conventional	-	-	-	-	-	-
	Soil 2, sample 1					-		-		-	
	White variety 2, sample 2	Riesling	Shell limestone	6	Conventional	-	-	-	-	-	-
	Soil 2, sample 2					-		-		-	
	White variety 3, sample 1	Sauvignon blanc	Shell limestone	5	Conventional	-	-	-	-	-	-
	Soil 3, sample 1					-		-		-	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Baden-Wuerttem-berg, Bavaria	White variety 3, sample 2	Sauvignon blanc	Shell limestone	5	Conventional	-	-	-	+	-	-
	Soil 3, sample 2					-		-		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Silvaner	ns	ns	ns	+		+		+	
	Must sample 2	Riesling	ns	ns	ns	+		+		+	
	Must sample 3	Sauvignon blanc	ns	ns	ns	+		+		+	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Hesse	White variety 1, sample 1	Riesling	Sandy loam	24	Integrated + Regalis, Teldor, Switch	+	-	+	+	-	-
	Soil 1, sample 1					-		-		-	
	White variety 1, sample 2	Riesling	Sandy loam	24	Integrated + Regalis, Teldor, Switch	+	-	+	+	-	-
	Soil 1, sample 2					-		+		-	
	White variety 2, sample 1	Gewürztra-miner	Sandy loam	22	Integrated + Regalis, Teldor, Switch	+	+	+	-	-	-
	Soil 2, sample 1					+		-		-	
	White variety 2, sample 2	Gewürztra-miner	Sandy loam	22	Integrated + Regalis, Teldor, Switch	+	-	+	+	-	-
	Soil 2, sample 2					+		+		-	
	Red variety 1, sample 1	Cabernet Franc	Sandy loam	22	Integrated + Teldor	-	-	-	+	-	-
	Soil 1, sample 1					-		-		-	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Red variety 1, sample 2	Cabernet Franc	Sandy loam	22	Integrated + Teldor	+	-	+	-	-	-
	Soil 1, sample 2					-		-		-	
	Red variety 2, sample 1	Pinot Noir	Sandy loam	22	Integrated + Teldor	-	-	+	+	-	-
	Soil 2, sample 1					+		+		-	
	Red variety 2, sample 2	Pinot Noir	Sandy loam	22	Integrated + Teldor	-	-	-	+	-	-
	Soil 2, sample 2					+		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Pinot Noir	Hand-pressed	2.75 kg	0.87 L	-		+		+	
	Must sample 2	Riesling	Hand-pressed	3.07 kg	1.20 L	-		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Hesse	Control, sample 1	Riesling	Sandy loam	5	No fungicides	+	-	+	+	-	-
	Soil, sample 1					-		+		-	
	Control, sample 2	Riesling	Sandy loam	5	No fungicides	+	-	+	-	-	-
	Soil, sample 2					-		+		-	
	Integrated 1, sample 1	Riesling	Sandy loam	5	Integrated + Teldor, Switch	+	+	+	+	-	-
	Soil 1, sample 1					+		+		-	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Integrated 1, sample 2	Riesling	Sandy loam	5	Integrated + Teldor, Switch	-	-	-	-	-	-
	Soil 1, sample 2					-		+		-	
	Integrated 2, sample 1	Riesling	Sandy loam	5	Integrated + Regalis, Teldor, Switch	-	-	-	-	-	-
	Soil 2, sample 1					+		-		+	
	Integrated 2, sample 2	Riesling	Sandy loam	5	Integrated + Regalis, Teldor, Switch	-	-	-	-	-	-
	Soil 2, sample 2					-		+		-	
	Organic 1, sample 1	Riesling	Sandy loam	5	Organic + Vitisan, Wetcit	+	-	+	+	-	-
	Soil 1, sample 1					-		-		+	
	Organic 1, sample 2	Riesling	Sandy loam	5	Organic + Vitisan, Wetcit	-	-	-	-	-	-
	Soil 1, sample 2					+		+		+	
	Organic 2, sample 1	Riesling	Sandy loam	5	Organic + Kumar	-	-	+	+	-	-
	Soil 2, sample 1					+		+		+	
	Organic 2, sample 2	Riesling	Sandy loam	5	Organic + Kumar	-	-	-	+	-	-
	Soil 2, sample 2					+		+		-	
	Organic 3, sample 1	Riesling	Sandy loam	5	Organic + Vintec, Regalis	-	+	-	+	-	-
	Soil 3, sample 1					+		+		-	
	Organic 3, sample 2	Riesling	Sandy loam	5	Organic + Vintec, Regalis	+	+	+	+	-	-
	Soil 3, sample 2					-		-		-	

Table 51 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Baden-Wuerttem-berg	White variety 1, sample 1	Riesling	Marl, gypsum, Keuper	30	Conventional	-	-	**+**	-	-	-
	Soil 1, sample 1					-		-		-	
	White variety 1, sample 2	Riesling	Marl, gypsum, Keuper	30	Conventional	-	-	-	**+**	-	-
	Soil 1, sample 2					-		-		-	
	White variety 2, sample 1	Riesling	Marl, gypsum, Keuper	20	Conventional	-	-	**+**	**+**	-	**+**
	Soil 2, sample 1					-		-		-	
	White variety 2, sample 2	Riesling	Marl, gypsum, Keuper	20	Conventional	-	-	**+**	-	-	-
	Soil 2, sample 2					-		-		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Healthy	Sedimentation	1600 kg	960 L	-		**+**		**+**	
	Must sample 2	Healthy	Sedimentation	4800 kg	3360 L	-		**+**		**+**	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
The Nether-lands, North Brabant	White variety 1, sample 1	Chardonnay	Sand	6	ns	-	n.a.	**+**	n.a.	-	n.a.
	Soil 1, sample 1					-		**+**		**+**	
	White variety 1, sample 2	Chardonnay	Sand	6	ns	-	n.a.	-	n.a.	-	n.a.
	Soil 1, sample 2					-		**+**		-	

Table 51 (continued)

Source	**Labeling of samples**	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
The Nether-lands, North Brabant	Must sample 1	Yes	Hand-picked, sorted whole bunch, sediment	6200 kg	4600 L	-		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
France, Bourgogne-Franche-Comté	Red variety 1, sample 1	Pinot Noir	Clay over limestone	ns	Lutte raisonée	-	-	+	-	-	-
	Soil 1, sample 1					-		-		-	
	Red variety 1, sample 2	Pinot Noir	Clay over limestone	ns	Lutte raisonée	-	-	+	+	-	-
	Soil 1, sample 2					-		+		-	
	Red variety 2, sample 1	Pinot Noir	Clay over limestone	20	Lutte raisonée	-	-	-	-	-	-
	Soil 2, sample 1					-		-		-	
	Red variety 2, sample 2	Pinot Noir	Clay over limestone	20	Lutte raisonée	-	-	+	-	-	-
	Soil 2, sample 2					-		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Yes	100 % destemmed	1650 kg	1140 L	-		+		+	
	Must sample 2	Yes	100 % destemmed	1660 kg	1140 L	-		+		+	
	Must sample 3	Yes	100 % destemmed	1650 kg	1140 L	-		+		+	
	Must sample 4	Yes	100 % destemmed	1650 kg	1140 L	-		+		+	

Table 52: LAMP results of the screening 2020

The results of the LAMP assays for the detection of *P. expansum*, patulin-producing *Penicillium* species (Pat. *Penicillium*), *B. cinerea*, and *P. oxalicum* by testing sample material from Italy, Luxembourg, Germany, and Greece of the harvest 2020 are listed. + = positive result, - = negative result, ns = not specified.

Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Italy, Emilia-Romagna	White variety 1, sample 1	Trebbiano	Clay	19	Yes	-	-	-	-	**+**	**+**	**+**	**+**
	Soil 1, sample 1					-		-		-		-	
	White variety 1, sample 2	Trebbiano	Clay	19	Yes	-	-	-	-	**+**	-	**+**	-
	Soil 1, sample 2					-		-		**+**		-	
	Red variety 1, sample 1	Sangiovese	Clay	19	Yes	-	-	-	-	-	-	-	-
	Soil 1, sample 1					-		-		-		-	
	Red variety 1, sample 2	Sangiovese	Clay	19	Yes	-	-	-	-	-	-	**+**	**+**
	Soil 1, sample 2					-		-		-		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Trebbiano	Grape crusher, wine press	100 kg	80 L	-		-		**+**		-	
	Must sample 2	Ciliegiolo	Grape crusher, wine press	100 kg	78 L	-		-		**+**		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Luxembourg, Remich canton	White variety 1, sample 1	Pinot gris	Keuper	16	Organic	-	-	-	-	**+**	**+**	-	-
	Soil 1, sample 1					-		-		-		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Luxembourg, Remich canton	White variety 1, sample 2	Pinot gris	Keuper	16	Organic	-	-	-	-	+	+	-	-
	Soil 1, sample 2					-		-		+		-	
	White variety 2, sample 1	Chardonnay	Keuper	7	Organic	-	-	-	-	+	+	-	-
	Soil 2, sample 1					-		-		+		-	
	White variety 2, sample 2	Chardonnay	Keuper	7	Organic	-	-	-	-	+	+	-	-
	Soil 2, sample 2					-		-		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Pinot gris	Pressed	85.70 kg	60 L	-		-		+		-	
	Must sample 2	Chardonnay	Pressed	40.15 kg	29 L	-		-		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Baden-Wuerttemberg	Red variety 1, sample 1	Pinot noir	ns	ns	ns	-	-	-	-	-	-	-	-
	Soil 1, sample 1					-		-		+		-	
	Red variety 1, sample 2	Pinot noir	ns	ns	ns	-	-	-	-	+	-	-	-
	Soil 1, sample 2					-		-		+		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Baden-Wuerttemberg	White variety 1, sample 1	Pinot blanc	ns	ns	ns	-	-	-	-	+	+	-	-
	Soil 1, sample 1					-		-		-		-	
	White variety 1, sample 2	Pinot blanc	ns	ns	ns	-	-	-	-	+	+	-	-
	Soil 1, sample 2					-		-		-		-	
	White variety 2, sample 1	Riesling	ns	ns	ns	-	-	-	-	-	+	-	-
	Soil 2, sample 1					-		-		-		-	
	White variety 2, sample 2	Riesling	ns	ns	ns	-	-	-	-	+	+	-	-
	Soil 2, sample 2					-		-		+		-	
	White variety 3, sample 1	Chasselas	ns	ns	ns	-	-	-	-	+	+	-	-
	Soil 3, sample 1					-		-		-		-	
	White variety 3, sample 2	Chasselas	ns	ns	ns	-	-	-	-	+	+	-	-
	Soil 3, sample 2					-		-		-		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Chasselas	Without yeast, not filtrated	ns	ns	-		-		-		-	
	Must sample 2	Pinot blanc	Without yeast, not filtrated	ns	ns	-		-		+		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Bavaria	White variety 1, sample 1	Silvaner	Shell limestone	4	ns	-	-	-	-	-	+	-	-
	Soil 1, sample 1					-		-		-		-	
	White variety 1, sample 2	Silvaner	Shell limestone	4	ns	-	-	-	-	+	+	-	-
	Soil 1, sample 2					-		-		-		-	
	White variety 2, sample 1	Silvaner	Shell limestone	10	ns	-	-	-	-	-	-	-	-
	Soil 2, sample 1					-		-		+		-	
	White variety 2, sample 2	Silvaner	Shell limestone	10	ns	-	-	-	-	-	-	-	-
	Soil 2, sample 2					-		-		-		-	
	White variety 3, sample 1	Riesling	Shell limestone	8	ns	-	-	-	-	-	-	-	-
	Soil 3, sample 1					-		-		+		-	
	White variety 3, sample 2	Riesling	Shell limestone	8	ns	-	-	-	-	-	-	-	-
	Soil 3, sample 2					-		-		-		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Silvaner	ns	1960 kg	1300 L	-		-		+		-	
	Must sample 2	Silvaner	ns	3550 kg	2200 L	-		-		-		-	

Table 52 (continued)

Source	Labeling of samples	Selection of grapes for must	Processing of must	Input (weight of grapes)	Output (liters of must)	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
Germany, Bavaria	Must sample 3	Riesling	ns	672 kg	450 L	-		-		-		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Germany, Hesse	White variety 1, sample 1	Riesling	Sandy loam	25	Integrated + Vitisan	-	-	-	-	+	+	-	-
	Soil 1, sample 1					-		-		+		-	
	White variety 1, sample 2	Riesling	Sandy loam	25	Integrated + Vitisan	-	-	-	+	+	+	-	-
	Soil 1, sample 2					-		-		+		-	
	White variety 2, sample 1	Gewürztraminer	Sandy loam	23	Integrated + Vitisan, Regalis	+	-	+	-	+	+	-	-
	Soil 2, sample 1					-		-		+		-	
	White variety 2, sample 2	Gewürztraminer	Sandy loam	23	Integrated + Vitisan, Regalis	-	-	-	-	+	+	-	-
	Soil 2, sample 2					+		+		+		-	
	Red variety 1, sample 1	Cabernet Franc	Sandy loam	23	Integrated + Vitisan	+	-	+	-	+	+	-	-
	Soil 1, sample 1					+		+		+		-	
	Red variety 1, sample 2	Cabernet Franc	Sandy loam	23	Integrated + Vitisan	-	-	-	-	+	+	-	-
	Soil 1, sample 2					+		+		+		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Red variety 2, sample 1	Pinot noir	Sandy loam	23	Integrated + Vitisan	+	-	+	´-	+	+	-	-
	Soil 2, sample 1					-		-		+		-	
	Red variety 2, sample 2	Pinot noir	Sandy loam	23	Integrated + Vitisan	+	+	+	+	+	+	-	-
	Soil 2, sample 2					-		+		+		-	
	Labeling of samples	**Selection of grapes for must**	**Processing of must**	**Input (weight of grapes)**	**Output (liters of must)**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
	Must sample 1	Riesling	Hand-pressed	1.19 kg	0.6 L	-		-		-		-	
	Must sample 2	Pinot noir	Hand-pressed	ns	ns	-		-		+		-	
	Must sample 3	Riesling	Hand-pressed	ns	ns	-		+		+		-	
	Must sample 4	Pinot noir	Hand-pressed	ns	ns	+		+		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Control, sample 1	Müller-Thurgau	Sandy loam	12	No fungicides	+	+	+	+	+	+	-	-
	Soil, sample 1					-		-		+		-	
	Control, sample 2	Müller-Thurgau	Sandy loam	12	No fungicides	+	+	+	+	+	+	-	-
	Soil, sample 2					-		+		+		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Integrated 1, sample 1	Müller-Thurgau	Sandy loam	12	Integrated + Teldor, Switch	+	+	+	+	-	-	-	-
	Soil 1, sample 1					+		+		+		-	
	Integrated 1, sample 2	Müller-Thurgau	Sandy loam	12	Integrated + Teldor, Switch	+	+	+	+	-	+	-	-
	Soil 1, sample 2					-		+		+		-	
	Integrated 2, sample 1	Müller-Thurgau	Sandy loam	12	Integrated + Regalis, Teldor, Switch	+	-	+	-	-	-	-	-
	Soil 2, sample 1					-		-		+		-	
	Integrated 2, sample 2	Müller-Thurgau	Sandy loam	12	Integrated + Regalis, Teldor, Switch	+	+	+	+	-	-	-	-
	Soil 2, sample 2					-		+		+		-	
	Organic 1, sample 1	Müller-Thurgau	Sandy loam	12	Organic + Kumar	+	+	+	+	+	-	-	-
	Soil 1, sample 1					-		-		+		-	
	Organic 1, sample 2	Müller-Thurgau	Sandy loam	12	Organic + Kumar	+	+	+	+	-	-	-	-
	Soil 1, sample 2					-		-		+		-	
	Organic 2, sample 1	Müller-Thurgau	Sandy loam	12	Organic + Vitisan	+	-	+	+	+	+	-	-
	Soil 2, sample 1					-		-		+		-	
	Organic 2, sample 2	Müller-Thurgau	Sandy loam	12	Organic + Vitisan	+	+	+	+	+	+	-	-
	Soil 2, sample 2					-		-		+		-	

Table 52 (continued)

Source	Labeling of samples	Variety of the grapes	Soil type at vineyard location	Age of the vine [years]	Application of pest control and fertilization	LAMP *P. expansum*		LAMP Pat. *Penicillium*		LAMP *B. cinerea*		LAMP *P. oxalicum*	
						Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2	Bunch 1	Bunch 2
Germany, Hesse	Integrated 3, sample 1	Riesling	Sandy loam	6	Integrated, no botryticide	-	-	-	-	+	+	-	-
	Soil 3, sample 1					-		+		+		-	
	Integrated 3, sample 2	Riesling	Sandy loam	6	Integrated, no botryticide	+	+	+	+	+	+	-	-
	Soil 3, sample 2					-		+		+		-	
Source	**Labeling of samples**	**Variety of the grapes**	**Soil type at vineyard location**	**Age of the vine [years]**	**Application of pest control and fertilization**	**LAMP *P. expansum***		**LAMP Pat. *Penicillium***		**LAMP *B. cinerea***		**LAMP *P. oxalicum***	
						Bunch 1	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**	**Bunch 1**	**Bunch 2**
Greece, Western Macedonia	White variety 1, sample 1	Xinomavro	Sandy loam	103	Copper and sulfur, manure	-	-	-	-	-	+	-	-
	White variety 1, sample 2	Xinomavro	Sandy loam	103	Copper and sulfur, manure	-	-	-	-	+	+	-	-

12 List of publications and student projects

Published articles

Frisch, Lisa M., Hackhofer, Magdalena A., Niessen, Ludwig (2019): Entwicklung von LAMP-Assays zur Detektion von Schimmelpilzen in Lebensmitteln. LABO 12 (51).

Frisch, Lisa M., Mann, Magdalena A., Marek, David N., Baudrexl, Melanie, Vogel, Rudi F., Niessen, Ludwig (2021): Studies on the gushing potential of *Penicillium expansum*. Food Research International 139.
Parts of this publication are constituents of the results and discussion chapters 3.1 and 3.2 as well as 4.1 and 4.2 and revisited in the introduction and methods parts.

Frisch, Lisa M., Mann, Magdalena A., Marek, David N., Niessen, Ludwig (2021): Development and optimization of a loop-mediated isothermal amplification (LAMP) assay for the species-specific detection of *Penicillium expansum*. Food Microbiology 95.
Parts of this publication are constituents of the results and discussion chapters 3.4.1 as well as 4.4.1 and revisited in the introduction and methods parts.

Mann, Magdalena A., Frisch, Lisa M., Vogel, Rudi F., Niessen, Ludwig (2021): Influence of fermentation conditions on the secretion of seripauperin 5 (PAU5) by industrial sparkling wine strains of *Saccharomyces cerevisiae*. Food Research International 139.

Oral presentations

Frisch, Lisa M., Vogt-Hrabak, Elisabeth I., Niessen, Ludwig (2019): LAMP- a fast method for on-site detection of fungal infections in grapes. Presented at “Science & Wine World Congress”, 08.05.2019-10.05.2019, Porto, Portugal.

Poster presentations

Frisch, Lisa M., Niessen, Ludwig (2019): Development and optimization of a group-specific loop-mediated isothermal amplification (LAMP) assay for the detection of patulin-producing *Penicillium* species. Presented at “Conference of the International Commission on Food Mycology”, 03.06.2019-05.06.2019, Freising, Germany.

Frisch, Lisa M., Mann, Magdalena A., Vogel, Rudi F., Niessen, Ludwig (2021): LAMP- a modern tool for the detection of fungal infections in the vineyard. Presented at “Macrowine 2021”, 23.06.2021-30.06.2021, virtual.

Student theses and projects

The following students supervised by the author gave the permission to partially incorporate the obtained raw data into this thesis:

Marek, David (2019): Investigations on the gushing potential of *Penicillium expansum* and development of a detection method. Master's Thesis.

Pauli, Sarah (2019): Untersuchungen zur Rolle des Proteins ns-LTP1 aus der Weintraube (*Vitis vinifera*) beim Gushing von Sekt und Schaumwein. Bachelor's Thesis.

Hinrichs, Katharina (2020): Vorkommen der Gushing-relevanten Pilze *Penicillium oxalicum*, *Penicillium expansum* und *Botrytis cinerea*. Literature research project.

Diana Neif (2021): Detektion des Vorkommens Gushing-relevanter Pilze in europäischen Weinbergen durch *Loop-mediated isothermal amplification* (LAMP) Assays. Research internship.

David Cepeda (2021): Development of an ELISA assay for the relative quantitative detection of ns-LTP1 from *Vitis vinifera* in sparkling wine. Research internship.

Mauer, Sebastian (2021): Einfluss von Proteinen auf die Schaumeigenschaften von Sekt. Literature research project.