TECHNISCHE UNIVERSITÄT MÜNCHEN

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

# Influence of grape-associated filamentous fungi and their exoproteome on the gushing in sparkling wine

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# 1 Introduction

## 1.1 Wine

Winemaking is a very old technology, which dates back to thousands of years before Christ (bC). For this esteemed and noble beverage, both the ancient Greeks and Romans even had their own divinities, Dionysos and Bacchus, respectively. The main raw material for this product are the berries of the vine plant, *Vitis vinifera*. The juice of these fruits is released by pressing and by subsequent yeast fermentation of the fruit's sugars, upon which ethanol and aroma compounds are formed (Vogt *et al.*, 1979).

Initially by accident, but later (around the year of 1700) strongly desired, wine can undergo a transformation to sparkling wine. If the liquid contains (residual) sugar and living yeasts, a second fermentation can take place introducing carbon dioxide into the beverage. In principle, also during the first fermentation of the must the resulting beverage can be enriched with carbon dioxide. This production process termed "méthode rurale" was mainly applied to formerly regional products like *Asti spumante* in Italy or *Blanquette de Limoux* in France. Today however, most sparkling wine producers prefer the fermentation of base wine to obtain products like Champagne or German *Sekt*, since base wine has a higher microbial stability than musts so they can be used on demand (Troost *et al.*, 1995).

The quality of sparkling wine greatly depends on the quality of the raw material: the grapes produced in the vineyard. Obviously, insects (see Figure 1) or birds as well as the weather in terms of strong rain, hail or highly intensive irradiation can cause mechanical damage to the fruits (Curtis *et al.*, 1994; Gadoury *et al.*, 2007; Vinet, 2001). The resulting microfractures may serve as entry points for saprophytic microorganisms (Belin, 1972; Renouf *et al.*, 2005; Serra *et al.*, 2005): Fungal spores can germinate and grow on poor quality grapes, lactic- and acetic acid bacteria assert themselves against other bacteria on the grape skin in the presence of leaking grape juice, and yeasts concentrate and grow around cracks on the grape surface.

**Figure 1:** Life in the vineyard. As an example different insects (*Apis mellifera*, *Halyzia sedecimguttata*) on *Vitis vinifera* variety Riesling are displayed. Pictures obtained with permission from Kimmo Siren (Weincampus, Neustadt).

### 1.1.1 Basic procedure of winemaking

An overview about the different production steps in winemaking is given in Figure 2.

Winemaking starts already with the harvest of grapes. As the quality of the harvested grapes is essential for the quality of the final wine (Vogt *et al.*, 1979), sound grapes are carefully selected. However, some grapes are unavoidably broken during harvest requiring a quick way of working to avoid oxidation. By destemming extraneous material like leaves or stems are completely removed from the grapes. The next processing step, entitled as crushing, involves the rupturing of the grape skin to release some of the juice. The resulting mash is treated differently, depending on the desired color of the wine. (Jackson, 2000)

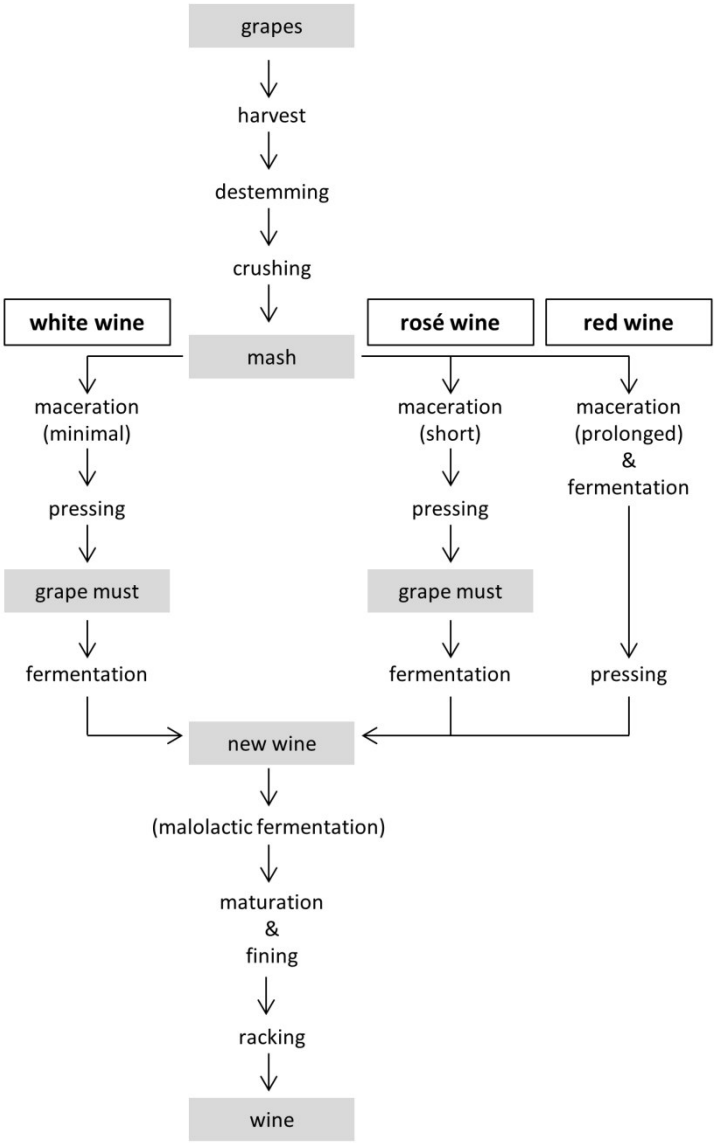


**Figure 2:** Flowchart of basic winemaking.

In particular, the process of maceration includes the leaching of phenolic compounds, pigments or enzymes from the solid constituents of the grape into the juice. Consequently, for white wine maceration is preferably short and followed immediately by pressing to remove the pomace and to obtain grape must. Subsequently, the must is fermented most commonly with *S. cerevisiae* wine

starter strains, resulting in new wine. In contrast, red wines macerate for hours till days or even weeks often accompanied by simultaneous heating. Therefore, several coloring compounds like anthocyanins are extracted along with tannins and other polyphenols. Furthermore, fermentation occurs at the same time spontaneously or induced by the addition of yeast cultures. By pressing of the mash the new wine is obtained. (Jackson, 2000; Vogt *et al.*, 1979) Maceration of rosé wines is kept very short to leach only a few pigments into the must. After pressing, fermentation of the must is performed similar to the procedure for white wines. (Jackson, 2000)

After the production of the new wine, all sorts of wine are treated in the same way. Malolactic fermentation can be induced to reduce the acidity of wine. Natural maturation occurs by aging at best under avoidance of air. During aging cells of microorganisms, remainder of the grapes or precipitates of proteins and phenolic compounds start to form a sediment. By racking, this sediment is separated after several weeks. Furthermore, several fining treatments as well as filtrations to stabilize the wine or to alter the aroma can be applied. Afterwards the wine is bottled or stored as base wine for sparkling wine production. (Jackson, 2000)

### 1.1.2 Methods for sparkling wine production

A second fermentation of base wine results in the production of a sparkling wine. Therefore, a *cuveé* is prepared by the blending of various base wines often from different varieties, vintages or origins. Yeast cultures are added along with a special sucrose solution, the so-called *tirage*, to supply enough sugar (~24 g/l) for a second fermentation including carbon dioxide production. (Jackson, 2000)

Depending on the production method employed for the sparkling wine production, further processing steps differ as depicted in Figure 3.

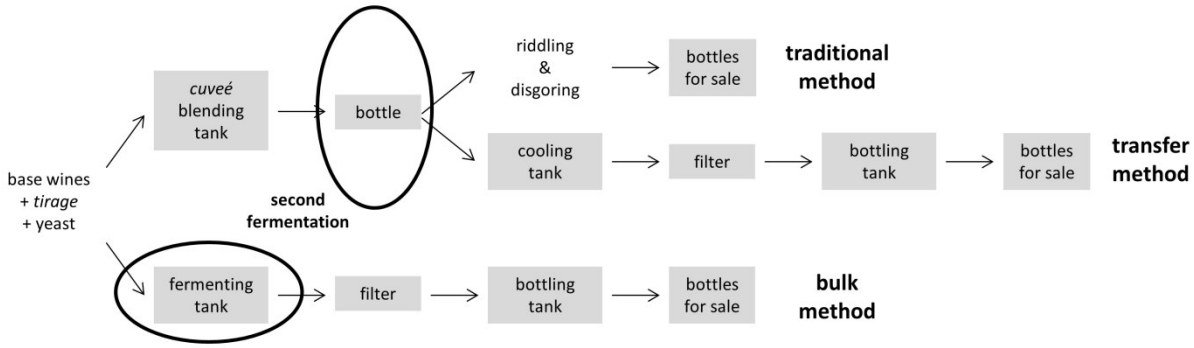


**Figure 3:** Flowchart of different methods for the production of sparkling wine.

According to the traditional method the *cuveé* is bottled and undergoes a second fermentation inside the bottle. By neck-down riddling the sediment consisting of yeast cells and other solid compounds is transferred as well as condensed at the bottle neck. The resulting sediment can be removed by disgorging, in which the bottle neck is frozen and the icy plug is removed by the pressure building up in the bottle after turning head up. Subsequently, bottles are amended with a *dosage* solution (concentrated sucrose solution, dissolved in wine) to compensate sparkling wine loss during disgorging. (Jackson, 2000; Troost *et al.*, 1995)

In the transfer method the second fermentation also occurs inside a bottle, but the previous time-consuming and cumbersome work of riddling and disgorging is omitted. Instead, after the second fermentation the new sparkling wine is transferred into a cooling tank pressurized with carbon

dioxide. Subsequently, the sediment is removed via filtration or centrifugation. Before bottling, the new sparkling wine is also added a *dosage* solution. (Jackson, 2000; Troost *et al.*, 1995)

In contrast, in the bulk method the second fermentation takes place in the *cuveé* blending tank, which is consequently called fermenting tank. After its completion, the new sparkling wine is filtered or centrifuged to remove the sediment. In bottling tanks a *dosage* can be added before bottling. (Jackson, 2000; Troost *et al.*, 1995)

### 1.1.3 Microbiota in the vineyard with emphasis on filamentous fungi

Grapes in the vineyard get commonly infected by various microorganisms. Besides different bacteria such as e.g. acetic acid bacteria, lactic acid bacteria or species of *Enterobacter*, *Staphylococcus* or *Bacillus* also many yeasts such as *Rhodotorula* spp., *Cryptococcus* spp. or *Sporodiobolus* spp. belong to the natural microbiota of grapes. As recently described in the detailed reviews by Loureirol *et al.* (2012) and by Barata *et al.* (2012), also several filamentous fungi occur regularly on grape berries. Usually, these opportunists act as saprophytes living in microfractures or other wounds of the berry skin. In contrast to the saprophytic filamentous fungi, a few fungi like the pathogen for downy mildew (*Plasmapara viticola*) or for powdery mildew (*Erysiphe necator*) act as obligate parasites and penetrate the intact grape skin causing devastating harvest losses (Barata *et al.*, 2012).

**Table 1:** Overview of *Aspergillus* and *Penicillium* species found in different European vineyards. Presence of a fungus in the corresponding country is marked with an x on gray background. [a] = data obtained from Bau *et al.* (2005); Bragulat *et al.* (2008); Gómez *et al.* (2006), [b] = Bejaoui *et al.* (2006); Sage *et al.* (2002), [c] = Abrunhosa *et al.* (2001); Serra *et al.* (2006a); Serra *et al.* (2006b), [d] = Battilani *et al.* (2003); Battilani *et al.* (2006); Lorenzini *et al.* (2016), [e] = Varga *et al.* (2007), [f] = (Ostry *et al.*, 2007); Varga *et al.* (2007) [g] = Felšöciová *et al.* (2015a); Felšöciová *et al.* (2015b); Mikušová *et al.* (2010); Mikušová *et al.* (2014); Santini *et al.* (2014)

| | Spain[a] | France[b] | Portugal[c] | Italy[d] | Hungary[e] | Czech Republic[f] | Slovakia[g] |
|---|---|---|---|---|---|---|---|
| ***Aspergillus carbonarius*** | x | x | x | x | | | x |
| ***A. clavatus*** | | | | | | x | x |
| ***A. flavipes*** | | | x | | | | |
| ***A. flavus*** | x | x | x | x | x | | x |
| ***A. fumigatus*** | x | x | x | x | x | | |
| ***A. ibericus*** | | | x | | | | x |
| ***A. japonicus var. aculeatus*** | x | x | x | x | | | x |
| ***A. melleus*** | x | | | | | | |
| ***A. nidulans*** | x | x | | | | | |
| ***A. niger*** | x | x | x | x | x | | x |
| ***A. ochraceus*** | x | | x | x | | | |
| ***A. ostianus*** | | | x | | | | x |
| ***A. parasiticus*** | x | x | | | | | x |
| ***A. sydowii*** | | | | x | | | |
| ***A. tamarii*** | x | | | | | | |
| ***A. terreus*** | x | x | x | | | | |
| ***A. tubingiensis*** | | | | x | x | | |
| ***A. ustus*** | x | x | x | | | | |
| ***A. uvarum*** | | | | x | | | x |
| ***A. variecolor*** | x | | | | | | |
| ***A. versicolor*** | x | | x | | | | x |

| | Spain[a] | France[b] | Portugal[c] | Italy[d] | Hungary[e] | Czech Republic[f] | Slovakia[g] |
|---|---|---|---|---|---|---|---|
| ***A. welwitschiae*** | | | | x | | | |
| ***A. wentii*** | x | | x | | | | |
| ***Penicillium adametzoides*** | | x | | | | | |
| ***P. atramentosum*** | | x | | | x | | |
| ***P. aurantiogriseum*** | | | x | | | x | x |
| ***P. bilaii*** | | | x | | | | |
| ***P. brevicompactum*** | x | x | x | | x | | x |
| ***P. canescens*** | x | x | | | | | |
| ***P. chermesinum*** | | | x | | | | |
| ***P. chrysogenum*** | x | x | | | x | | x |
| ***P. citreonigrum*** | | x | | | | | |
| ***P. citrinum*** | x | x | x | | | | x |
| ***P. coprophylum*** | | | | | | | x |
| ***P. corylophylum*** | x | | x | | | | x |
| ***P. crustosum*** | | x | x | x | | | x |
| ***P. decumbens*** | x | | | | | | |
| ***P. echinulatum*** | | x | x | | | | |
| ***P. expansum*** | x | x | x | x | x | x | x |
| ***P. funiculosum*** | | | x | x | | | x |
| ***P. glabrum*** | x | x | x | x | | | x |
| ***P. glandicola*** | | x | | | | | |
| ***P. griseofulvum*** | x | x | | x | | | x |
| ***P. implicatum*** | | | x | | | | |
| ***P. italicum*** | x | | | | | | |
| ***P. miczynskii*** | | x | x | | | | |
| ***P. minioluteum*** | | x | x | | | | |
| ***P. novae-zelandiae*** | | | x | | | | |
| ***P. oxalicum*** | x | x | x | x | | | x |
| ***P. palitans*** | | | | | | | x |
| ***P. paxili*** | | x | | | | | |
| ***P. pinophilum*** | x | | | | | | |
| ***P. purpurogenum*** | x | x | x | | x | | x |
| ***P. raistrickii*** | | | x | | | | |
| ***P. restrictum*** | | | x | | | | |
| ***P. roquefortii*** | | | x | | | | x |
| ***P. rugulosum*** | | x | | | | | |
| ***P. sclerotiorum*** | x | | x | | | | |
| ***P. simplicissimum*** | | | x | | | | |
| ***P. solitum*** | | | | | | | x |
| ***P. spinulosum*** | x | x | x | x | | x | |
| ***P. thomii*** | x | x | x | x | | | x |
| ***P. ubiquetum*** | | | | x | | | |
| ***P. variabile*** | x | | x | | | | |
| ***P. verrucosum*** | x | | | | | | x |
| ***P. waksmanii*** | | | x | | | | |

Despite differences in the diversity of the mycobiota depending on climatic and regional factors, the following saprophytic fungal genera have been frequently found in the vineyard in several studies all over Europe: *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium* and *Penicillium* (Abrunhosa *et al.*, 2001; Bau *et al.*, 2005; Belli *et al.*, 2006; Felšöciová *et al.*, 2015a; Lorenzini *et al.*, 2016; Sage *et al.*, 2002; Serra *et al.*, 2006a; Varga *et al.*, 2007). As typical producers of several different mycotoxins, especially the occurrence of *Aspergillus* and *Penicillium* species in European vineyards has been assessed. A European-wide survey about the "risk assessment and integrated ochratoxin A management in grape and wine" focused on the identification of those two genera, in particular. In Table 1 the results of this and similar follow-up studies all over Europe are qualitatively summarized. While *Aspergillus* spp. are the predominant species in Spain and Italy (Battilani *et al.*, 2003; Bau *et al.*, 2005; Gómez *et al.*, 2006), *Penicillium* spp. occur predominantly in Hungary, Czech Republic, and Slovakia (Felšöciová *et al.*, 2015b; Ostry *et al.*, 2007; Varga *et al.*, 2007). Nevertheless, both genera were found in all assessed vineyards. Depending on the geographical position of the vineyards within Portugal or France, either *Penicillium* spp. or *Aspergillus* spp. were found to be more dominant (Abrunhosa *et al.*, 2001; Bejaoui *et al.*, 2006; Sage *et al.*, 2002; Serra *et al.*, 2006a). No data have been published yet about the occurrence of different *Aspergillus* and *Penicillium* species in German, Austrian or Swiss vineyards.

Beside species of *Aspergillus* and *Penicillium* it should be mentioned here that a wide variety of filamentous fungi from other genera occur infrequently on grapes. Just to mention a few: *Acremonium* spp., *Epicoccum* spp., *Mucor* spp., *Fusarium* spp. as well as many others were detected during the aforementioned studies (Felšöciová *et al.*, 2015b; Sage *et al.*, 2002; Serra *et al.*, 2006a).

## 1.2 Gushing of carbonated beverages

Gushing is a phenomenon known to the beverage industry worldwide. It is described as the spontaneous overfoaming of a carbonated beverage upon opening of a bottle, despite correct handling. Besides substantial economic losses, gushing causes damage to the image of affected companies. Most research has been focused on gushing in beer with currently more than 10,000 publications available. However, other beverages like fruit spritzers, cider or sparkling wine are also prone to this unwanted phenomenon (Bach, 2001; Christian *et al.*, 2010; Liger-Belair *et al.*, 2013a; Schumacher, 2002; Wilson, 1999) but have been analyzed to a much lesser extent.

### 1.2.1 Definition and classification

Gushing is known since English brewers first filled beer into bottles at around 1650 (Beattie, 1951). However, the first research paper dealing with this phenomenon dates back to the early 20$^{th}$ century (Kastner, 1909). The phenomenon is described as a sudden, explosive degassing upon opening of a bottle of a carbonated beverage with the formation of a vast number of gas bubbles inside the liquid. They rise immediately to the surface causing an eruptive overfoaming of the drink (Pellaud, 2002). An even more pictorial and logical definition for gushing is given by Liger-Belair *et al.* (2013a): The volume of the ascending gas bubbles exceeds the volume of the bottle neck.

The most popular classification of gushing was proposed for beer by the Carlsberg research group (Gjertsen *et al.*, 1963; Gjertsen, 1967). According to their classification, primary gushing occurs epidemically and is associated with the raw materials used for beer production. In contrast, secondary gushing is associated with technical failure or hygienic problems during the production process. Long time before this, Vogel (1949) already proposed three different types of gushing beers: mistreated beer (e.g. by severe shaking), wild beer (e.g. due to over-carbonation or an access of air inside the liquid) and real gushing beer, where the reasons for gushing are not clearly assignable to any of the mentioned factors. A more recent classification by Pellaud (2002) differentiates the causes for beer gushing into three categories. Factors affecting microbubbles by increasing their stability (e.g. surface-active proteins), factors which increase the number of microbubbles (e.g. mechanical stress) and factors influencing the potency of microbubbles (e.g. carbon dioxide level).

In sparkling wine, German researchers speak of “echten” (German for true) and “unechten” (German for false) overfoaming (Bach, 2001; Würdig & Müller, 1979). True overfoaming is caused by factors coming from the beverage itself, whereas false overfoaming is caused by solid particles e.g. of lees, filtration- and fining agents (Bach, 2001). Consequently, true overfoaming and primary gushing as well as false overfoaming and secondary gushing seem to be equivalent terms for the same phenomena in different beverages. French researchers differentiate the reasons for “le gerbage” (French for gushing) in sparkling wine into organic and mineral (e.g. calcium tartrate) (Liger-Belair *et al.*, 2013a). This classification is more narrowly defined but it still resembles the definition of primary and secondary gushing in beer. An overview of the different gushing classifications in beer and sparkling wine is given in Table 2.

**Table 2:** Classification of gushing-types in beer and sparkling wine.

| beverage | gushing classification | reference |
|---|---|---|
| **beer** | primary vs. secondary gushing | Gjertsen *et al.* (1963); Gjertsen (1967) |
| | stability vs. number vs. potency of microbubbles | Pellaud (2002) |
| | mistreated vs. wild vs. gushing beer | Vogel (1949) |
| **sparkling wine** | “echtes” vs. “unechtes” overfoaming | Bach (2001); Würdig and Müller (1979) |
| | organic vs. mineral induced | Liger-Belair *et al.* (2013a) |

Further studies by Liger-Belair *et al.* (2013b) described a (matrix independent) mathematical model for the explanation of possible gushing affecting factors. In this article the authors showed by mathematical derivations that gushing depends on the volume of a gas bubble, the frequency with which bubbles rise from the liquid and the average lifespan of a bubble at the surface. The higher these factors are the higher is the risk for the occurrence of gushing in sparkling wine. Even though these factors mainly depend on physical parameters (temperature, concentration and solubility of carbon dioxide, viscosity, hydrostatic pressure and travel distance of a gas bubble in the liquid) – the lifespan of a bubble at the surface also depends on the drainage rate, which is influenced among others by amphiphilic molecules.

In sparkling wine these surface-active molecules can have their origin in the grape berry, the yeast (Dambrouck *et al.*, 2003) as well as in filamentous fungi or bacteria (Cilindre *et al.*, 2008; Kwon, 2004). Consequently, their composition differs in each wine displaying the one mysterious and unknown parameter for the induction of gushing.

### 1.2.2 Basic principles of bubble formation

Upon opening of a bottle of a carbonated beverage, carbon dioxide changes from a dissolved into a gaseous form due to pressure release. This process usually takes place slowly without any overfoaming. In case of gushing, a big quantity of $CO_2$ is transformed into its gaseous form almost instantaneously resulting in bubble formation and volume multiplication, which forces large quantities of liquid out of the open bottle. As carbon dioxide is the driving force of the phenomenon, over-carbonation was demonstrated to favor gushing of beer (Dachs & Nitschke, 1977; Pellaud, 2002; Wershofen, 2004). Nevertheless, an elevated level of carbonation alone does not trigger gushing: Despite an about two times higher $CO_2$ content in sparkling wine (sparkling wine up to 12 g/l, bottom-fermented beer 4-5 g/l) (Fischer, 2001), this beverage is no more prone to gushing than beer. Nevertheless, there is a correlation between the pressure inside the bottle and the volume of overfoaming of a beverage (Würdig & Müller, 1979). However, to induce gushing, the presence of gas nuclei is necessary for formation and growth of bubbles in a solution. According to the theory of microbubbles established by Guggenberger and Kleber (1963) for gushing in beer, gas bubbles enter the liquid by mechanical agitation (e.g. turbulence during filling, shaking during transport). After their formation, bubbles with a diameter exceeding 10-100 µm will rapidly rise to the surface due to buoyancy. Smaller bubbles remain in the liquid but will vanish very soon due to dissolution of their gaseous content depending on surface tension and hydrostatic pressure. An exception to this rule is bubbles filled with air. The main components of air are nitrogen (78.08 % v/v) and oxygen (20.95 % v/v). Carbon dioxide is a very minor gas component (0.04 % v/v). It is well established that the solubility of oxygen in aqueous alcohol solutions is lower than that of carbon dioxide. Nitrogen has by far the lowest solubility of those three gases (Tokunaga, 1975). Air bubbles introduced into the beverage during filling will therefore rapidly get devoid of carbon dioxide and, more slowly, of oxygen. In contrast, due to the minimal solubility of nitrogen, small nitrogen-filled gas bubbles will remain inside the liquid. They are too small to ascend to the surface, consequently serving as potential nucleation sites for carbon dioxide during gushing (Loitsch, 2000; Schumacher, 2002). Another exception is the presence of surface-active molecules in the surrounding liquid causing an accumulation of those molecules at the gas/water interface to stabilize the gas bubble. For such coated bubbles the surface tension is dramatically reduced, resulting in the loss of their spheroidal shape. However, such gas bubbles have an extended life span, do not get dissolved and are able to persist in in the liquid. Such microbubbles can than act as nucleation sites, although a consistent agreement on the exact mechanism has yet to be reached among experts (Deckers *et al.*, 2012; Draeger, 1996; Franke *et al.*, 1995; Liger-Belair *et al.*, 2013a; Niessen *et al.*, 2006). Insight into the slightly different theories is given in the following section.

### 1.2.3 Suggested mechanisms for primary gushing in beer

Different mechanisms for the occurrence of primary gushing have been proposed. As previously described in section 1.2.2, theories for the underlying mechanism for gushing of beer have been based on the presence of microbubbles as described by Guggenberger and Kleber (1963). However, almost all theories are also based on the varying-permeability model (VMP) described by Yount (1982). According to this model, surface-active molecules accumulate around gas nuclei, thus stabilizing them by a reduction of surface tension. However, the permeability of this thin film of surface-active molecules varies depending on the ambient pressure. Therefore, in a closed bottle with high pressure the diffusion of gas from the bubble into the surrounding liquid is reduced to zero. Opening a bottle of a carbonated beverage, however, results in a crucial pressure drop with the membrane of surface-active molecules becoming permeable again. As a result, the volume of the gas bubble multiplies abruptly. However, Yount worked on decompression sickness, therefore, he never intended to explain gushing in carbonated beverages with his theory.

On the basis of the two mentioned theories, several authors discussed the generation of bubbles as well as the gushing phenomenon from a physical point of view (Draeger, 1996; Franke *et al.*, 1995; Pellaud, 2002). They agreed on a heterogeneous bubble formation with already present gas nuclei. According to these authors, bubble formation during primary gushing occurs ubiquitous in the liquid and must therefore be based on microbubbles and not on solid particles as nucleation sites. These authors confirm the presence of a large number of stabilized microbubbles in the liquid as a necessary prerequisite for bubble formation, growth and thus gushing.

A more recently proposed gushing mechanism of Niessen *et al.* (2006) implies that gushing can be traced back to a shift in the relation of gushing-inducing and gushing-suppressing substances. The proposed mechanism assumes that the main component of surface-active substances in beer, the non-specific lipid transfer protein 1 (nsLTP1), is drastically reduced or even absent in gushing samples (Zapf *et al.*, 2005). At the same time, fungal surfactants like hydrophobins are present. Representatives of both kinds of proteins accumulate at the interface of microbubbles in order to stabilize them, but accumulate to patches within their protein species. As a result, rafts between patches of e.g. hydrophobins and the surrounding nsLTP1 occur on the bubble skin. During the sudden pressure drop upon opening of the bottle the coated bubbles grow but the bubble skin is not very elastic due to its heterogeneity and the missing adhesion between the different groups of amphiphilic proteins. Therefore it disrupts easily into smaller fragments which exhibit their gas contaminated hydrophobic surface freely to the surrounding aqueous solution serving as nucleation sites for the release of dissolved carbon dioxide. As a result, large numbers of bubbles are formed in short time, leading to gushing of the beverage.

Hippeli and Elstner (2002) and Hippeli and Hecht (2009) assumed that not only hydrophobins but also nsLTP1 or at least cleavage products of this protein are responsible for the induction of gushing by serving as nucleation points for dissolved $CO_2$ in beer. However, they postulate the production of such cleavage products as a result of fungal infection of the grain accompanied by the secretion of fungal proteases.

Christian *et al.* (2009) proposed a mechanism were cationic, aliphatic surfactants such as hexadecyltrimethylammonium chloride (CTAC) forms micelles. This process takes time and depends on the structure of the surfactant as well as the critical micellar concentration (CMC) but could be one reason for the explanation of gushing without shaking. Upon diffusion of $CO_2$ into the inner part of the micelle the aggregate grows and forms stabilized microbubbles. Upon opening of the bottled beverage these microbubbles finally induce gushing.

According to Deckers *et al.* (2012) gushing is connected to the presence of "nanobombs" in a beverage. Their theory implies an interaction of the hydrophobic patch of fungal hydrophobins with carbon dioxide. Consequently, an aggregation of hydrophobins at the interface between beer and $CO_2$ in the bottle neck forms a crystalline monolayer. By shaking this monolayer folds burying $CO_2$ molecules inside and, thus, creating stabilized nanobubbles for which they coined the term "nanobombs". Upon opening of the bottle the nanobubbles expand explosively providing sufficient energy for new nucleation sites and the transition of dissolved carbon dioxide into its gaseous form in the close vicinity of such a bubble. Bubbles resulting from the new nucleation site are filled with gaseous carbon dioxide and ascend to the surface by buoyancy and cause vigorous overfoaming.

All approaches to explain the mechanism of primary gushing are based on surface-active molecules stabilizing small gas quantities in the beverage. At the same time, several theories accuse these surfactants to act as nucleation sites for the sudden release of dissolved carbon dioxide in beer, thus, to result in gushing of the beverage.

### 1.2.4 Surface-active proteins involved in beer gushing

Several classes of plant or fungus derived proteins have been investigated in regard to their influence on gushing in beer.

Two proteins, Z4 and nsLTP1 from barley present the largest proportion of surface-active proteins in beer and, in particular, its foam (Gibson *et al.*, 1996; Sorensen *et al.*, 1993). Since nsLTP1 is a plant protein serving in defense reactions of the plant against microbial attack its expression is upregulated upon infection of the plant with filamentous fungi. As mentioned earlier, Hippeli and Elstner (2002) have suggested that nsLTP1 induces gushing in beer when present in concentrations above a certain threshold. In contrast, Zapf *et al.* (2006) showed that a transgenic yeast strain producing vast amounts of barley nsLTP1 did not induce gushing in experimental beer. Later on, Hippeli and Hecht (2009) set up a new hypothesis when they found that gushing beers had a significantly lower concentration of nsLTP1 as compared to non-gushing beers of comparable make: According to their theory, fungal infection of brewing malt is accompanied by the production of fungal proteases which cleave nsLTP1 that has been produced in high amounts as a reaction to the fungal attack. Using this mechanism, the attacking fungus neutralizes the defense reaction of the barley plant. According to the authors, the resulting cleavage products of nsLTP1 can induce gushing in beer. However, this hypothesis has never been verified experimentally. In contrast, Lutterschmid *et al.* (2011) gave experimental evidence that recombinant nsLTP1 had a gushing reducing effect when added to beer that had been supplemented with a gushing-inducing concentration of the *Fusarium* hydrophobin

FcHyd5p. In this experiment, also recombinant Z4 protein as the second predominant beer foam component showed some potential to impair FcHyd5p-induced gushing (Specker *et al.*, 2014). Albeit the exact nature of the influence which plant derived proteins, nsLTP1 and Z4, have on the gushing phenomenon in beer is still a matter of debate, the unmodified proteins seem to have an "anti-gushing" effect as demonstrated by the experiments.

In contrast to the gushing-reducing effect of plant proteins there is no doubt that gushing-inducing molecules can be produced by fungi. Already in the '70s of the last century, several research groups identified gushing factors produced by filamentous fungi such as *Nigrospora*, *Stemphylium*, *Rhizopus* or *Fusarium* (Amaha *et al.*, 1973; Kitabatake & Amaha, 1974; Kitabatake & Amaha, 1977; Yoshida *et al.*, 1975). Even though the identity of these gushing-inducing factors was not established at the time, all inducing molecules have been described as (poly)peptides or peptidoglycans (Amaha *et al.*, 1973; Kitabatake & Amaha, 1977). At about the same time, also species of *Penicillium* and *Aspergillu*s were found to induce gushing in beer (Fukushima *et al.*, 1976; Gyllang & Martinson, 1976; Yoshida *et al.*, 1975). The gushing-inducing factor of *Penicillium chrysogenum* was isolated and characterized as a cyclic tetrapeptide (Kitabatake *et al.*, 1980). Nevertheless, the major cause of primary gushing in beer is an infection of barley with species of the genus *Fusarium*, especially *F. graminearum, F. culmorum* or *F. crookwellense* (Donhauser, 1994; Gjertsen *et al.*, 1965; Niessen *et al.*, 1992; Schwarz *et al.*, 1996). By addition of uncharacterized acidic proteins concentrated by preparative isoelectric focusing from wheat grain heavily infected with *F. culmorum,* Weideneder (1992) was able to induce gushing in beer. Nowadays these acidic proteins would probably be identified as belonging to the well-established class of hydrophobins, which were still unpublished at the time when Weideneder did his experiments. Hydrophobins are small, hydrophobic, cysteine-rich, highly surface-active proteins produced and secreted by all filamentous fungi that produce aerial structures (Schuren & Wessels, 1990; Wessels *et al.*, 1991). They are involved in a variety of functions during the fungal life cycle and different hydrophobins may be produced by a fungal colony in different stages of its development. A major task of proteins of the hydrophobin type is to decrease the surface tension of the water surrounding a fungal hypha in order to overcome the air-water interface and allow the production of aerial structures (Wösten *et al.*, 1994a; Wösten *et al.*, 1999). However, these molecules have been demonstrated to also induce gushing when present in beer or in carbonated water. This was proven by addition of several different hydrophobins isolated from culture supernatants of *T. resei*, *F. poae* and *Nigrospora* sp. to formerly non-gushing beer (Sarlin *et al.*, 2005). Yeast transformed with the hydrophobin Fchyd5p from *F. culmorum* resulted in gushing beer when used in brewing experiments (Zapf *et al.*, 2006). Also recombinant hydrophobins such as FcHyd5p of *F. culmorum* or Hfb2 of *T. resei* were demonstrated to have a gushing-inducing effect (Lutterschmid *et al.*, 2010; Lutterschmid *et al.*, 2011; Stübner *et al.*, 2010). A list of gushing-inducing fungi with corresponding gushing factors described in the last decades is given in Table 3.

Besides hydrophobins, a further class of surface-active proteins produced and secreted by *F. culmorum* was detected by Zapf *et al.* (2007). These alkaline proteins were termed fungispumins and possess the ability to form aggregates but not to induce gushing. However, among them is AfpA,

the role of which in the gushing induction was contradictorily discussed. According to Zapf *et al.* (2007) AfpA increases the volume of over-foaming in gushing beer samples, whereas Lutterschmid *et al.* (2011) stated that AfpA has a gushing-reducing effect similar to nsLTP1. As AfpA is only detected in malts resulting in gushing beer when used for brewing but is not found in gushing-negative malts it was proposed as a useful marker protein for gushing potential in malt quality control (Zapf *et al.*, 2007).

**Table 3:** Some gushing-inducing fungi with partial characterization of corresponding gushing factors. n.d. = not determined

| gushing inducing organism | gushing inducing molecule | molecular weight [kDa] | concentration needed for gushing [ppm] | reference |
|---|---|---|---|---|
| ***Rhizopus* sp.** | polypeptide | n.d. | 0.06 | Amaha *et al.* (1973) |
| ***Stemphylium* sp.** | peptidoglycan | n.d. | 4 | |
| ***Fusarium* sp.** | peptide | n.d. | 0.4 | |
| ***Nigrospora* sp.** | polypeptide | 16.5 | 0.05 | Kitabatake and Amaha (1974) |
| | | | | Yoshida *et al.* (1975) |
| | | | | Kitabatake and Amaha (1977) |
| ***Penicillium chrysogenum*** | cyclic tetrapeptide | n.d. | 0.05 | Kitabatake *et al.* (1980) |
| | | | | Yoshida *et al.* (1975) |
| ***Aspergillus fumigatus*** | n.d. | n.d. | n.d. | Gyllang and Martinson (1976) |
| ***Aspergillus amstelodami*** | n.d. | n.d. | n.d. | |
| ***Fusarium culmorum* infected malt** | protein | 10.0 | n.d. | Weideneder (1992) |
| ***Fusarium poae*** | hydrophobin | 8.5 | 0.1 | Sarlin *et al.* (2005) |
| ***Nigrospora* sp.** | Hydrophobin | 8.5 | 0.03 | |
| ***Trichoderma resei*** | hydrophobin | 7.5 | 0.003 | |
| **Transgenic yeast expressing *Fusarium culmorum* protein** | recombinant hydrophobin FcHyd5p | 8.4 | (brewing experiments with yeast) | Zapf *et al.* (2006) |
| **Transgenic yeast expressing *Fusarium culmorum* protein** | purified, recombinant hydrophobin FcHyd5p | 8.4 | 1 | Lutterschmid *et al.* (2010) |
| | | | 0.43 | Stübner *et al.* (2010) |
| **Transgenic yeast expressing *Trichoderma resei* protein** | purified, recombinant hydrophobin Hfb2 | 7.2 | 0.1 | Lutterschmid *et al.* (2011) |

### 1.2.5 Prevention strategies for gushing in beer

As causes for gushing are diverse they cannot be overcome by a single solution. Consequently various measures have been applied to prevent gushing in beer. A small selection is described in the following paragraphs and summarized in Table 4. Nevertheless, it must be noted here that there have been even more approaches suggested to cope with the gushing phenomenon as described in the review of Postulkova *et al.* (2016).

One prevention strategy focuses on the avoidance of fungal contamination, in particular *Fusarium* spp., of barley in the field, already. Various possibilities for the generation of transgenic barley with resistance to fungi or insects as main transmitters of fungal spores exist (Linko *et al.*, 1998).

Furthermore, crop rotation is taken into consideration to suppress fungal activity as long as no maize is cultivated (Dill-Macky & Jones, 2000). As various fungicides are active against *Fusarium* spp., a chemical treatment of barley is also possible (Edwards *et al.*, 2001). By prolonged dry storage of crops fungal viability is reduced, in general (Beattie *et al.*, 1998). Physical destruction of fungi by static magnetic field (sMF) or electron beam irradiation has been successfully assessed for application during storage of cereal grain (Albertini *et al.*, 2003; Kottapalli *et al.*, 2003).

In the malt house, fungal contamination and hence the gushing risk can be reduced as well. Chlorine dioxide or ozone are effectively used against *Fusarium* spp. during the steeping process (Basar *et al.*, 2014; Kottapalli *et al.*, 2005). As *Fusarium* species are heat-sensitive, heating of steeping water as well as its acidification reduce *Fusarium* contamination (Kottapalli & Wolf-Hall, 2008). Biological control of *Fusarium* on the malting barley with different *Lactobacillus* species, *Geotrichum candidum* or *Pichia anomala* was also applied (Boivin & Malanda, 1997; Haikara *et al.*, 1993; Laitila *et al.*, 2002; Laitila *et al.*, 2007). An antifungal protein from *Aspergillus giganteus* is also classified as efficient to prevent growth of *Fusarium* spp. during malting (Barakat *et al.*, 2010).

**Table 4:** Prevention of fungal growth as well as other treatments to reduce the gushing risk in beer.

| site of action | methods to cope with gushing | reference |
|---|---|---|
| **in the field and during storage** | use of transgenic barley | Linko *et al.* (1998) |
| | crop rotation | Dill-Macky and Jones (2000) |
| | application of fungicides | Edwards *et al.* (2001) |
| | prolonged storage of crops | Beattie *et al.* (1998) |
| | physical destruction of fungi | Albertini *et al.* (2003); Kottapalli *et al.* (2003) |
| **in the malting house** | chemicals e.g. $O_3$ or $ClO_2$ for inhibition of fungal growth | Basar *et al.* (2014); Kottapalli *et al.* (2005) |
| | heating/acidification of steeping water to prevent fungal contamination | Kottapalli and Wolf-Hall (2008) |
| | biological control of *Fusarium* | Barakat *et al.* (2010); Boivin and Malanda (1997); Haikara *et al.* (1993); Laitila *et al.* (2002); Laitila *et al.* (2007) |
| **during the brewing process** | addition of anti-gushing reagents e.g. hop products, proteases etc. | Aastrup *et al.* (1996); Carrington *et al.* (1972); Hudson (1962); Müller *et al.* (2010) |
| | blending of gushing beer with non-gushing beer | Gjertsen (1967) |
| | early precipitation of $CaC_2O_4$ | Müller *et al.* (2013) |

Even during the brewing process different methods for gushing prevention have been applied. Adsorbents such as nylon powder show a gushing reducing effect when added to gushing beer (Hudson, 1962). Furthermore, several hop compounds are known as gushing suppressants and thus are added to beer to reduce the gushing risk (Carrington *et al.*, 1972; Müller *et al.*, 2010). However,

also the addition of different proteases showed a reduction in the overfoaming volume of faulty beer (Aastrup *et al.*, 1996). Moreover, a measure easily to apply is the blending of gushing beer with non-gushing beer (Gjertsen, 1967). Gushing-inducing substances are simply diluted to such a low concentration that they have no gushing-inducing effect anymore. Such a measure, however, would imply that a method for the detection of gushing previous to filling must be available. Moreover, to prevent precipitation of calcium oxalate serving as an additional nucleation site in gushing beer, calcium ions in form of calcium chloride, calcium sulphate or the commercially available product AnGus$^{1516®}$ have been added in excess before filtration. Due to an aggregation of oxalic acid and calcium ions a precipitate is formed which can be separated from the beer before bottling. (Müller *et al.*, 2013)

Despite all these different measures to reduce the gushing risk in beer, a method fully preventing beer gushing is still not available.

### 1.2.6 Factors inducing secondary gushing in carbonated beverages

In addition to technical failure during the production process of a carbonated beverage, also inorganic matter present in the beverage can induce secondary gushing (see Table 5).

**Table 5:** Partial list of factors associated with secondary gushing in beer and sparkling wine.

| factors for secondary gushing | example in beer | example in sparkling wine |
|---|---|---|
| **particles from bottles' closure** | Ferdinandus *et al.* (1962); Weideneder (1992) | Bach (2001); (Schanderl, 1964) |
| **filtration aids** | Donhauser (1994) | Bach (2001); Würdig and Müller (1979) |
| **cleansing agents** | Dachs and Nitschke (1977) | Würdig and Müller (1979) |
| **metal ions** | Gray and Stone (1958); Hudson (1962); Zepf (1998) | Schanderl (1964) |
| **hop compounds** | Rudin and Hudson (1958) | - |
| **crystals** | Zepf and Geiger (2000) | Liger-Belair *et al.* (2013a); Schanderl (1964) |
| **bottle surface** | Dachs and Nitschke (1977) | Hennig (1963) |
| **tannin precipitation** | - | Hennig (1963) |
| **fungal spores** | - | Schanderl (1964) |
| **air inside the beverage** | Fischer *et al.* (1997) | Schumacher (2002) |
| **high air headspace** | Dachs and Nitschke (1977) | - |
| **over-carbonation** | Dachs and Nitschke (1977) | Würdig and Müller (1979) |

It is a general assumption that alien particles in a carbonated beverage can serve as nucleation sites for the release of dissolved carbon dioxide in its gaseous form and thus induce gushing (Pellaud, 2002; Wershofen, 2004; Zarnkow & Back, 2001). Such particles can originate from the crown caps in beer (Ferdinandus *et al.*, 1962; Weideneder, 1992) or respectively from the cork in a bottle of sparkling wine (Bach, 2001; Schanderl, 1964). Moreover, in both beverages filtration aids such as diatomite (Bach, 2001; Donhauser, 1994; Würdig & Müller, 1979), cleansing agents (Dachs &

Nitschke, 1977; Würdig & Müller, 1979), metal ions (Rudin & Hudson, 1958; Schanderl, 1964) or crystals of organic or inorganic matter (Liger-Belair *et al.*, 2013a; Schanderl, 1964; Zepf & Geiger, 2000) can act as nucleation sites. Also a rough interior bottle surface can represent a source for foreign particles (Dachs & Nitschke, 1977; Hennig, 1963). Moreover, it was reported that tannin precipitations (Hennig, 1963) or fungal spores (Schanderl, 1964) can provoke gushing in sparkling wine. As already stated above, physical factors such as e.g. air inside the beverage, a high headspace of the bottle or an over-carbonation are also known to be triggers for secondary gushing (Dachs & Nitschke, 1977; Fischer *et al.*, 1997; Schumacher, 2002; Würdig & Müller, 1979).

Nevertheless, this type of gushing can be handled by a strict control of the production process and appropriate handling of the products (see also section 1.2.5).

### 1.2.7 Comparison of beer and sparkling wine

Sparkling wine and beer are both yeast fermented carbonated beverages prone to the gushing phenomenon. In beer, primary as well as secondary gushing have been intensively investigated and research is still in progress. Whereas in sparkling wine only potential reasons for secondary gushing are known (see Table 5). The surface-active trigger-substances for the induction of primary gushing in sparkling wine are yet unidentified.

Apart from a few common features, sparkling wine and beer differ in many respects (see Table 6). Both are made from plant materials: wine from grapes and beer from wheat, barley or other cereals or pseudocereals. As a result, the mycobiota of the raw materials differ widely due to water activity and pH. *Fusarium* spp., as the major cause for primary beer gushing (see section 1.2.4) frequently infect cereal grain (Niessen *et al.*, 1992). In contrast to this, *Fusarium* spp. are quite rare on grapes, whereas other fungi such as certain species of *Penicillium* and *Aspergillus* or *Botrytis cinerea* occur considerably more often on grapes than on cereals (see section 1.1.1).

In addition, at least German beer is subjected to the German Purity Law which prohibits the use of any fining except filtration, while wine as the basic ingredient for sparkling wine production is extensively treated with a variety of different fining agents. Fining agents such as bentonite or charcoal will extract several proteins from wine (Marchal *et al.*, 2002; Vanrell *et al.*, 2007) whereas other proteinaceous fining agents such as lysozyme, ovalbumin or casein are added during fining and remain in the beverage (Bresson *et al.*, 2007; Weber *et al.*, 2007). Therefore, the protein composition of the final product sparkling wine is heavily influenced by the production process. Another factor exclusively affecting the protein composition of sparkling wine is bacteria (D'Amato *et al.*, 2010; Kwon, 2004). In wine, a variety of bacterial species occur regularly (LAB, AAB, *Bacillus* spp.) without spoiling the beverage (Alexandre *et al.*, 2004; Bae *et al.*, 2004). In contrast, bacteria growing in beer (e.g. LAB) are undesired beer-spoiling organism (Sakamoto & Konings, 2003) so that their presence is associated with spoilage of the product. Furthermore, yeast autolysis is desired in sparkling wine to improve the wine's stability regarding tartrate precipitation (wine stone) (Gerbaud *et al.*, 1997; Lubbers *et al.*, 1993; Moine-Ledoux *et al.*, 1997) as well as haze formation (Dupin *et al.*, 2000; Ledoux *et al.*, 1992; Moine-Ledoux & Dubourdieu, 1999). Even foaming characteristics of sparkling wine can

be increased by addition of thermally extracted yeast cell wall compounds as demonstrated by Núñez *et al.* (2006). However, in beer yeast autolysis is unwanted due to the production of unpleasant, yeasty off-flavors as well as a possible formation of haze or a removal of foam stabilizing proteins by the yeast's proteases (Barnes, 2011; Staudt, 2017). It is therefore obvious that surface-active proteins in a flawless sparkling wine can originate from a variety of different sources, whereas in a flawless beer brewed according to the German Law of Purity the protein composition comes mainly from the malt and from the yeast.

Further differences between sparkling wine and beer include for example the before mentioned difference in the $CO_2$ content (see 1.2.2) or the addition of hops or hop extracts in beer. All these differences illustrate that not everything in gushing research can be transferred from one beverage to the other.

**Table 6:** Comparison of beer and sparkling wine.

| | beer | sparkling wine |
|---|---|---|
| **raw material** | wheat/barley | grapes |
| **filamentous fungi on raw material influencing gushing** | *Fusarium* spp. etc. | *Penicillium*, *Aspergillus*, *Botrytis* etc. |
| | *Fusarium* spp. (see Table 3) | unknown |
| **fining application** | no (forbidden by German Purity Law) | yes |
| **bacteria in the beverage** | no (only as spoilage organism) | yes (e.g. for malolactic fermentation) |
| **yeast autolysis** | unwanted | desired |
| **$CO_2$ content** | 4-5 g/l | ~12 g/l |
| **hop** | yes | no |

## 1.3 Motivation, hypotheses and objectives

Besides the aromatic components, the effervescence and foam formation in sparkling wine are the most important and most apparent characteristics of this product for the consumer. However, batches are observed that show vigorous overfoaming of the beverage referred to as gushing. Affected lots are unsellable. In particular years, the number of complaints related to gushing may account for up to 30 % of the total number of complaints in Germany. This corresponds to an annual quantity of up to 8 million bottles in a total annual production of about 450 million bottles (according to the Association of German Sparkling Wine Producers). In addition to the costs incurred by the removal of the products from the market and the disposal of the affected batch, the occurrence of gushing means an overall damage to the brand's image. At worst, gushing can ruin small and medium-sized enterprises. Therefore, the understanding of the mechanisms involved in the gushing phenomenon in sparkling wine is essential in order to tackle this economic problem analytically or technically.

Gushing research in beer displayed a multifactorial phenomenon (Pellaud, 2002). Nevertheless, it is known that especially primary gushing is induced by an infection of barley with filamentous fungi of the genus *Fusarium* (Gjertsen *et al.*, 1965). Hydrophobins are one class of surface-active proteins, which are able to induce gushing in beer (Lutterschmid *et al.*, 2010; Sarlin *et al.*, 2005). Besides them, other plant-derived amphiphilic proteins have been suggested to influence the gushing mechanism in beer (Hippeli & Hecht, 2009).

The hypotheses underlying the current dissertation were the following:

- Filamentous fungi found on grapes can produce surface-active proteins.
- These fungal proteins induce gushing in sparkling wine with a mechanism similar to the one suggested for gushing in beer.
- Consequently, respective fungal proteins are present in gushing sparkling wine but absent in non-gushing sparkling wine.
- These fungal proteins are secreted in the surrounding medium, also in laboratory cultures.
- Therefore surface-active proteins can be isolated and identified *in vitro*.
- Furthermore, the potential of these surface-active proteins to induce gushing can be demonstrated.
- Consequently, these proteins can be used for the development of an assay for the monitoring of these proteins already in base wine.

From these working hypotheses the following approaches and objectives were derived:

- Comparative proteomics should be performed of gushing and non-gushing sparkling wine in order to detect gushing-influencing proteins.
- Gushing-influencing proteins should be identified.
- Respective fungi that produce these proteins should be grown in the laboratory to prove the production and especially the secretion of gushing-influencing factors.
- The ability of the fungi to alter the surface activity of culture supernatant should be monitored.
- The possibility to enrich surface-active proteins by foam fractionation should be demonstrated.
- Surface-active proteins of the fungal exoproteome should be characterized by various protein chemical methods.
- Artificial gushing should be induced by addition of fungal proteins to sparkling water as a model system to prove the gushing-inducing potential of these proteins.
- Antibodies should be produced targeting the respective gushing-inducing proteins.
- An immunochemical assay should be developed and established allowing the detection of gushing-inducing proteins in base and sparkling wine.

# 2 Materials and methods

## 2.1 Materials

### 2.1.1 Chemicals

Chemicals used in this work are listed in Table 7 including reference to their source and purity.

**Table 7:** List of chemicals used in this study.

| chemical | purity grade | manufacturer |
|---|---|---|
| **2,5-dihydroxybenzoic acid (DHBA)** | | Bruker Daltonics GmbH, Bremen, Germany |
| **2,6-dichloro-4-nitroaniline (dichloran)** | for synthesis | Merck KGaA, Darmstadt, Germany |
| **2-butanol** | ≥ 98.5 %, for synthesis | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **2-iodoacetamide** | BioChemica | AppliChem GmbH, Darmstadt, Germany |
| **2-mercaptoethanol** | BioReagent, 99 % | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **2-propanol** | ≥ 99.5 %, for synthesis | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **3-(N-morpholino)-propanesulfonic acid (MOPS)** | | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)** | | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **5-bromo-4-chloro-3-indolyl phosphate (BCIP) toluidine salt** | | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **acetic acid** | Rotipuran®, 100 %, p.a. | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **acetone** | ≥ 99 % | FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany |
| **acetonitrile (ACN)** | Rotisolv®, HPLC gradient grade | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **acrylamide /Bis solution 29:1** | (30 % w/v), 3.3 % C | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **agarose Biozym LE** | for electrophoresis | Biozym Scientific GmbH, Hessisch Oldendorf Germany |
| **albumin fraction V (bovine serum albumin (BSA))** | ≥ 98 % | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **ammonium acetate** | ≥ 96 %, research grade | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **ammonium hydrogen carbonate** | purum, p.a., ≥ 99 % | FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany |
| **ammonium persulphate (APS)** | analytical grade | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **ammonium sulphate** | high purity | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **ampicillin sodium salt** | | GERBU Biotechnik GmbH, Heidelberg, Germany |

| chemical | purity grade | manufacturer |
|---|---|---|
| **antifoam B Emulsion** | aqueous-silicone emulsion | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **bengal rose B** | | FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany |
| **bentonite** | pure | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **Berol 532** | | Julius Hoesch GmbH & Co. KG, Düren, Germany |
| **Berol 840** | | Akzo Nobel Surface Chemistry AB, Stenungsund, Schweden |
| **bromphenol blue** | for electrophoresis | AppliChem GmbH, Darmstadt, Germany |
| **boric acid** | | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **calcein** | for calcium analysis | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **chloramphenicol** | ≥ 98.5 %, Ph. Eur. | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **dimethylformamide (DMF)** | ≥ 99 % | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **dimidium bromide** | ≥ 95 % | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **disodium hydrogen phosphate dihydrate** | ≥ 98 %, Ph. Eur., USP | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **dipotassium hydrogen phosphate trihydrate** | for analysis, Emsure® | Merck KGaA, Darmstadt, Germany |
| **dithiothreitol (DTT)** | high purity | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **DNA loading dye** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **dNTP Mix (for PCR)** | | MP Biomedicals GmbH, Eschwege, Germany |
| **dNTPs (for LAMP)** | | Fermentas GmbH, Thermo Fisher Scientific Inc., St. Leon-Roth, Germany |
| **dodecyl maltoside (DDM)** | ultrapure | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **E.Z.N.A. cycle pure kit** | | Omega Bio-tek Inc., Norcross, USA |
| **ethanol** | ≥ 94 %, completely denaturated | CLN GmbH, Niederhummel, Germany |
| **ethanol absolute** | ACS Reag., Ph. Eur. | VWR International GmbH, Darmstadt Germany |
| **ethylendiaminetetra-acetic acid (EDTA) disodiumsalt dihydrate** | | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **FireSilver staining kit** | compatible with mass spectrometry | Proteome Factory AG, Berlin, Germany |
| **formaldehyde (37 %)** | ≥ 37 %, for synthesis | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **GeneJET gel extraction kit** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **GeneJET plasmid miniprep kit** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **GeneRuler 100 bp plus DNA ladder** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **glucose monohydrate** | for microbiology | Merck KGaA, Darmstadt, Germany |
| **glycerol (87 %)** | high purity, Ph. Eur. | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **glycerol (99 %)** | high purity | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **glycine** | for mol. biology & electrophoresis | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **hydrochloric acid, fuming (37 %)** | 37 %, technical | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |

| chemical | purity grade | manufacturer |
|---|---|---|
| **magnesium chloride** | ≥ 98.5 %, anhydrous | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **magnesium sulphate heptahydrate** | for analysis, ACS, Reag. Ph. Eur., Emsure® | Merck KGaA, Darmstadt, Germany |
| **methanol** | Rotisolv®, HPLC gradient grade | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **neutral red** | research grade | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **nitrotetrazolium blue chloride (NBT)** | ≥ 98 %, p.a. | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **organic solvent (ACN 50 %, water 47.5 %, TCA 2.5 %)** | for mass spectrometry | Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany |
| **ortho phosphoric acid (85 %)** | Rotipuran®, p.a., ACS, ISO | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **peqGold fungal Mini kit** | | PEQLAB, VWR International GmbH, Ismaning, Germany |
| **perchloric acid** | 70 % | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **polyethylene glycol 20000** | Rotipuran®, Ph. Eur. | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **polyethylene glycol 8000** | | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **polyvinylpolypyrrolidone (PVPP)** | ~110 µm particle size | FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany |
| **polyvinylpyrrolidone (PVP) 360** | | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **potassium chloride** | ≥ 99 %, Cellpure® | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **potassium dihydrogen orthophosphate** | for analysis, Emsure®, ISO | Merck KGaA, Darmstadt, Germany |
| **protease inhibitor cocktail tablets** | for life science research only | Roche diagnostics GmbH, Unterhaching, Germany |
| **protein marker, broad range** | (2-212 kDa) | New England BioLabs GmbH, Frankfurt am Main, Germany |
| **QIAGEN genomic DNA kit** | | QIAgen GmbH, Hilden, Germany |
| **QIAquick® PCR purification kit** | | QIAgen GmbH, Hilden, Germany |
| **Roti®-aqua-phenol** | for RNA extraction | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **Roti®-Blue 5x concentrate** | | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **servalyte™ 3-10 Iso-Dalt** | 40 % w/v solution in water | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **silver nitrate** | ≥ 99.9 %, p.a. | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **sinapinic acid (SA)** | | Bruker Daltonics GmbH, Bremen, Germany |
| **sodium carbonate** | ≥ 99.9 %, p.a., ACS | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **sodium chloride** | ≥ 99.9 %, 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 %, p.a., ISO | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |

| chemical | purity grade | manufacturer |
|---|---|---|
| **sodium thiosulphate pentahydrate** | p.a., ACS, ISO | Merck KGaA, Darmstadt, Germany |
| **spectra™ multicolor low range protein ladder** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **tetramethylethylenediamine (TEMED)** | ~99 % | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **thio urea** | p.a., ACS, Reag. Ph. Eur. | Merck KGaA, Darmstadt, Germany |
| **trichloroacetic acid (TCA)** | p.a. ACS, Reag. Ph. Eur., Emsure® | Merck KGaA, Darmstadt, Germany |
| **tricine** | Pufferan®, ≥ 99 % | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **trifluoroacetic acid (TFA)** | Chromasolv®, ≥ 99 %, for HPLC | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **tris-HCl** | for mol. biology | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **tris-X** | ultra pure | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **triton®X-100** | for mol. biology | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **Tween 20** | cell culture and bacteriology grade | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **urea** | ultra pure | GERBU Biotechnik GmbH, Heidelberg, Germany |
| **water** | HPLC gradient grade | J.T. Baker, Center Valley, USA |
| **zeocin™ solution (100 mg/ml)** | | Thermo Fisher Scientific Inc., Waltham, USA |
| **zink sulphate heptahydrate** | ≥ 99.5 %, p.a., ACS, ISO, Reag. Ph. Eur. | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **α-Cyano-4-hydroxycinnamic acid (HCCA)** | | Bruker Daltonics GmbH, Bremen, Germany |

In the following the composition of buffers used for HPLC analysis are described in detail.

**buffer A**

| | |
|---|---|
| TFA | 0.1 % v/v |
| In water (HPLC grade) | |

**buffer B**

| | |
|---|---|
| TFA | 0.1 % v/v |
| In acetonitrile | |

**buffer D**

| | |
|---|---|
| Acetonitrile | 65 % w/v |
| water (HPLC grade) | 35 % w/v |

Buffers and solutions used for SDS-PAGE analysis and 2D gel electrophoresis as well as for staining of acrylamide gels in general were as follows:

**anode puffer (SDS-PAGE)**

| | |
|---|---|
| Tris-X | 1 M |

pH adjusted to 8.9

**application buffer (SDS-PAGE)**

| | |
|---|---|
| tris-HCl | 250 mM |
| SDS | 7.5 % w/v |
| Glycerin | 25 % v/v |
| 2-mercaptoethanol | 12.5 % v/v |
| bromphenol blue | 0.25 mg/ml |

pH adjusted to 8.46

**cathode buffer (SDS-PAGE)**

| | |
|---|---|
| tris-X | 0.5 M |
| Tricine | 0.5 M |
| SDS | 0.5 % w/v |

pH adjusted to 8.25

**gel buffer**

| | |
|---|---|
| tris-X | 3 M |

pH adjusted to 8.25

**running buffer (2D-GE) 6x**

| | |
|---|---|
| SDS | 0.595 % w/v |
| Glycine | 8.99 % w/v |
| tris-X | 1.74 % w/v |

**lysis buffer (2D-GE)**

| | |
|---|---|
| Urea | 6.71 M |
| Thiourea | 1.79 M |
| CHAPS/DDM | 65.06 mM |
| DTT | 1 % w/v |
| Servalyte | 0.5 % v/v |

**SDS buffer (2D-GE)**

| | |
|---|---|
| SDS | 0.9 % w/v |
| tris-X | 0.1 M |
| protease inhibitor | 1 piece |

**rehydration buffer (2D-GE)**

| | |
|---|---|
| Urea | 6.71 M |
| Thiourea | 1.79 M |
| CHAPS/DDM | 8.13 mM |
| DTT | 0.2 % w/v |
| Servalyte | 0.2 % v/v |

**buffer T (2D-GE)**

| | |
|---|---|
| tris-X | 18.2 % w/v |
| SDS | 0.4 % w/v |

pH adjusted to 8.8

**equilibration buffer (2D-GE)**

| | |
|---|---|
| Urea | 36 % w/v |
| glycerol (99 %) | 30 % w/v |
| SDS | 2.5 % w/v |
| buffer T | 3.3 % v/v |
| DTT or iodoacetamide | 2 % or 4 % w/v |

**buffer D (2D-GE)**

| | |
|---|---|
| tris-X | 18.2 % w/v |
| SDS | 0.4 % w/v |

pH adjusted to 8.6

**fixation & staining solution (coomassie staining)**

| | |
|---|---|
| ortho phosphoric acid | 1 % v/v |
| Methanol | 20 % v/v |
| Roti®-Blue 5x concentrate | 20 % v/v |

**washing solution (coomassie staining)**

| | |
|---|---|
| ortho phosphoric acid | 1 % v/v |
| Methanol | 20 % v/v |

**developing solution (silver staining)** 3 % w/v

| | |
|---|---|
| sodium carbonate | 3 % w/v |
| sodium thiosulphate pentahydrate | 0.5 % w/v |
| formaldehyde (37 %) | 0.02 % v/v |

**fixation solution (silver staining)**

| | |
|---|---|
| Ethanol | 40 % v/v |
| acetic acid | 10 % v/v |

**silver solution (silver staining)**

| | |
|---|---|
| silver nitrate | 0.2 % w/v |

**stop solution (silver staining)**

| | |
|---|---|
| Glycine | 0.5 % w/v |

**thiosulphate solution (silver staining)**

| | |
|---|---|
| sodium thiosulphate pentahydrate | 0.02 % w/v |

**washing solution (silver staining)**

| | |
|---|---|
| Ethanol | 30 % v/v |

Buffers used for AGE are described in the following. The concentrated buffer was diluted with $dH_2O$ result in 0.5x (TAE) and 1x (TBE) solutions prior to electrophoresis.

**TAE buffer 5x**

| | |
|---|---|
| Tris-HCl | 2 M |
| EDTA | 63.6 mM |
| acetic acid | 5.71 % v/v |

pH adjusted to 8.2

**TBE buffer 5x**

| | |
|---|---|
| Tris-HCl | 0.89 M |
| EDTA | 25.3 mM |
| boric acid | 0.89 M |

pH adjusted to 8.0

Subsequently buffers and other solution used for western blot methods are noted.

**AP buffer**

| | |
|---|---|
| tris-HCl | 100 mM |
| sodium chloride | 100 mM |
| magnesium chloride | 5 mM |

pH adjusted to 8.8

**BCIP solution**

| | |
|---|---|
| BCIP | 60 mg/ml |
| DMF | 100 % v/v |

**Blocking solution**

| | |
|---|---|
| Tris | 0.02 M |
| sodium chloride | 0.2 M |
| BSA | 3 % w/v |

pH adjusted to 7.4

**NBT solution**

| | |
|---|---|
| NBT | 75 mg/ml |
| DMF | 70 % v/v |

**PBS buffer**

| | |
|---|---|
| potassium dihydrogen orthophosphate | 4 mM |
| disodium hydrogen phosphate dihydrate | 16 mM |
| sodium chloride | 115 mM |

pH adjusted to 7.4

**PBS-T buffer**

| | |
|---|---|
| Tween 20 | 0.10 % v/v |
| in PBS buffer | |

**transfer buffer**

| | |
|---|---|
| Tris | 50 mM |
| glycin | 190 mM |
| SDS | 0.1 % |
| methanol | 20 % |

### 2.1.2 Equipment

Laboratory equipment used in this work is listed in Table 8 with details of the manufacturer.

**Table 8:** List of equipment used in this study.

| equipment | type | manufacturer |
|---|---|---|
| **agarose gel chamber 13.8 x 12 cm** | Easy cast electrophoresis system | Owl Seperation Systems, Thermo Fisher Scientific Inc., Portsmouth, USA |
| **binocular microscope** | Nikon SMZ-2T | Nikon Corporation, Tokyo, Japan |
| **camera (hand held)** | P8 lite | Huawei Technologies GmbH, Shenzen, China |
| **camera (inside UV cabinet)** | | Intas Science Imaging, Instruments GmbH, Göttingen, Germany |
| **centrifuge** | Z 383K | HERMLE Labortechnik GmbH, Wehingen, Germany |
| **centrifuge** | Z 382K | HERMLE Labortechnik GmbH, Wehingen, Germany |
| **centrifuge** | Z 216K | HERMLE Labortechnik GmbH, Wehingen, Germany |
| **cold light source** | Intralux 4000-1 | Volpi AG, Schlieren, Switzerland |
| **cryothermostate** | Fisherbrand™ | Fisher Bioblock Scientific, Thermo Fisher Scientific Inc., Schwerte, Germany |
| **freeze dryer** | FreeZone 2,5 Plus | Labconco Corp., Kansas City, USA |
| **glass cassette 1 mm** | SE9102-1-10 | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **glass plates** | Mini-PROTEAN® glas plates with 1.00 mm spacer | Bio-Rad Laboratories GmbH, München, Germany |
| **glass plates** | Mini-PROTEAN® glas plates without spacer | Bio-Rad Laboratories GmbH, München, Germany |
| **homogenizer** | Fastprep®-24 | MP Biomedicals Germany GmbH, Eschwege, Germany |
| **HPLC autosampler** | Ultimate 3000 | Dionex, Thermo Fisher Scientific Inc., Waltham, USA |
| **HPLC column** | Aeris PEPTIDE 3.6u XB-C18 | Phenomenex Inc., Aschaffenburg, Germany |
| **HPLC column compartment** | TCC-100 (thermostatted) | Dionex, Thermo Fisher Scientific Inc., Waltham, USA |
| **HPLC pump** | Ultimate 3000 | Dionex, Thermo Fisher Scientific Inc., Waltham, USA |
| **HPLC variable wavelength** | Ultimate 3000 | Dionex, Thermo Fisher Scientific Inc., Waltham, |

| equipment | type | manufacturer |
|---|---|---|
| **detector** | | USA |
| **imaging station** | colony Doc-it™ | UVP, Ultra-Violet Products Ltd, Cambridge, UK |
| **incubator** | Certomat R | B. Braun Biotech International, Melsungen, Germany |
| **incubator** | Certomat BS-1 | B. Braun Biotech International, Melsungen, Germany |
| **isoelectric focusing unit** | IEF 100 | Hoefer Inc., Holliston, USA |
| **MALDI-TOF MS** | microflex LT | Bruker Daltonics GmbH, Bremen, Germany |
| **microScout target (for MALDI-TOF MS)** | MSP 96 polished steel | Bruker Daltonics GmbH, Bremen, Germany |
| **microscope** | Axiostar | Carl Zeiss AG, München, Germany |
| **microscope** | Axiolab | Carl Zeiss AG, München, Germany |
| **microscope objective** | A-Plan 40x/0.65 | Carl Zeiss AG, München, Germany |
| **microscope objective** | A-Chroplan 100x/1.25 oil, Ph3 | Carl Zeiss AG, München, Germany |
| **microscope camera** | Axiocam ICc1 | Carl Zeiss AG, München, Germany |
| **orbital shaker** | Unitwist 300 | Uniequip Laborgerätebau- und Vertriebs GmbH, Planegg, Germany |
| **PCR thermocycler** | mastercycler gradient | Eppendorf AG, Hamburg, Germany |
| **pH electrode** | 6161857 | Mettler-Toledo GmbH, Gießen, Germany |
| **pH meter** | 761 Calimatic | Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany |
| **plate reader** | Emax | Molecular Devices GmbH, Biberach an der Riss, Germany |
| **power supply (for 2D-GE)** | EPS 3501 XL | Amersham Pharmacia Biotech Inc., New Jersey, USA |
| **power supply (for agarose gel electrophoresis, western blot)** | Power Pack P25 | Biometra GmbH, Göttingen, Germany |
| **power supply (for SDS-PAGE)** | Power Pac™ Basic | Bio-Rad Laboratories GmbH, München, Germany |
| **SDS-PAGE chamber** | Mini-PROTEAN® Tetra Cell | Bio-Rad Laboratories GmbH, München, Germany |
| **semi-dry electroblotting system** | Hep-1 the Panther™ | Owl Seperation Systems, Thermo Fisher Scientific Inc., Portsmouth, USA |
| **separation tank (for 2D-GE)** | SE900-1.0 | Hoefer Inc., Holliston, USA |
| **spectrophotometer** | NanoDrop 1000 | PEQLAB, VWR International GmbH, Ismaning, Germany |
| **Thoma hemocytometer** | 0.01 mm depth | Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany |
| **UV lamp (portable)** | minUVIS | Desaga GmbH, Wiesloch, Germany |
| **UV table** | UVT-28 M | Herolab GmbH Laborgeräte, Wiesloch, Germany |

### 2.1.3 Proteins

Enzymes with corresponding buffers as well as antibodies used in the current study are listed in Table 9 with details of the manufacturer.

**Table 9:** List of proteins used in this study.

| proteins and buffers | | manufacturer |
|---|---|---|
| **anti-chicken-IgY-AP antibody (produced in rabbit)** | affinity isolated | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **anti-FcHyd5p(C-term)-IgG (polyclonal antibody directed against Hyd5 produced in rabbit)** | affinity isolated | GenScript, Piscataway, USA |
| **anti-FcHyd5p(N-term)-IgG (polyclonal antibody directed against Hyd5 produced in rabbit)** | affinity isolated | GenScript, Piscataway, USA |
| **anti-rabbit-IgG-AP F(ab´)$_2$ fragment (produced in goat)** | affinity isolated | Sigma-Aldrich GmbH, Schnelldorf, Germany |
| **anti-VOG-APA-IgG (polyclonal antibody directed against PDE_04519 produced in chicken)** | affinity isolated | Davids Biotechnology GmbH, Regensburg, Germany |
| **anti-VOG-EVA-IgG (polyclonal antibody directed against PDE_07106 produced in chicken)** | affinity isolated | Davids Biotechnology GmbH, Regensburg, Germany |
| ***Bst* polymerase** | 8,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| **CutSmart™ Buffer** | 10x | New England BioLabs GmbH, Frankfurt am Main, Germany |
| **NEBuffer 2** | 10x | New England BioLabs GmbH, Frankfurt am Main, Germany |
| ***Nco*I-HF®** | 20,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| ***Not*I** | 10,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| ***Pml*I** | 20,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| ***Sal*I-HF®** | 20,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| **shrimp alkaline phosphatase rSAP** | 1,000 U/ml | New England BioLabs GmbH, Frankfurt am Main, Germany |
| **T4-DNA ligase** | 500,000 U/ml | Fermentas GmbH, Thermo Fisher Scientific Inc., St. Leon-Roth, Germany |
| **T4-DNA ligase buffer** | 10x | Fermentas GmbH, Thermo Fisher Scientific Inc., St. Leon-Roth, Germany |
| ***Taq* buffer** | 10x | MP Biomedicals GmbH, Eschwege, Germany |
| ***Taq* polymerase** | 250 U/ µl | MP Biomedicals GmbH, Eschwege, Germany |

### 2.1.4 Consumables

Other materials used in the current study are listed in Table 10 under provision of the manufacturer's name.

**Table 10:** List of consumables used in this study.

| consumable | | manufacturer |
|---|---|---|
| **blotting paper sheets** | | Munktell & Filtrak GmbH, Bärenstein, Germany |
| **cannula, Sterican®** | 0.60 x 30 mm | B. Braun Biotech International, Melsungen, Germany |
| **cover glass** | 20 x 20 mm | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **dialysis tube, Membra-Cel®** | MWCO: 3.5 kDa | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **folded filter** | grade: 3hw | Munktell & Filtrak GmbH, Bärenstein, Germany |
| **glass beads** | Ø 0.5 mm | Scientific industries Inc., Bohemia, USA |
| **glass beads** | Ø 1.25-1.65 mm | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **IPG strip *Blue*Strips** | pH 3-10, 18 cm | SERVA Electrophoresis GmbH, Heidelberg Germany |
| **microscope slides** | 76 x 26 mm | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **microtube** | 1.5 ml, 2 ml | Sarstedt AG & Co, Nürnbrecht, Germany |
| **muslin bandage** | 100 % Polyester, elastic | Altapharma Naturprodukte GmbH, Hamburg, Germany |
| **Nalgene™ Rapid-Flow™ bottle top filter** | 0.45 µm | Thermo Fisher Scientific Inc., Waltham, USA |
| **Parafilm®** | | Bemis Company Inc., Rheinbach, Germany |
| **peqGOLD Safeguard™ filter tips** | | PEQLAB, VWR International GmbH, Ismaning, Germany |
| **petri dish w/o cams** | 92 x 16 mm | Sarstedt AG & Co, Nürnbrecht, Germany |
| **PVDF membrane** | Immun-Blot® | Bio-Rad Laboratories GmbH, München, Germany |
| **round filter** | grade: GF/A | Whatmann, GE Healthcare GmbH, München, Germany |
| **screw cap micro tube** | 2 ml | Sarstedt AG & Co, Nürnbrecht, Germany |
| **sea sand** | p.a. | Merck KGaA, Darmstadt, Germany |
| **syringe filtration unit Filtropur S 0.45** | 0.45 µm | Sarstedt AG & Co, Nürnbrecht, Germany |
| **syringe filtration unit, Phenex-NY** | 0.2 µm | Phenomenex Inc., Aschaffenburg, Germany |
| **TC plate, 24 well** | standard F | Sarstedt AG & Co, Nürnbrecht, Germany |
| **tube** | 15 ml, 50 ml | Sarstedt AG & Co, Nürnbrecht, Germany |
| **verex HPLC vials** | 2 ml | Phenomenex Inc., Aschaffenburg, Germany |

### 2.1.5 Oligonucleotides and plasmids

Plasmids used in this work are listed in Table 11.

**Table 11:** List of vectors used in this study.

| plasmid | features | supplier |
|---|---|---|
| **pPICZαA** | AOX1 promotor, α-factor secretion signal, Zeocin resistance for selection in *E. coli* and *Pichia pastoris*, His-tag (6x), c-myc epitope tag | Thermo Fisher Scientific Inc., Carlsbad, USA |
| **pBAD/Myc-His C** | *araBAD* promoter, Ampicillin resistance for selection in *E. coli*, His tag (6x), c-myc epitope tag | Thermo Fisher Scientific Inc., Carlsbad, USA |

Oligonucleotides used in this work are listed in Table 12. If primers are used to create a restriction site within the amplificate the corresponding restriction enzyme is stated in square brackets behind the oligonucleotide name.

**Table 12:** List of oligonucleotides used in this study.

| oligoname | sequence (5' → 3') | $T_M$ [°C] | target gen |
|---|---|---|---|
| **Bt2a** | GGTAACCAAATCGGTGCTGCTTTC | 62.7 | β-tubulin for identification of *Penicillium* species |
| **Bt2b** | ACCCTCAGTGTAGTGACCCTTGGC | 66.1 | |
| **PDE07106_for [*Pml*I]** | CACGTGAGCTCCTGCCAGCGGC | 69.6 | PDE_07106 for cloning |
| **PDE07106_for [*Kpn*I]** | GGTACCGCTCCTGCCAGCGGC | 69.6 | |
| **PDE07106_for [*Nco*I]** | TATACCATGGCTCCTGCCAGCGGC | 67.8 | |
| **PDE07106_rev_T [*Not*I]** | GCGGCCGCCTAAGGGAGAGCGTAGGGAG | 75.4 | |
| **PDE07106_rev_HisTag_T [*NotI*]** | GCGGCCGCCTAATGATGATGATGATGATGA GGGAGAGCGTAGGGAG | 78.3 | |
| **PDE07106_rev [*Not*I]** | GCGGCCGCAGGGAGAGCGTAGGGAG | 74.5 | |
| **PDE07106_rev [*Sal*I]** | ATTAGTCGACAGGGAGAGCGTAGGGAG | 68.0 | |
| **PDE04519_for [*Pml*I]** | CACGTGAGCGCCCACCAGCCAG | 69.6 | PDE_04519 for cloning |
| **PDE04519_for [*Kpn*I]** | GGTACCGCGCCCACCAGCCAG | 69.6 | |
| **PDE04519_for [*Nco*I]** | TATACCATGGCGCCCACCAGCCAG | 67.8 | |
| **PDE04519_rev_T [*NotI*]** | GCGGCCGCCTAGATGTACTGCCAGGCAGG | 75.2 | |
| **PDE04519_rev_HisTag_T [*Not*I]** | GCGGCCGCCTAATGATGATGATGATGATG GATGTACTGCCAGGCAGG | 78.2 | |
| **PDE04519_rev [*Not*I]** | GCGGCCGCGATGTACTGCCAGGCAGG | 74.3 | |
| **PDE04519_rev [*Sal*I]** | ATTAGTCGACGATGTACTGCCAGGCAGG | 68.0 | |
| **pPICZaA_for** | GCTGCTAAAGAAGAAGGGGTATCTC | 63.0 | multiple cloning site of pPICZαA |
| **pPICZaA_rev** | GGCGCTATTCAGATCCTCTTCTGAG | 64.6 | |
| **FIP-RET21** | TCACCGCAGTTGACGGGTCCCCTTGCACAC TCGTCGTGAC | 78.7 | PDE_07106 for detection of *P. oxalicum* via LAMP assay |
| **BIP-RET21** | CCTCAGGCTGGAGCGGTCAATCTGGCGGCT CTT GTTGTTGA | 77.4 | |
| **F3-RET21** | CTGGACCTTTGGCATCTACC | 59.4 | |
| **B3-RET21** | TGTCGGTGTAAGCAGGGTAG | 59.4 | |
| **LF-RET21** | TGGACTGGGAGGCCTTTTGG | 61.4 | |
| **LB-RET21** | GTCCCGGCAATGGCTTCACC | 63.5 | |

### 2.1.6 Organisms

Organisms used for preparation or construction of plasmids are listed in Table 13. Also three *Penicillium oxalicum* isolates on which the current study is focused are listed. All other microorganisms used in this study are displayed in the appendix (see Table 30 and Table 35).

**Table 13:** List of selected organisms used in this study.

| organism | TMW ID | supplier |
|---|---|---|
| ***Escherichia coli* TOP 10** | 2.580 | Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, USA |
| ***Escherichia coli* TOP 10 [pPICZαA]** | 2.651 | Bacterial strain collection TMW |

| organism | TMW ID | supplier |
|---|---|---|
| ***Escherichia coli* DH5α [pBAD/Myc-His C]** | 2.584 | Bacterial strain collection TMW |
| ***Penicillium oxalicum*** | 4.2539 | Micoteca da Universidade do Minho, Braga, Portugal |
| ***Penicillium oxalicum*** | 4.2553 | Micoteca da Universidade do Minho, Braga, Portugal |
| ***Penicillium oxalicum*** | 4.2553 | Micoteca da Universidade do Minho, Braga, Portugal |

### 2.1.7 Nutrients

Media and additives used in this work are listed in Table 14 with reference to their source.

**Table 14:** List of nutrients used in this study.

| nutrient | for | manufacturer |
|---|---|---|
| **agar-agar** | | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **calcium caseinate agar** | microbiology | FLUKA, Sigma-Aldrich GmbH, Steinheim, Germany |
| **Difco™YCB (yeast carbon base)** | laboratory use | BD – Becton, Dickinson and Company, Heidelberg, Germany |
| **malt extract** | microbiology | AppliChem GmbH, Darmstadt, Germany |
| **peptone from casein (trypton) pancreatic digested** | microbiology | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **peptone from soy, papainic digested** | microbiology | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |
| **skimmed milk powder** | microbiology | Merck KGaA, Darmstadt, Germany |
| **yeast extract** | bacteriology | Carl Roth GmbH + Co. KG, Karlsruhe, Germany |

In the following the composition of growth media used during the study is listed.

**DRBC agar**

| | |
|---|---|
| peptone from soy | 0.50 % w/v |
| glucose monohydrate | 1.00 % w/v |
| potassium dihydrogen orthophosphate | 0.10 % w/v |
| magnesium sulphate heptahydrate | 0.05 % w/v |
| chloramphenicol | 0.01 % w/v |
| bengal rose B | 0.025 ‰ w/v |
| agar | 1.50 % w/v |

pH adjusted to 5.6
addition of 1 ml EtOH (70 %) supplemented with
2 mg dicloran after autoclaving

**calcium caseinate agar**

| | |
|---|---|
| ready-to-use-medium (FLUKA) | 3.07 % w/v |
| skimmed milk powder | 1.00 % w/v |
| agar | 1.35 % w/v |

**CYA medium/agar**

| | |
|---|---|
| Czapek concentrate | 1 % v/v |
| dipotassium hydrogen phosphate trihydrate | 0.12 % w/v |
| yeast extract | 0.5 % w/v |
| sucrose | 3 % w/v |
| agar (if necessary) | 1.50 % w/v |

pH adjusted to 6.0-6.5

**LB medium/agar**

| | |
|---|---|
| peptone from casein (trypton) | 1.00 % w/v |
| yeast extract | 0.50 % w/v |
| sodium chloride | 0.50 % w/v |
| agar (if necessary) | 1.50 % w/v |

pH adjusted to 7.5

| | |
|---|---|
| addition of zeocin™ for selection is possible | 25 µg/ml |
| addition of ampicillin for selection is possible | 100 µg/ml |

**malt extract medium/agar**

| | |
|---|---|
| malt extract | 2.00 % w/v |
| peptone from soy | 0.20 % w/v |
| agar (if necessary) | 1.50 % w/v |

pH adjusted to 5.6

**SOC medium**

| | |
|---|---|
| peptone from casein (trypton) | 2 % w/v |
| yeast extract | 0.5 % w/v |
| magnesium sulphate heptahydrate | 0.48 % w/v |
| glucose monohydrate | 0.36 % w/v |
| sodium chloride | 0.05 % w/v |
| potassium chloride | 0.02 % w/v |

| | |
|---|---|
| **YCB medium** (component I: component II) | (9:1) |
| <u>component I</u> | autoclaved |
| ammonium sulphate | 0.55 % w/v |
| <u>component II</u> | sterile-filtered |
| Difco™YCB | 11.7 % w/v |
| glucose monohydrate | 5.5 % w/v |

**YES medium/agar**

| | |
|---|---|
| yeast extract | 2.00 % w/v |
| sucrose | 4.00 % w/v |
| agar (if necessary) | 1.50 % w/v |

pH adjusted to 5.8

**YPG medium**

| | |
|---|---|
| yeast extract | 0.50 % w/v |
| peptone from casein | 1.00 % w/v |
| glucose monohydrate | 2.00 % w/v |

pH adjusted to 6.5

### 2.1.8 Software and databases

Various programs and different databases were used in this work as listed in Table 15.

**Table 15:** List of programs and databases used in this study.

| program/database | source | use |
|---|---|---|
| **Adobe Photoshop® CS2** | installed on a computer of TMW | calculation/comparison of growth areas of fungal cultures |
| **BLAST** | http://blast.ncbi.nlm.nih.gov/Blast.cgi | sequence alignment |
| **CBS** | http://www.westerdijkinstitute.nl/ in the past http://www.cbs.knaw.nl/ | identification of fungal species |
| **Chromeleon** | | analysis of HPLC data |
| **Clustal Ω** | http://www.ebi.ac.uk/Tools/msa/clustalo/ | multiple sequence alignment |
| **Flexanalysis 3.3** | installed on a computer of TMW | analysis of MALDI-TOF MS data |
| **MALDI Biotyper 2.0** | installed on a computer of TMW | controlling of MALDI-TOF MS |
| **Mascot** | http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF | identification of proteins by use of peptide mass fingerprints |
| **Microsoft Excel** | installed on a computer of TMW | graphical depiction of results |
| **NCBI** | https://www.ncbi.nlm.nih.gov/ | research about proteins, genes |
| **Octave 4.0** | installed on a computer of TMW | numerical calculation of potential biomarkers |
| **Primer Explorer V.4** | http://primerexplorer.jp/e/ | design of primers for LAMP |
| **Protein molecular weight** | http://www.bioinformatics.org/sms/prot_mw.html | calculation of the molecular weight of a protein sequence |
| **ProtParam** | http://web.expasy.org/protparam/ | computing of physicochemical properties of a protein sequence |
| **ProtScale** | http://web.expasy.org/protscale/ | drawing of Kyte & Doolittle plots |
| **REPFIND** | https://zlab.bu.edu/repfind/form.html | finding of clustered repeats in nucleotide sequences |
| **Scan Wizard Bio** | installed on a computer of TMW | scanning of acrylamide gels and blotting membranes |
| **SignalP 4.1** | http://www.cbs.dtu.dk/services/SignalP/ | prediction of signal cleavage sites in a protein sequence |
| **Tandem Repeats Finder** | https://tandem.bu.edu/trf/trf.html | finding of tandem repeats in DNA sequences |
| **Uniprot** | http://www.uniprot.org/ | research about proteins |
| **Yaspin** | http://www.ibi.vu.nl/programs/yaspinwww/ | secondary structure prediction by use of amino acid sequence |

## 2.2 Methods

### 2.2.1 Protein chemical methods

#### *2.2.1.1 Protein preparation from wine and sparkling wine*

Different preparation techniques for the wine and sparkling wine samples were applied. If not otherwise stated, a protocol based on the method of Hurkman and Tanaka (1986) was conducted to extract interfering phenolic compounds. Therefore, aliquots (50 ml) of (sparkling) wine were dialyzed against 20 times their volume of deionized water (see section 2.2.1.2). The retentates were freeze dried overnight and the obtained lyophilizates were resuspended in extraction puffer (0.1 M Tris-HCl (pH 8.8), 10 mM EDTA, 0.4 % (v/v) β-mercaptoethanol, 10 % (w/v) DTT, 100 mM KCl). An equal volume of water-saturated phenol was added to the extraction puffer followed by 30 min of shaking at 4 °C and a subsequent phase separation by centrifugation (6,000 x g, 15 min, 4 °C). The lower phenolic phase was recovered and washed once with extraction buffer. Protein precipitation was achieved with three times the volume of 0.1 M ammonium acetate in methanol overnight at -20 °C. Following centrifugation (20,000 x g, 40 min, 4 °C) the protein pellet was washed with 0.1 M ammonium acetate, 10 mM DTT in methanol and subsequently with 10 mM DTT in 80 % ice cold acetone (both times incubation: 60 min, 4 °C; centrifugation: 13,000 x g, 30 min, 4 °C). The supernatant was discarded and the protein pellet air-dried and stored at -20 °C until further use. The protein pellets obtained with this method were suspended in Laemmli application buffer (0.25 M Tris-HCl (pH 8.46), 7.5 % (w/v) SDS, 25 % (v/v) glycerine, 0.25 mg/ml bromophenol blue, 12.5 % (v/v) β -mercaptoethanol) for SDS-PAGE or in organic solvent (50 % ACN, 2.5 % TFA) for MALDI-TOF MS analysis.

#### *2.2.1.2 Dialysis and freeze-drying of samples*

In order to remove interfering salts, ethanol or other small compounds samples were dialyzed for three days against twenty times their volume of $dH_2O$. For that, dialysis tube (MEMBRA-CEL®, SERVA Electrophoresis GmbH, Heidelberg, Germany) with a molecular weight cut-off (MWCO) of 3.5 kDa was used to maintain most proteins inside the tubing. Water was changed twice a day to get rid of as much phenolic compounds as possible. Subsequently, samples were freeze-dried. Therefore, liquid samples were completely frozen with greatest possible surface area and placed under vacuum to allow the sublimation of water. Consequently, a concentration of the samples was achieved.

#### *2.2.1.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with staining methods*

According to the method of Schägger and von Jagow (1987), vertical SDS-PAGE (separating gel = 16 % T, stacking gel = 4 % T) was performed in a Mini-PROTEAN® Tetra Cell Electrophoresis System (Bio-Rad Laboratories GmbH, München, Germany). The electrophoresis was conducted under constant voltage of 100 V for 120 min at room temperature. A molecular marker (if not otherwise stated: Spectra Multicolor Low Range Protein Ladder; Thermo Fisher Scientific Inc., St Leon-Rot, Germany) was loaded simultaneously with the samples in each run. Prior to the application onto the gel, samples were diluted in 6x Laemmli buffer. Following electrophoresis gels were usually silver stained according to the method of Blum *et al.* (1987). The individual steps of the staining procedure

are listed in Table 16 and all chemicals used are described in section 2.1.1. In rare cases colloidal coomassie brilliant blue (Roti®-Blue 5x concentrate, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was used for staining of polyacrylamide gels. For that, gels were incubated overnight in coomassie fixation solution (see section 2.1.1) and subsequently washed in washing solution until the background was destained.

**Table 16:** Procedure of silver staining.

| step | reagent | duration |
|---|---|---|
| **fixation** | fixation solution | > 3 h or overnight |
| **washing** | washing solution | 2 x ~20 min |
| **washing** | deionized water | ~20 min |
| **sensitization** | thiosulphate solution | 1 min |
| **washing** | deionized water | 3 x 5 s |
| **labeling** | silver solution | ~20 min |
| **washing** | deionized water | 3 x 5 s |
| **development** | developer | until bands occur |
| **washing** | deionized water | 3 x 5 s |
| **stop** | stop reagent | ~5 min |
| **storage** | deionized water | ∞ |

#### *2.2.1.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)*

The matrix for MALDI-TOF MS analysis was prepared by dissolving 10 mg of SA in 1 ml organic solvent. HCCA and DHBA were also tested as MALDI-TOF MS matrices. For measurements 1 µl of matrix was applied onto the target and successively coated after air-drying with 1 µl of sample and additionally 1 µl of matrix. Analyses were performed using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm) operating in linear positive ion detection mode using the MALDI Biotyper 2.0 software (Bruker Daltonics GmbH, Bremen, Germany). Mass spectra (2000-20000 m/z) were acquired manually at each spot position by accumulating 240 laser shots per sample. Processing of spectra was done in FlexAnalysis 3.3 (Bruker Daltonics GmbH, Bremen, Germany).

#### *2.2.1.5 Reversed-phase high-performance liquid chromatography (RP-HPLC)*

Protein samples were analyzed with reversed-phase high performance liquid chromatography (RP-HPLC) using an UltiMate3000 system (Dionex/Thermo Fisher Inc.) equipped with a C18 column (Aeris PEPTIDE 3.6u XB-C18 250 x 2.1 mm, Phenomenex) and a matching pre-column. Proteins were eluted from the column with a linear gradient of buffer B (0.1 % trifluoroacetic acid in acetonitrile) against buffer A (0.1 % trifluoroacetic acid in dd$H_2O$). The content of buffer B in the mobile phase was raised from 3 to 35 % within 35 min at a flow rate of 0.4 ml per min. Data evaluation was done with the Chromeleon 6.8 software package. Protein fractions were collected manually, dialyzed against deionized $H_2O$ (MWCO = 3.5 kDa) and freeze-dried before further analysis.

### 2.2.1.6 Western blot analysis

By use of specific antibodies, fungal proteins could be detected immunochemically. Therefore, a SDS-PAGE (see section 2.2.1.3) with fungal culture supernatants as sample was conducted to separate proteins according to their apparent molecular weight. Subsequently, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane by semi-dry electro blotting. For the blotting procedure the membrane was saturated with methanol, before it was saturated along with the filter papers as well as the polyacrylamide gel in transfer buffer. The gel and the blotting membrane were assembled with the filter papers as shown in Figure 4 and a current of 50 mA was applied for 1 h. Following electrotransfer, the membrane was incubated with blocking solution at 4 °C for > 12 h to diminish unspecific reactions in later steps. After three washing steps (10 min each with PBS-T buffer), the membrane was incubated with the primary antibody diluted 1:2000 in PBS-T for 1.5 h. To remove residual unbound antibody the membrane was washed again three times with PBS-T buffer, before the secondary antibody (dilution 1:5000 in PBS-T) was added to the membrane for further incubation for 1.5 h. Subsequently, the membrane was washed twice with PBS-T buffer, twice with PBS buffer and once with AP buffer each time for 5 min. For the development of a color reaction 7.5 µl NBT solution and 45 µl BCIP solution were added to 15 ml AP buffer. The alkaline phosphatase bound to the secondary antibody catalyzes a chromogenic reaction of these substrates resulting in a blue coloration. This reaction allows a sensitive indirect detection of the specific fungal proteins.

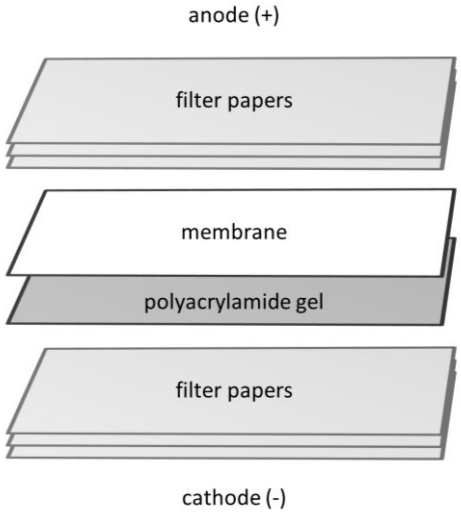


**Figure 4:** Schematic assembly of the blotting sandwich.

### 2.2.1.7 Two-dimensional gel electrophoresis (2D-GE)

2D-GE combines isoelectric focusing with SDS-PAGE. Proteins get separated in two dimensions regarding their isoelectric point (pI) and, subsequently, their apparent molecular weight (MW). For the separation in the first dimension IPG strips (SERVA Electrophoresis GmbH, Heidelberg Germany) were first rehydrated in 0.5 ml rehydration buffer for > 12 h. Freeze-dried culture supernatant of *Penicillium* was resuspended in 0.5 ml SDS buffer and 0.5 ml lysis buffer to achieve a tenfold concentration of the sample. Half of the sample (2 x 250 µl) was applied onto the strip by double anodic cup loading. The focusing was conducted as displayed in Table 17.

**Table 17:** Conditions for isoelectric focusing.

| | voltage | duration | |
|---|---|---|---|
| **gradient** | 250 V | 6 h | first loading step |
| **gradient** | 250 V | 6 h | second loading step |
| **gradient** | 500 V | 4 h | |
| **gradient** | 1000 V | 5 h 20 min | |
| **gradient** | 8000 V | 1 h 06 min | |
| **constant** | 8000 V | 6 h | |

Following isoelectric focusing, strips were equilibrated with DTT and iodoacetamide equilibration buffers each for 15 min to irreversibly break disulfide bonds within the proteins. For the separation in the second dimension the strips were placed horizontally onto polyacrylamide gels. By application of voltage (see Table 18) the proteins migrate through the gel and are separated according to their MW.

**Table 18:** Conditions for gel electrophoresis.

| | voltage | current | duration |
|---|---|---|---|
| **gradient** | 180 V | 50 mA | 1 h |
| **gradient** | 200 V | 80 mA | 1 h |
| **gradient** | 200 V | 165 mA | 16 h |

Following electrophoresis, silver staining of the polyacrylamide gels was performed as described in 2.2.1.3.

#### *2.2.1.8 Protein identification*

Previously to protein identification, SDS-PAGE with subsequent silver staining was conducted (see section 2.2.1.3). In this case, the FireSilver staining Kit (Proteome Factory AG, Berlin, Germany) was used, as it is compatible with mass spectrometric analysis. Protein bands were excised from the polyacrylamide gel and delivered to the Protein Analysis Unit of the Ludwig-Maximilians-University of Munich (LMU). The department of the LMU performed a tryptic in-gel digestion followed by nanoESI-LC-MS/MS analysis. By use of the MASCOT as well as the X!Tandem algorithms they were able to correlate the obtained spectra with entries in the UniProt database. The generated data were used for protein identification during the current study.

### 2.2.2 Molecular biological methods

#### *2.2.2.1 Isolation of DNA*

For preparation of fungal genomic DNA, fungi were grown in ME broth for 5-7 d at ambient temperature (± 23 °C). Mycelia were filtered (folded filters, grade: 3 hw, Munktell, Bärenstein, Germany), washed twice with sterile tap water and finally air dried on a filter disc (grade: 3 hw, Munktell, Bärenstein, Germany). The peqGOLD Fungal Mini Kit (PEQLAB, Erlangen, Germany) was used for isolation of genomic DNA according to the manufacturer's recommendation with slight modifications of the cell disruption protocol. In particular, cell lysis was achieved by addition of lysis buffer (PL1), 0.5 g sterile sea sand (Merck, Darmstadt, Germany) and 0.1 g glass beads (Ø 1.25-1.65 mm; Carl Roth, Karlsruhe, Germany) followed by severe shaking in a FastPrep®-24 homogenizer

(MP Biomedicals; 45 sec, 24*2, 5.5 m/s). Bacterial genomic DNA was prepared using the QIAGEN Genomic DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. For the preparation of plasmid DNA, bacteria were grown over night in LB media supplemented with the respective antibiotic for selection at 37 °C. Bacteria were harvested by centrifugation (6,800 x g, 10 min). By use of the GeneJET plasmid miniprep kit (Thermo Fisher Scientific Inc., Waltham, USA) cells were disrupted and plasmid DNA was isolated according to the manufacturer's instructions. All isolated DNA was stored at 4 °C at the highest possible concentration. DNA quantification was performed in a Nano-Drop 1000 spectrophotometer (PEQLAB, Erlangen, Germany).

#### *2.2.2.2 Sequencing of DNA*

DNA sequencing was conducted by GATC Biotech AG (Konstanz, Germany). For sequencing of PCR products (see section 2.2.2.3), amplified fragments were purified using the E.Z.N.A. cycle pure kit. 20 µl of purified PCR product (~20-30 ng/µl) as well as the corresponding primers (10 pmol/µl) were sent to GATC for Sanger Sequencing. Sequencing results were usually available within 48 h. For identification of microorganisms the obtained sequences were compared with online databases (see 2.1.8). For a positive identification sequence homology with database entries was assumed to be ≥ 99 %.

#### *2.2.2.3 In vitro modification of DNA*

DNA was isolated from living microorganisms (see section 2.2.2.1) in order to perform different experiments *in vitro* such as polymerase chain reaction (PCR), digestion of DNA with restriction enzymes, dephosphorylation of plasmid DNA or ligation of two DNA fragments.

PCR was used for the amplification of a selected DNA sequence. *Taq*-polymerase was used throughout the study and the pipetting scheme shown in Table 19 was used to set up reactions.

**Table 19:** Pipetting scheme for PCR-reactions with a total volume of 25 µl.

| reagent | volume [µl] |
|---|---|
| **$dH_2O$** | 20.25 |
| **10x buffer with MgCl** | 2.5 |
| **dNTP mix** | 0.5 |
| **primer (*forward*) [50 pmol/µl]** | 0.25 |
| **primer (*reverse*) [50 pmol/µl]** | 0.25 |
| ***Taq*-polymerase** | 0.25 |
| **DNA template** | 1<br>(one colony in case of colony-PCR) |

The accuracy of PCR-amplified sequences was checked by sequencing (see section 2.2.2.2). Reactions were conducted in a total volume of 25 µl as displayed in Table 19. Different types of DNA such as genomic or plasmid DNA as well as bacterial colonies were used as template using the standard cycling program described in Table 20. Annealing temperatures and extension times were individually adjusted according to the selected primers and the size of the target sequence.

**Table 20:** Standard PCR-cycling program.

| step | temperature [°C] | duration [min:s] | number of cycles |
|---|---|---|---|
| **heating of the lid** | 103 | - | - |
| **initial denaturation** | 94 | 4:00 | 1 |
| **denaturation** | 94 | 0:30 | 30 |
| **annealing** | individually determined for each pair of oligonucleotides | 0:30 | |
| **extension** | 72 | 1:00/kb amplified DNA | |
| **final extension** | 72 | 8:00 | 1 |
| **storage** | 4 | ∞ | - |

Selective enzymatic cleavage of DNA fragments or plasmids was achieved using restriction endonucleases. Depending on the cloning strategy, different endonucleases were used in a double digest (see Table 21) with buffers as recommended by the manufacturer. The first attempt was conducted with *Pml*I and *Not*I in NEB2 10x buffer. As *Pml*I generates blunt ends this enzyme was replaced by *Kpn*I to obtain sticky ends in further experiments. In a last cloning approach the two enzymes *Sal*I-HF® and *Nco*I-HF® were applied with CutSmart™ buffer. Reactions were incubated for at least 1 hour at 37 °C.

Digestion of plasmid DNA was followed by dephosphorylation to avoid self-ligation of the vector. Therefore, 2 µl of shrimp alkaline phosphatase (rSAP) were added to the restriction after half of the incubation time. This enzyme cleaves the phosphate residues at the 5' end of DNA that are necessary for ligation. As a result, the digested DNA fragments must not be treated with rSAP as no ligation of any DNA sequences would occur due to dephosphorylation. Restrictions were terminated by heat inactivation at 65 °C for 10 min.

**Table 21:** Pipetting scheme for digest with restriction enzymes.

| reagent | volume [µl] (total volume 20 µl) | (total volume 30 µl) |
|---|---|---|
| **$dH_2O$** | 6.5 | 4 |
| **10x buffer** | 2 | 3 |
| **each restriction enzymes** | 0.5-1 (20 U/10 U) | 1-2 (20 U/10 U) |
| **DNA** | 10 | 20 |

For the ligation reaction, a DNA fragment and a plasmid were combined that had previously been treated with the same endonucleases. 1 µl $T_4$-DNA ligase, 1 µl of corresponding buffer, 2 µl vector and 6 µl of the DNA sequence to be inserted were mixed. The mix was incubated at 10 °C in a water bath overnight to achieve a slow ligation. If a fast ligation within 2 h was desired, the temperature was raised to ambient conditions. The resulting vector constructs were used for the transformation of bacteria (see section 2.2.2.6).

#### 2.2.2.4 Agarose gel electrophoresis (AGE)

For separation of DNA fragments according to their size, horizontal AGE was performed in an easy cast electrophoresis system (Owl Seperation Systems, Portsmouth, USA). Agarose gels consisted of 1.0-1.3 % w/v agarose in TBE or TAE buffer (see section 2.1.1). Electrophoresis was conducted at a constant voltage of 110 V for 60 min at ambient temperature. Samples were mixed with 6x loading dye before application onto gel pockets. A molecular marker (usually: GeneRuler 100 bp plus DNA ladder, Thermo Fisher Scientific Inc., Waltham, USA) was simultaneously loaded to estimate the length of DNA fragments. Following electrophoresis DNA was stained with dimidium bromide. Removal of any surplus staining solution from the gels was achieved by subsequent washing with deionized water before gels were analyzed under UV light. The AGE was performed in an analytical as well as a preparative scale where relevant DNA fragments were excised from the gel and purified with the GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., Waltham, USA) for further use.

#### 2.2.2.5 Preparation of competent E. coli TOP10 cells

Competent cells bear the ability to take up (plasmid) DNA from the surrounding medium. Within the context of transformation of a bacterial strain this capacity is essential. To make cells of *E. coli* TOP10 (TMW 2.580) competent for DNA-uptake, bacteria were grown in 3 ml LB broth (37 °C, 150 rpm) overnight. 0.5 ml of the culture was used to inoculate 50 ml of LB broth. This new culture was incubated at equal conditions until an $OD_{600}$ of ~0.5 was reached. By centrifugation (5,000 x g, 4 °C, 10 min) bacteria were harvested and the resulting cell pellet was suspended in 40 ml precooled 0.1 M $MgCl_2$-solution. After a further centrifugation step (5,000 x g, 4 °C, 10 min) the cell pellet was suspended in 20 ml of 0.05 M chilled $CaCl_2$-solution. Subsequently, the bacteria were incubated on ice for 30 min before the cells were separated from the solution by a final centrifugation step (5,000 x g, 4 °C, 10 min). The cell pellet was suspended in 2 ml precooled 0.05 M $CaCl_2$-solution with 15 % (v/v) glycerin and divided into 200 µl-aliquots. These aliquots were stored at -80 °C or immediately used for transformation experiments.

#### 2.2.2.6 Transformation of E. coli TOP10 with plasmid DNA

For the transformation of *E. coli* TOP10, isolated plasmid DNA (1 µl, see 2.2.2.1) or a finished ligation mixture (10 µl, see 2.2.2.3) was added to a 200 µl aliquot of competent bacteria (see section 2.2.2.5). Cells were incubated on ice for 30 min, at 37 °C for 5 min and finally on ice for 2 min before the bacteria were transferred to 1.9 ml of SOC medium. In this medium the cells were incubated (37 °C, 150 rpm) for recovery for 45 min. Subsequently, 100 µl of the transformed cells were plated on LB agar plates supplemented with a specific antibiotic for selection. The remaining solution was centrifuged (6,000 x g, 1 min) and the resulting pellet was plated as well. Agar plates were incubated overnight at 37 °C and checked for colonies after 24 h.

#### 2.2.2.7 Loop-mediated isothermal amplification (LAMP) assay

The loop-mediated isothermal amplification (LAMP) method was used for *in vitro* enzymatic DNA amplification under isothermal conditions. For indirect in-tube detection of DNA amplification during the reaction either calcein or neutral red were used as indicators (Tanner *et al.*, 2015; Tomita *et al.*, 2008). The master mix with calcein as indicator was prepared as described in Denschlag *et al.* (2012).

Primers were designed to bind to the gene coding for the PDE_07106 protein in *Penicillium oxalicum*. Sequences for LAMP primers are given in Table 12. Primers had the following concentrations in the master mix: 1.6 µM FIP-RET21-ID1, 1.6 µM BIP-RET21-ID1, 0.2 µM F3-RET21-ID1, 0.2 µM B3-RET21-ID1, 0.8 µM LF-RET21-ID4, 0.8 µM LB-RET21-ID4 (Eurofins MWG Operon, Ebersberg, Germany). In case of neutral red as indicator the master mix was modified by using only 1.9 µl 10x LAMP buffer and 1.5 µl aqueous neutral red (3 mM) instead of calcein per reaction. Master mixes were distributed into 200 µl Multiply®- µStrip Pro 8-strip PCR tubes (Sarstedt, Nümbrecht, Germany) in a separate location with a separate set of pipettes to avoid DNA carry over, before 5 µl of sample DNA solution were added with another set of pipettes in a different location. Sterile, deionized water was applied instead of DNA as negative control and DNA of *P. oxalicum* TMW 4.2539 (~100 ng/µl) was used as positive control. Sterile filter tips (peqGOLD Safeguard™ filter tips, PEQLAB, Erlangen, Germany) were used for all liquid handling throughout the study. After incubation at 63 °C for 60 min in a Mastercycler® Gradient thermal cycler (Eppendorf, Hamburg, Germany) or a water bath the reaction tubes were visually checked for a color change (in case of neutral red) or for the occurrence of green fluorescence under a 365 nm UV lamp (in case of calcein). Results were documented with a hand-held digital camera.

#### *2.2.2.8 Sample preparation for LAMP assay*

In case of artificial contamination, table grapes from a local supermarket were surface sterilized in 70 % ethanol for 5 min followed by sodium hypochlorite (1 % active chlorine) for 30 s with two washing steps with deionized water after each treatment. The infection of grapes was conducted by punching individual berries with a disposable needle (Sterican® Ø 0.60 x 80 mm, B. Braun, Melsungen, Germany) previously dipped into a conidial suspension (~$10^8$ conidia per ml). Grapes stung with a disposable needle dipped in deionized water were used as negative control. Infected grapes were individually incubated at ambient temperature in sterile 50 ml reaction tubes (Sarstedt, Nümbrecht, Germany) until assessment. For LAMP analysis, 1.5 ml of sterile tap water supplemented with 1 % (v/v) Tween 20 (GERBU, Heidelberg, Germany) were added to an artificially infected grape or a grape sample (3-6 grapes of a bunch randomly picked) from a vineyard. After manual shaking for 1 min, the supernatant was transferred to a 2 ml reaction tube (Sarstedt, Nümbrecht, Germany) and centrifuged for 5 min with 10,000 x g at ambient temperature. Two washing steps with sterile tap water were applied to remove the Tween 20 before the resulting pellet was suspended in 300 µl sterile deionized water. After addition of glass beads (0.1 g Ø 0.5 mm, Scientific industries, New York USA; 0.3 g Ø 1.25-1.65 mm, Carl Roth, Karlsruhe, Germany) samples were shaken for 10 min on a vortex mixer. Subsequently, 5 µl of the solution were added to a LAMP master mix. Incubation of reactions and readout of results was done as described under 2.2.2.7.

### 2.2.3 Mycological methods

#### *2.2.3.1 Fungal cultivation and isolation*

For fungal cultivation, malt extract (ME) agar plates were used at ambient temperature if not otherwise stated. In order to isolate one strain out of a mixed culture, the method of conidiophore separation under a binocular microscope with cold light source was conducted: A single conidiophore

was picked with a thin glass needle and placed onto a new ME agar plate. Glass needles were produced by melting two glass rods together and pulling the molten glass out to a thin thread. After cooling, the thread is broken in midways between the rods to result in a very thin glass tip. Pure cultures were identified microscopically using appropriate identification keys. Identifications of some *Penicillium* isolates were confirmed by DNA sequencing (see section 2.2.2.2) using primers targeting the β-tubulin gene in *Penicillium* spp. (see Table 12).

#### *2.2.3.2 Cryo-conservation of filamentous fungi*

Long-term conservation of filamentous fungi was achieved by cryo-conservation. 50 ml ME medium with expanded clay granules (diameter 2-4 mm) were inoculated with the respective fungus. The fungus was grown for 4-7 days at ambient temperature without shaking before clay granules were transferred to 0.8 ml 80 % glycerin. Cryo-conserves were stored at -80 °C until used. For inoculation from cry-conserves, one clay granule was transferred to an agar plate and the remaining cryo-conserve was subsequently stored again at -80 °C.

#### *2.2.3.3 Preparation of conidial suspensions*

Fungi were grown on ME agar plates at ambient temperature for several days until conidiation occurred. In order to ensure the presence of conidia the colonies were checked with a binocular microscope. In cases where cultures refused to form conidia, cultivation under UV light or in darkness was applied as well as mechanical stimuli by scraping the mycelium with a sterile steel needle to initiate the production of mitospores. Conidia were harvested from the agar plates by suspension of the culture in 1-4 ml sterile water. Total conidial numbers were counted in a hemocytometer (Thoma type, 0.1 mm chamber depth).

#### *2.2.3.4 Preparation of culture supernatants of filamentous fungi grown in protein-free medium*

Yeast carbon base (YCB) was used as protein-free liquid medium for fungal cultivation. The medium was composed of two components (YCB I = 0.55 % w/v ammonium sulfate, YCB II = 5.5 % w/v glucose monohydrate, 11.7 % w/v Difco yeast carbon base (Becton, Dickinson & Company, Heidelberg, Germany); YCBI:YCBII = 9:1) which were mixed after autoclaving of YCB I at 121 °C for 15 min and sterile filtration of YCB II through a 0.2 µm membrane. The inoculation of liquid cultures (300 ml) in 500 ml Erlenmeyer flasks was done with cryoconserved fungi on expanded clay granules (see 2.2.3.2). For upscaling, 600 ml medium in Fernbach flasks were inoculated with a conidial suspension ($10^6$ conidia) (see section 2.2.3.3). Liquid cultures were incubated at 80 rpm, ambient temperature (~23 °C) for one week before the mycelium was separated from the medium by filtration through a muslin bandage. Subsequently, sterile filters (Sarstedt AG & Co, Nümbrecht, Germany, 0.45 µm pore size) were used to obtain a cell-free supernatant.

#### *2.2.3.5 Surface activity assay of culture supernatants*

Fungal strains were grown in YCB-broth in sterile petri dishes (60 mm x 15 mm with notches) for 12 d at ambient temperature (~23 °C) without shaking. Starting on day 4, the influence on the surface activity of culture supernatants was tested every second day by placing a strip (1.5 cm x 3.5 cm) of highly hydrophobic Parafilm (Bemis Company, Inc., Neenah, USA) on the mycelium-free surface of

the medium. After one minute the strip was removed and drainage of the liquid from the hydrophobic surface was visually checked and classified according to the following scheme:

- ‘positive’, medium forms a stable liquid film over the complete surface of the Parafilm strip.
- ‘negative’, media drains off immediately and completely.
- ‘indifferent’, single drops of medium adhere to the strip.

#### *2.2.3.6 Protease activity assay of culture supernatants*

Cell-free culture supernatants (see section 2.2.3.4) of different filamentous fungi shown in Table 30 of the appendix were analyzed in order to determine their protease activity. Calcium caseinate agar plates were used for analysis. This agar medium is opaque due to its high protein content but clears up by proteolytic digestion of caseinate. Five cavities (Ø 0.7 cm) per plate were cut out from the agar and filled with 50 µl of cell-free culture supernatant. Relative proteolytic activity was determined by measurement of the diameter of the clear zone around the cavities after 4 h and 24 h.

#### *2.2.3.7 Foam fractionation of culture supernatants*

Cell-free supernatants of fungal liquid cultures (hereinafter referred to as ‘filtrate’) were foam fractionated. Therefor a filter flask was employed. By use of a sealed tube in the main opening of the flask, nitrogen was led into the filtrate through a porous glass frit in order to generate foam in the upper part of the vessel. The gas flow was regulated to 1-2 $l*h^{-1}$ for 10 min and subsequently increased to 4-5 $l*h^{-1}$. Foam was collected at the side opening of the filter flask. The collected foam was termed ‘spumate’ whereas the remaining liquid in the filter flask was referred to as ‘retentate’ in this study.

#### *2.2.3.8 Gushing tests with culture supernatants*

For testing of the gushing potential of foam fractions, 0.33 l-bottles of Bonaqa® mineral water (6.5-6.9 g $CO_2$ per liter) or commercial sparkling wines were precooled to 4 °C before opening and sample addition. If not otherwise stated, 1 ml of fungal spumate was added to a bottle before it was re-sealed with a new ethanol sterilized crown cap and horizontally incubated on a rotary shaker (18 h, 30 rpm, ambient temperature). Prior to opening, bottles were left standing upright without shaking for 2 h at ambient temperature. The amount of water or sparkling wine lost due to gushing was determined by weighing bottles before and after opening. The general loss of $CO_2$ during opening was 0.1 g. Therefore, the displayed gushing amount was always corrected by subtraction of this value throughout this study.

### 2.2.4 Bioinformatics

#### *2.2.4.1 Primer design*

For the loop-mediated isothermal amplification of *P. oxalicum* DNA, six primers were designed using the Primer Explorer V.4 software tool provided by Eiken Chemical Co., Ltd. (Tokyo, Japan) (see section 2.1.8). The gene coding for protein PDE_07106 (GenBank accession no. EPS32147.1) of *P. oxalicum* had a length of 438 bp. The nucleotide sequence was used to generate five primer sets for LAMP. From these the primer set RET21 ID1 was selected for external as well as internal primers. A set of loop primers termed RET21 ID4 was designed using the loop-primer design function of the

Primer Explorer V.4 with the primer information file generated for primer set RET21 ID1. The specificity of the designed primer set was examined by *in silico* testing with the nucleotide Blast search tool on the NCBI database revealing no significant hits (alignment score ≤ 40) to species other than *P. oxalicum* as to October 2016. The positioning and orientation of the used LAMP primers and their complementarity to their target DNA are displayed in Figure 5.

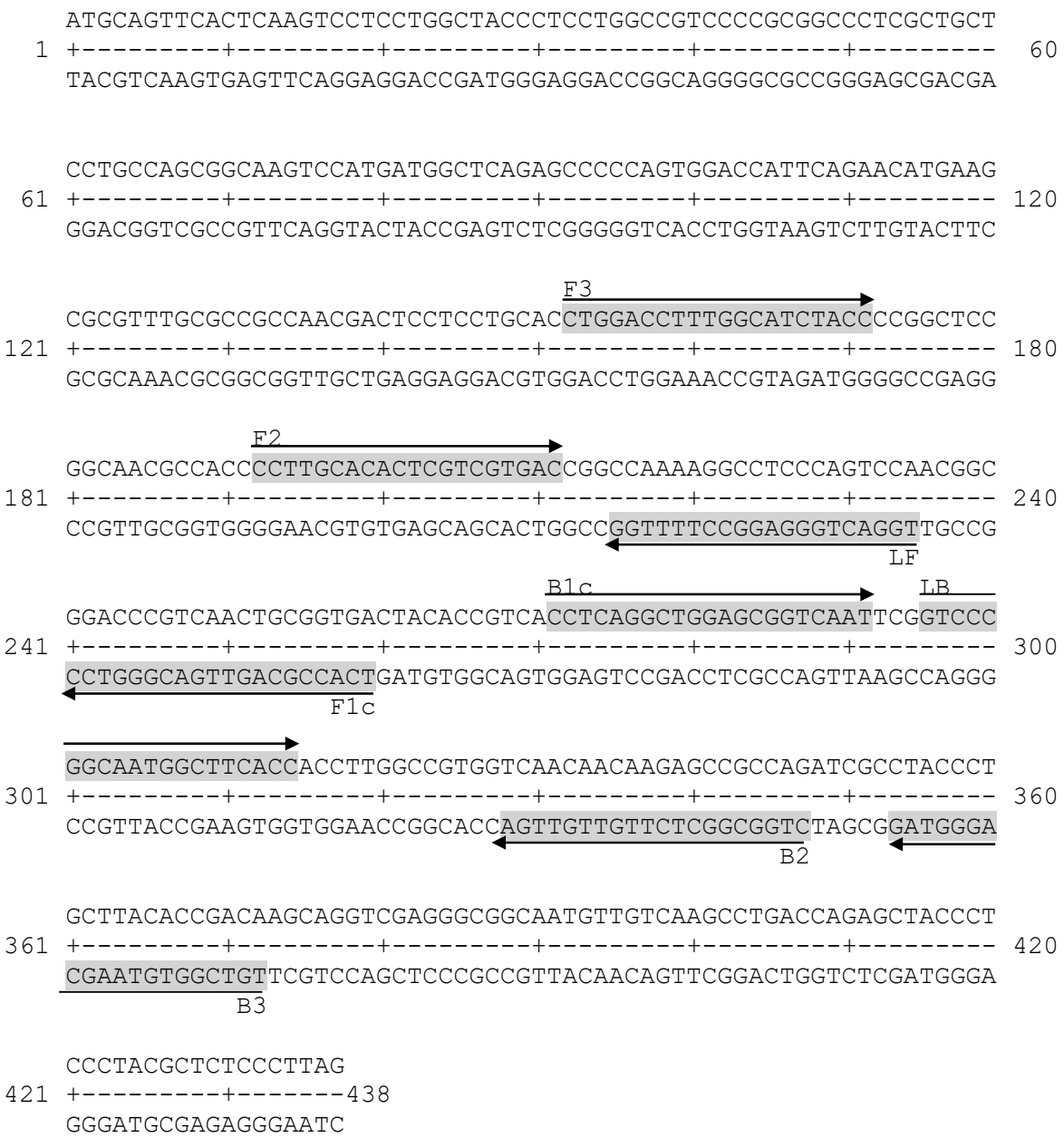


**Figure 5:** Double-stranded nucleotide sequence of the gene coding for protein PDE_07106 in *P. oxalicum* 114-2according to NCBI database. Binding sites of the LAMP primers used are marked in grey and their orientation is indicated by arrows. External primers = F3, B3; internal primers = F2-F1c, B2-B1c; loop primers = LF, LB

In contrast to LAMP primer design, the design of primers for PCR-based DNA amplification was conducted manually. Binding regions of the oligonucleotides were sometimes equipped with recognition sites of endonucleases (see section Table 12) to achieve the directed insertion of a PCR-

product into a vector. Moreover, in some cases additional non-binding nucleotides were added to the 5'-end of primers to ensure the correct binding of restriction enzymes or to shift the reading frame.

#### *2.2.4.2 Determination of global sequence features of protein or DNA sequences*

The nucleotide- as well as the amino acid sequences of proteins PDE_07106 and PDE_04519 were analyzed with the help of different software tools (see section 2.1.8). The DNA sequences were checked for the occurrence of inverted or tandem repeats with the programs REPFIND and Tandem Repeats Finder. The protein sequences were checked for various global sequence features. Signal cleavage sites were predicted by the online tool SignalP4.1. By use of other online tools like Protein molecular weight, ProtParam or ProtScale different physicochemical properties were computed. The secondary structure of amino acid sequences was predicted with the help of the program Yapsin.

# 3 Results

## 3.1 Development of a new technique for the preparation of proteins from (sparkling) wine

Red (sparkling) wine is more prone to gushing than white sparkling wine. But red sparkling wine is an uncommon, yet complex matrix for protein analysis. As wine has a low content of proteins, but at the same time a high content of interfering substances like e.g. salts, sugars, alcohols or phenols it was not possible to use it directly for analytical methods like SDS-PAGE or MALDI-TOF MS. Therefore, the first main task of this thesis was to find a suited method for the preparation of red sparkling wine proteins.

By dialysis (see section 2.2.1.2) small molecular weight compounds such as ethanol, glycerin or salts were removed during the current study. However, samples showed no detectable bands at all applied to SDS-PAGE gels with subsequent silver staining. Consequently, a concentration of the proteins needed to be performed by freeze drying. However, SDS-PAGE conducted with samples treated as described displayed an extremely strong background with the result that hardly any single band was distinguishable in gels (see Figure 6).

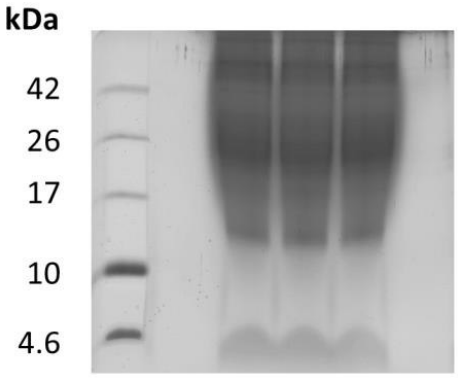


**Figure 6:** SDS-PAGE of red sparkling wine samples treated with dialysis and lyophilization in triplicates. Proteins are visualized by silver staining.

In order to reduce the background caused by phenolic compounds such as pigments or tannins, a broad arsenal of methods was tested. Known as a wine fining agent used to reduce phenols, the reagents PVP, PVPP as well as bentonite were assessed for their usefulness in sample preparation. However, no reduction of background in silver stained polyacrylamide gels was obtained with none of the three substances. Also attempts to isolate proteins by cloud point extraction or non-ionic detergents such as Berol 532, Berol 840 or Antifoam B Emulsion, failed. Testing of precipitation reactions to isolate proteins e.g. according to the protocols of Brovko and Zagranichnaya (1998) or Damerval *et al.* (1986) were only mildly successful in improving SDS-PAGE results. Furthermore, a technique was used in which freeze-dried samples were suspended in extraction puffer (0.1 M Tris-HCl (pH 8.8), 10 mM EDTA, 0.4 % (v/v) β-mercaptoethanol, 10 % (w/v) DTT, 100 mM KCl) and protein precipitation was achieved with three times the volume of 0.1 M ammonium acetate in methanol at -20 °C overnight. Protein pellets were separated from the supernatant by centrifugation (3,000 x g,

10 min, 4 °C) and washed with 80 % acetone. Subsequently the supernatant was discarded again after centrifugation (3000 g, 10 min, 4 °C) and the resulting pellets were air-dried and stored at -20 °C. An application of so treated samples onto SDS-PAGE resulted in silver stained gels with slightly improved backgrounds as compared to previously used protocols. Some protein bands became visible but a clear distinction between bands was still not possible (see Figure 7).

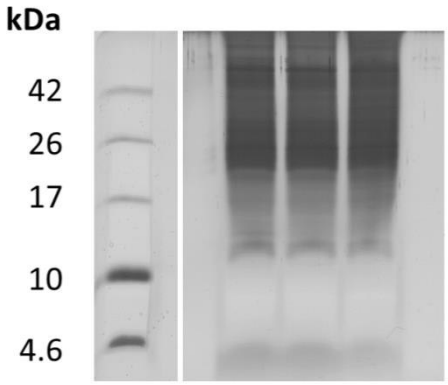


**Figure 7:** SDS-PAGE of red sparkling wine samples treated with a protein precipitation protocol in triplicates (same sample as in **Figure 6**). Proteins are visualized by silver staining.

Inspired by the method of Hurkman and Tanaka (1986) for extraction of interfering phenolic compounds from plant material, a new preparation technique was developed as described in 2.2.1.1. The protocol was composed of dialysis and lyophilization followed by a phenol extraction of proteins with water-saturated phenol and precipitation of proteins by addition of ammonium acetate in methanol. During the optimization of this protocol, application of different numbers of washing steps (0-4) as well as various organic solvents for extraction (hexane, butanol and phenol/chloroform/isoamyl alcohol) were tested. Best results were obtained by application of one washing step and the use of water-saturated phenol for extraction. Silver stained polyacrylamide gels of samples prepared by the proposed method resulted in minimal background and streaking as well as clearly resolved bands (see Figure 8). To demonstrate the reproducibility of this preparation technique several runs were conducted.

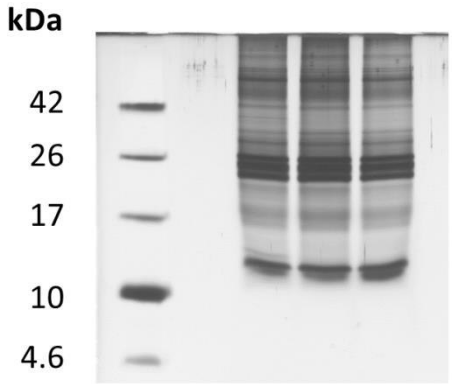


**Figure 8:** SDS-PAGE of red sparkling wine samples treated with the new preparation protocol in triplicates (same sample as in **Figure 6**). Proteins are visualized by silver staining.

The applicability of this method for the preparation of proteins of other grape-derived products was assessed on SDS-PAGE with subsequent silver staining. For example a silver stained polyacrylamide gel of different wines and red grape juice (see Figure 9) also displayed minimal background and clearly distinguished protein bands.

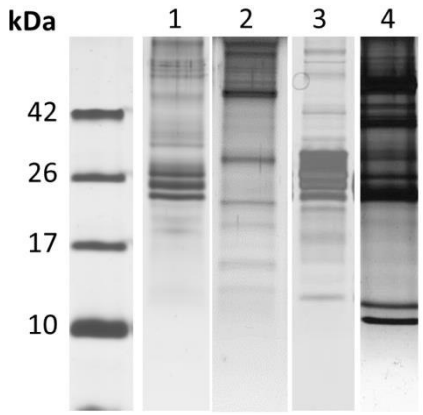


**Figure 9:** Application of the new preparation protocol for (1) white wine, (2) red wine (3), rosé wine Weißherbst and (4) red grape juice. SDS-PAGE of different samples treated with the new preparation protocol with subsequent silver staining for the visualization of proteins.

Therefore, the preparation protocol was found to be suitable for all kinds of non-sparkling wines (red, white, rosé) as well as grape juice. By use of this technique, protein patterns of grape-derived beverages could be compared with each other as the basis for proteomic analysis of samples.

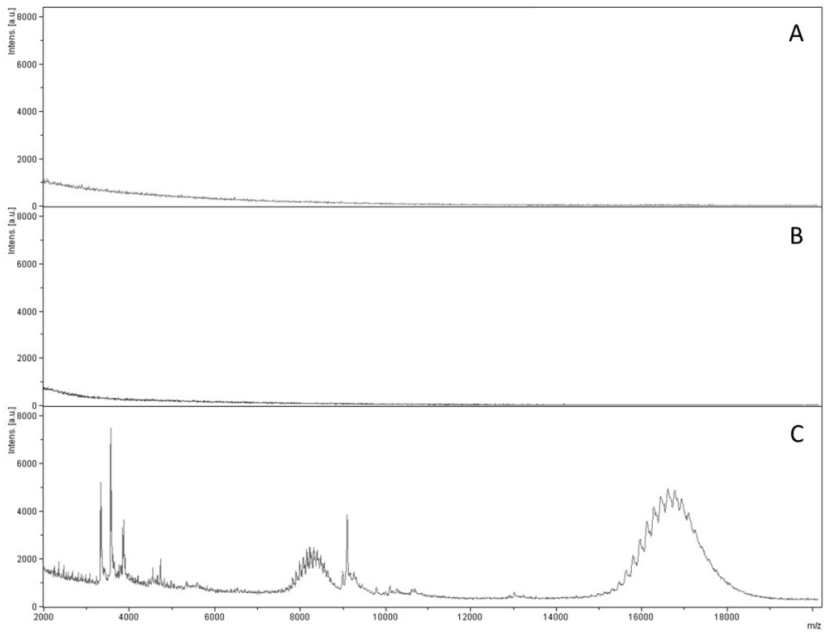


**Figure 10:** MALDI-TOF MS spectra of red sparkling wine obtained with three different preparation techniques for the same sample. (A) Dialysis and lyophilization. (B) Dialysis and lyophilization with subsequent protein precipitation with ammonium acetate. (C) Dialysis and lyophilization with extraction of phenols by use of water-saturated phenol and subsequent protein precipitation with ammonium acetate.

To elucidate whether this preparation method also fits for other protein analytical methods, MALDI-TOF MS analysis was performed with samples prepared according to the different protocols

previously described. The suitability of the different protocols was determined by evaluation of signal-to-noise-ratio and signal intensity. Samples treated with dialysis and lyophilization with or without subsequent protein precipitation with ammonium acetate showed low signal intensity and a low signal-to-noise ratio. In contrast, the same sample prepared with the newly developed preparation protocol displayed clear signals in MALDI-TOF MS analysis with a high signal to noise ratio (see Figure 10).

In order to further optimize MALDI-TOF MS analysis, different methods for the deposition of matrix (sinapinic acid (SA)) and samples were evaluated (see Figure 11). A single-layer method that involved covering of the sample with a single layer of matrix was tested. Furthermore, a protocol that included mixing of sample and matrix in a reaction tube before spotting onto the target was assessed as well. Both techniques revealed high signal-to-noise ratios but only modest signal intensities (see Figure 11 A and C). In contrast, by use of the sandwich method (matrix-sample-matrix), signal intensity increased with a consistent signal-to-noise ratio (see Figure 11 B). In addition to the loading strategy, various matrices (SA, DHBA, HCCA) were tested in MALDI-TOF MS analysis with wine proteins (Figure 11 lowercase letters). Best results in regard to signal-to-noise ratio and signal intensity were obtained by using SA as matrix (Figure 11 b).

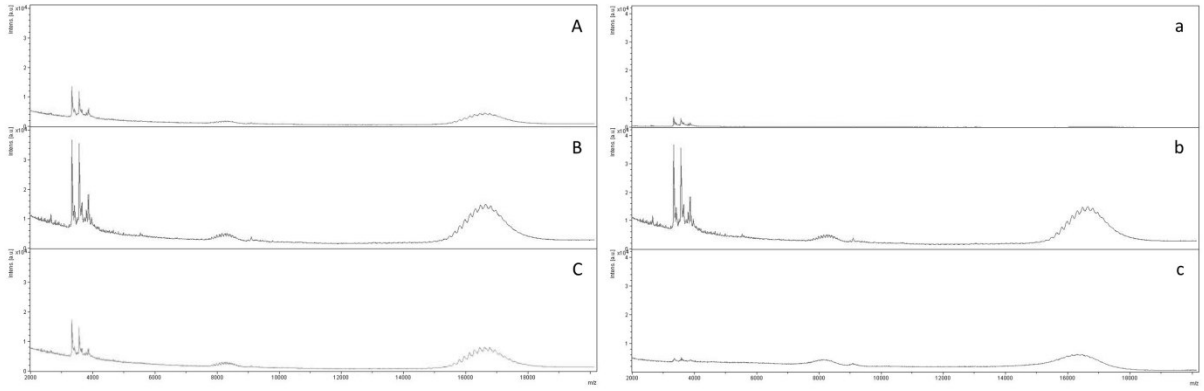


**Figure 11:** MALDI-TOF MS spectra of red sparkling wine. Spectra obtained with three different deposition methods for the same sample: (A) single layer method (B) sandwich method (C) previous mixing method; Spectra obtained with three different matrices for the same sample: (a) DHBA (b) SA (c) HCCA.

The results showed that the newly developed preparation protocol for proteins from grape-derived beverages is an effective tool for the removal of interfering substances. This preparation technique allowed subsequent high-resolution protein analysis via SDS-PAGE and MALDI-TOF MS by minimizing the background and increasing signal-to-noise ratio, respectively.

## 3.2 Comparison of gushing and non-gushing sparkling wines

In order to define marker proteins, the protein composition of several samples of gushing and non-gushing red sparkling wines was compared using SDS-PAGE and MALDI-TOF MS analysis.

In Figure 12 a compilation of different gushing and non-gushing red sparkling wines is shown. The samples were treated with the new preparation method (2.2.1.1) and applied onto SDS-PAGE with subsequent silver staining. For each sparkling wine several protein bands could be visualized.

However, no remarkable differences between the protein patterns of gushing and non-gushing red sparkling wine were detectable except for a bunch of proteins with a molecular weight of ~25 kDa which was strongly represented in all non-gushing samples and was barely visual in the gushing sparkling wine. In general, a more diverse protein pattern was seen in the non-gushing sparkling wine samples as compared to gushing sparkling wine.

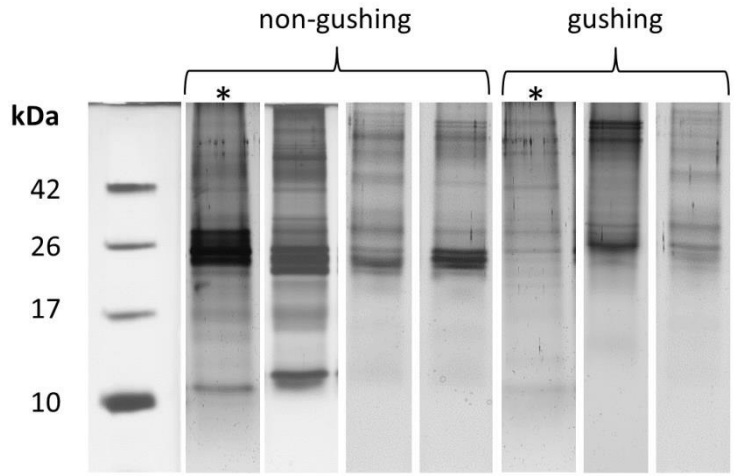


**Figure 12:** SDS-PAGE of different gushing and non-gushing red sparkling wine samples treated with the new preparation protocol with subsequent silver staining for the visualization of proteins. From lanes marked with * protein bands were excised for subsequent identification.

Two conspicuous protein bands (~13 kDa and ~25 kDa) were excised from gels of non-gushing sparkling wine samples and identified via sequencing analysis (see 2.2.2.2). Likewise, some protein bands (~42 kDa and ~55 kDa) more noticeable in one of the gushing samples were examined in the same way. Results revealed that the tested proteins in non-gushing sparkling wine could be identified as grape proteins. In contrast, fungal proteins were detected in the gushing sparkling wine sample (see Table 22).

**Table 22:** Identification of proteins from different sparkling wines. Data were obtained by nanoESI-LC-MS/MS followed by Mascot analysis as well as SDS-PAGE. (1) Theoretically calculated mol. mass of proteins (w/o signal sequence) in kDa. (2) Estimated mol. mass of proteins in kDa visualized via silver staining after SDS-PAGE.

| identified protein | organism | unique peptides | sequence coverage [%] | (1) | (2) | GenBank accession | sample |
|---|---|---|---|---|---|---|---|
| **unnamed protein product** | *Vitis vinifera* | 7 | 49.7 | 12.93 | 13 | CBI35210 | non-gushing sparkling wine |
| **hyp. protein VITISV_021587** | *Vitis vinifera* | 6 | 49.3 | 21.26 | 25 | CAN66515 | non-gushing sparkling wine |
| **probable pectin lyase A** | *Aspergillus niger* | 11 | 35.4 | 37.87 | 40 | UniProt A2R3I1.1 | gushing sparkling wine |
| **lcc2, laccase** | *Botrytis cinerea* | 13 | 19.4 | 61.44 | 55 | CCD44233 | gushing sparkling wine |

Nevertheless, the protein composition of sparkling wine seemed to be influenced by other factors more fundamentally than by the parameter “gushing vs. non-gushing”. A significant marker protein exclusively present in all gushing or all non-gushing samples was not detectable.

Similar results were obtained by MALDI-TOF MS analysis. As an example, MS-spectra of several red sparkling wine samples are displayed in Figure 13. Various protein peaks but no marker peak for the attributes “gushing” or “non-gushing” could be defined from the spectra. Consequently, also with this analytic method other parameters probably changed the protein pattern more decisively than the occurrence of gushing.

Neither by SDS-PAGE nor by MALDI-TOF MS a significant marker protein was found to distinguish between gushing and non-gushing sparkling wine. Obviously, the protein composition of sparkling wine samples depended on other external factors as well.

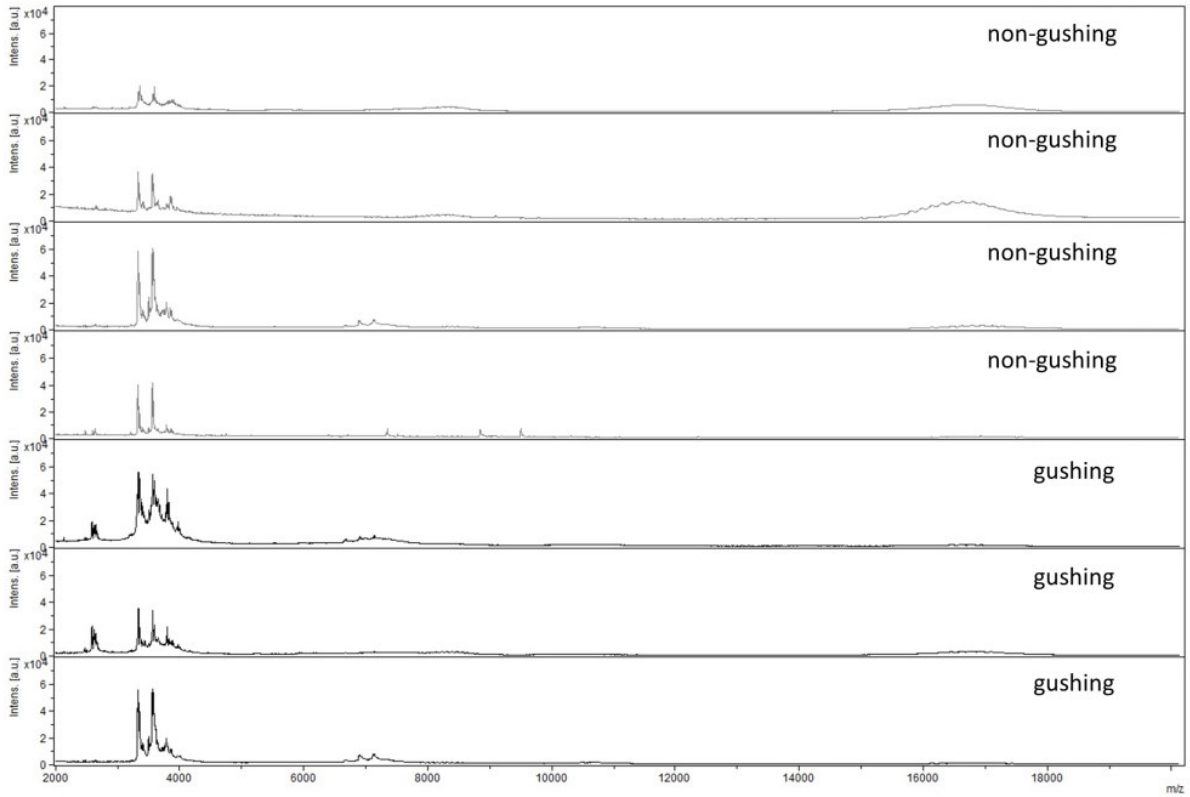


**Figure 13:** MALDI-TOF MS spectra of different gushing as well as non-gushing red sparkling wines treated with the new preparation protocol.

## 3.3 Influence of external factors on the protein composition of single-variety wines

In order to analyze the protein composition of different wines in regard to the influence of external factors, wines produced from different precisely defined grape varieties under clearly set conditions were analyzed. With these samples the level of influence of factors such as production process, harvest year, variety or growing region was determined via SDS-PAGE and MALDI-TOF MS.

Comparison of two Pinot noir wines from the same region (Geisenheim) and harvest year (2013) demonstrated the importance of the production process for the protein pattern of the resulting wine. While one wine was produced as red wine, the other one was treated to became a rosé. By SDS-PAGE analysis two clearly distinct protein patterns were revealed (see Figure 14 A). The mash fermentation during the red wine production obviously impaired the protein content as well as the protein variability in the resulting wine. Nevertheless, it must be pointed out that the manufacturer used for the fermentation of the red wine the yeast strain "Oenoferm Klosterneuburg" and for the fermentation of the rosé wine the yeast strain "Oenoferm Freddo". Consequently, two sets of two wines each were selected differing solely in the utilized yeast to assess the influence of yeast on the protein pattern of the fermented wines. One set of wine contained two Rieslings from location Weinsberg (Germany) harvested in 2013 and the other set consisted of two Pinot blanc from location Neustadt (Germany) harvested in the same year. Between the two wines of each set only marginal differences were detectable in silver stained gel of SDS-PAGE analysis (see Figure 14 B and C). Therefore, the influence of yeast on the protein pattern of the fermented wines was classified as low in this experiment.

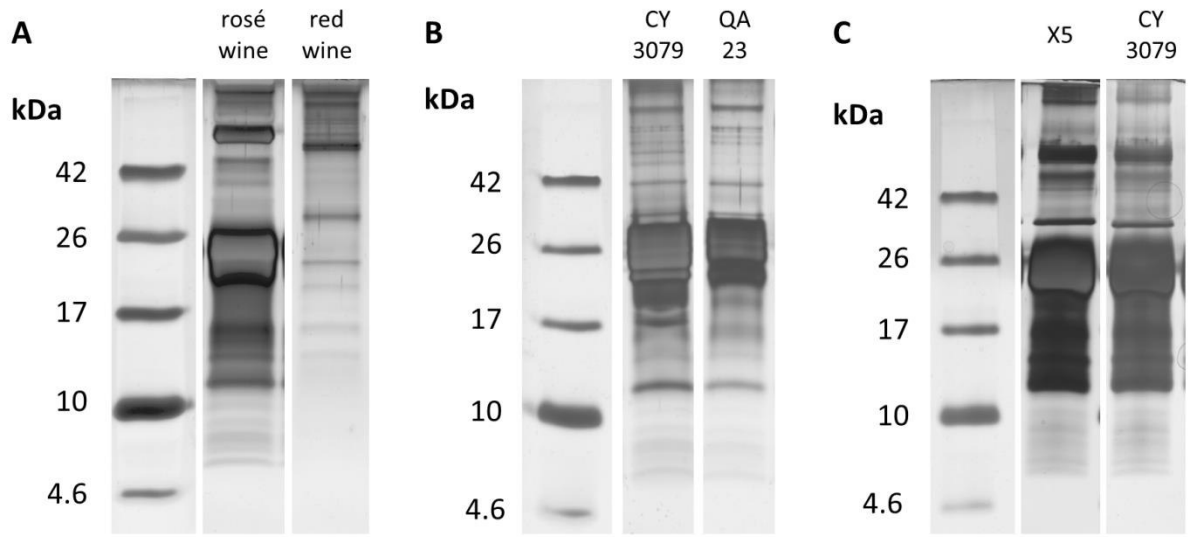


**Figure 14:** SDS-PAGE of different single-variety wine samples treated with the new preparation protocol with subsequent silver staining for the visualization of proteins. (A) Comparison of two Pinot noir wines (grapes harvested in the same year and region) produced as rosé or red wine. (B) Comparison of two Riesling wines (grapes harvested in the same year and region) fermented with two different *Saccharomyces cerevisiae* strains (Lalvin® CY 3079, Lalvin® QA 23). (C) Comparison of two Pinot blanc wines (grapes harvested in the same year and region) fermented with two different *Saccharomyces cerevisiae* strains (Zymaflore® X5, Lalvin® CY 3079).

In the next series of experiments the influence of the harvest year of the grapes on the protein pattern of the resulting wine was evaluated. Two examples of SDS-PAGE with subsequent silver staining of wine samples only differing in the harvest year are displayed in Figure 15 A and B. In part A of the figure, a comparison of two different Grüner Veltliner wines from location Krems (Austria) each from harvest years 2013 and 2014 are shown. In the protein pattern of wines made of grapes from the same variety and region fermented with the same yeast hardly any differences could be seen as long as the harvest year was the same, too. Comparing protein patterns of wines between the two harvest years revealed a considerable influence of the harvest year of the grapes on the

protein pattern of the resulting wines. This fact is underlined by the results shown in Figure 15 B. Three Riesling wines from the same region (Neustadt, Germany) but of different vintages displayed completely different protein patterns after SDS-PAGE analysis. However, within the same vintage the protein pattern was highly conserved.

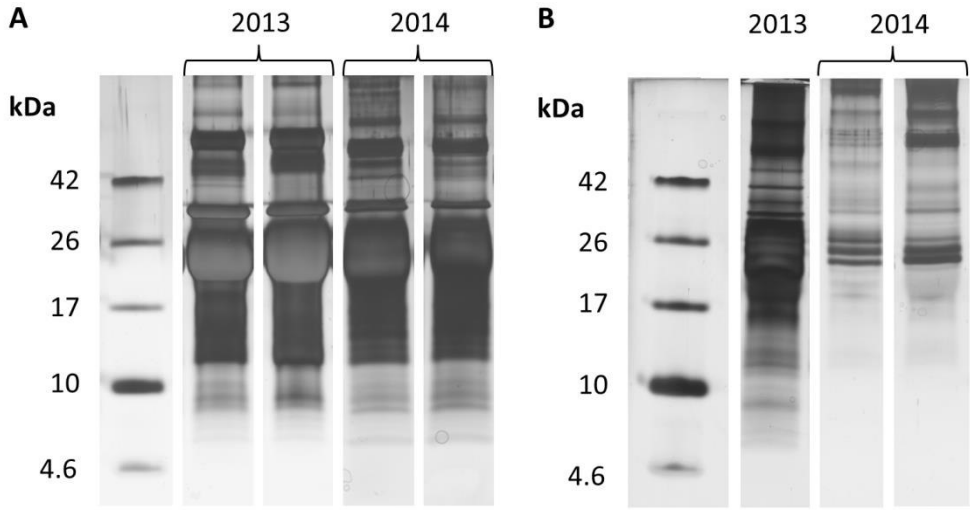


**Figure 15:** SDS-PAGE of different single-variety wine samples treated with the new preparation protocol with subsequent silver staining for the visualization of proteins. (A) Comparison of two times two Grüner Veltliner wines produced from grapes of the same region but of different years. (B) Comparison of three Riesling wines produced from grapes of the same region but of different years.

The influence of grape variety on the protein patterns of wines was investigated as illustrated in Figure 16 A.

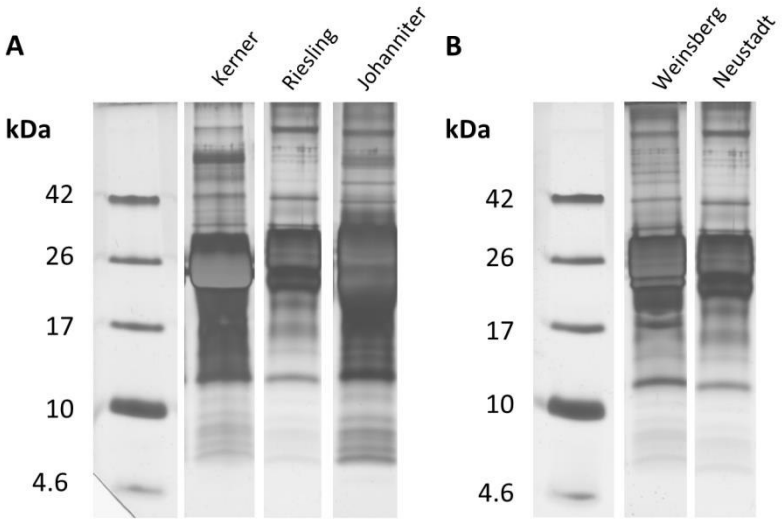


**Figure 16:** SDS-PAGE of different single-variety wine samples treated with the new preparation protocol with subsequent silver staining for the visualization of proteins. (A) Comparison of wines of different grape varieties produced of grapes from the same region and vintage year. (B) Comparison of two Riesling wines produced from grapes of different regions but of the same vintage year.

All three wines were made of grapes from location Weinsberg (Germany) in 2013 and were fermented with the same yeast strain. Considerable differences in the protein pattern between different varieties could be detected via SDS-PAGE analysis. Nevertheless, also some similarities

between related species (Kerner and Johanniter are both Riesling hybrids) became visible. The results obtained confirmed the assumption according to which also the grape variety has an influence on the protein pattern of the final wine.

For the assessment of the influence of the growing region on the wine's protein pattern no perfectly suiting samples were available to the study. Only two Riesling wines from the same vintage (2013) which were fermented with different yeast strains were available. Hardly any differences could be detected in SDS-PAGE analysis (see Figure 16 B). Consequently, the growing region did not seem to be decisive for the resulting protein pattern of wine.

This chapter of the thesis obviously showed the crucial influence of external factors such as fermentation condition, harvest year or variety on the protein pattern of the final wine. These findings were displayed in silver stained polyacrylamide gels, but MALDI-TOF MS analysis revealed similar results: The wine's protein pattern depends on external factors. With this information in mind, the comparison of the protein patterns of gushing and non-gushing sparkling wine as a means of finding responsible proteins was not considered appropriate. Therefore, a new approach for the investigation of the gushing phenomenon in sparkling wine was followed as described in the next chapters.

## 3.4 Screening of filamentous fungi isolated from grapes

As gushing research in beer repeatedly identified filamentous fungi growing on malt as main trigger for gushing (see section 1.2.4) and fungal proteins were identified in gushing sparkling wine (see section 3.2), further investigations focused on grape-associated filamentous fungi. Therefore, filamentous fungi isolated from grapes were assessed in regard to their secretion of protein-degrading substances on calcium caseinate agar. Furthermore, their ability to alter surface activity of culture supernatants as well as to secrete low molecular weight proteins was evaluated via their growth in protein-free medium. Finally, the potential of numerous selected fungal culture supernatants to induce gushing was tested in gushing-tests with sparkling water used as a model system. The screening procedure resulted in the selection of a test set of grape-associated fungi as potential candidates for gushing induction.

### 3.4.1 Screening for protease activity

In order to evaluate the ability of grape-associated filamentous fungi to secrete protein-degrading substances a test set of 36 isolates (27 species) of *Penicillium* and 22 isolates (13 species) of *Aspergillus* was assessed. The degradation of milk proteins was used as indirect evidence for an existing proteolytic activity in culture supernatants of the tested strains.

Except three *Penicillium* isolates, all fungi secreted some kind of proteases in their culture supernatants as was demonstrated by the clear-up of the caseinate agar after 4 h of incubation at ambient temperature. The culture supernatants of six *Aspergillus* species showed a strong proteolytic effect. However, most isolates from both genera were able to induce a moderate but noticeable degradation of caseinate (see Table 31 in the appendix). Almost no differences between an incubation time of 4 or 24 h could be detected.

This assay showed that almost all tested grape-associated fungi produced and secreted some kind of proteolytic substances. No obvious differences could be detected within or between the genera.

### 3.4.2 Screening for surface activity

The same set of 36 isolates (27 species) of *Penicillium* and 22 isolates (13 species) of *Aspergillus* isolated from grapes or grape musts was tested for their influence on the surface activity of culture supernatants. The secretion of surface-active compounds during a 12 d interval was monitored via a surface activity assay.

Results given in Figure 17 showed that the majority of tested strains had either a positive or negative influence on the wettability of a hydrophobic surface. Only a low percentage showed indifferent behavior in the test. Furthermore it was salient that much more strains of the genus *Penicillium* compared to *Aspergillus* had a positive influence on the wettability of the hydrophobic film used for testing. On day 8 of the experiment more than half of the *Penicillium* strains (53 %) were able to change the nature of the test-surface from hydrophobic to hydrophilic (Figure 17 A). In contrast, 82 % of *Aspergillus* strains did not exhibit this ability (Figure 17 B). Detailed results of the complete surface activity screening are provided in Table 32 in the appendix.

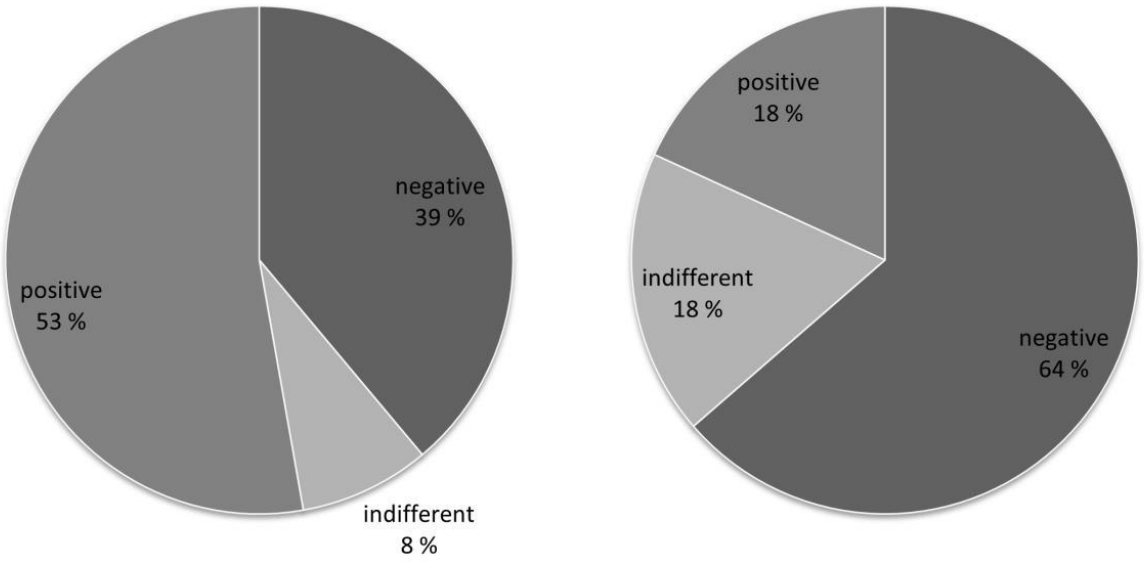


**Figure 17:** Influence of grape-associated fungi on the surface activity of their culture supernatants after 8 days of incubation. Isolates from genera *Penicillium* (A) and *Aspergillus* (B) have been compared.

This evaluation showed that *Penicillium* spp. had a very high potential to influence surface characteristics of supernatants.

### 3.4.3 Secretion of low molecular weight proteins

The test set of 58 isolates of grape-associated filamentous fungi was analyzed in regard to the secretion of small proteins into the culture supernatant. Fungi were grown in protein-free liquid medium in order to detect only secreted fungal proteins by application of culture supernatants onto SDS-PAGE with subsequent silver staining.

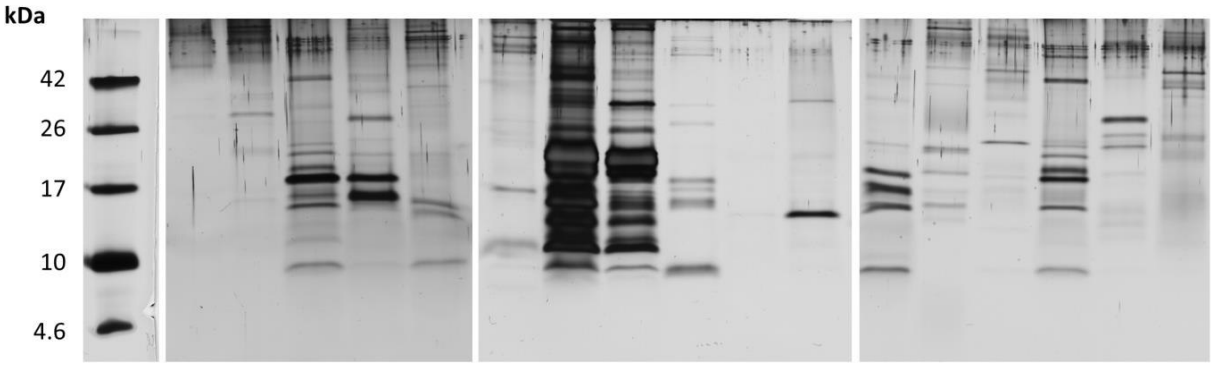


**Figure 18:** SDS-PAGE with subsequent silver staining of the culture supernatants of various grape-associated fungi.

The obtained polyacrylamide gels displayed a variety of different protein patterns for the analyzed fungal species. Some isolates secreted many different proteins, whereas other fungi produced apparently no proteins at all in the culture supernatant. Also the size of the secreted proteins varied from species to species. To provide a brief insight into the diversity of various protein patterns of different filamentous fungi a compilation of randomly selected polyacrylamide gels are displayed in Figure 18.

SDS-PAGE analysis of culture supernatants revealed a high diversity of protein patterns between different filamentous fungi. No general tendency for one of the genera or special anomalies for a species were conspicuous.

### 3.4.4 Induction of gushing in sparkling water as model system

All 36 isolates of the genus *Penicillium* were tested twice for their ability to induce gushing in sparkling water. As *Aspergillus* spp. performed poorly in section 3.4.2, no gushing tests were done with the strains of this genus.

Fungi were cultured for 8 d in Erlenmeyer flasks before 5 ml of each filtrate were assessed for their gushing inducing potential in a sparkling water model system. No gushing could be induced after direct addition of any of the culture filtrates in sparkling water. Therefore, foam fractionation was applied to enrich surface-active molecules from the culture supernatants. A spumate could be generated (see section 2.2.3.7) from only 19 out of the 36 tested strains. Culture supernatants of 17 of the strains gave no foam at all under the applied conditions. Gushing potential of the spumates was assessed in a sparkling water model system. Gushing was defined as severe (> 15 g), medium (> 5 g, < 10 g) or slight (< 5 g) according to the amount of lost water due to overfoaming. The results displayed in Table 23 showed that in the first run five spumates of *Penicillium* isolates induced severe gushing. From the remaining spumates one could induce medium, seven slight and six no gushing. The gushing potential changed slightly in the second run as in this case spumates of more strains were able to induce severe (seven strains) or medium (three strains) gushing. In the second run of the experiment, only three spumates provoked slight and another five no gushing at all. However, many strains influencing the gushing potential severely did it in a not consistent manner. There were spumates of nine different isolates from seven different species, which induced severe gushing. However, five of the strains were able to do so in only one of the two repetitions of the experiment.

Only the isolate of *P. vagum* as well as three isolates of *P. oxalicum* were able to induce severe or at least medium gushing in a reproducible manner. The strongest gushing inducing potential on average could be detected for *P. oxalicum* TMW 4.2553 with a gushing amount of > 30 g, closely followed by another *P. oxalicum* strain (TMW 4.2552) with a gushing amount of > 22 g.

**Table 23:** Gushing potentials of spumate of *Penicillium* isolates from grapes or grape musts. Values given in run 1 and run 2 are averages of technical triplicates. n.f. = no spumate could be generated by foam fractionation.

| mould | clone ID | strain no | difference in weight [g] | |
|---|---|---|---|---|
| | | | run 1 | run 2 |
| ***P. adametzioides*** | TMW 4.2529 | MUM 14.26 | n.f. | n.f. |
| ***P. aurantiogriseum*** | TMW 4.2536 | MUM 14.24 | 0.00 ± 0.00 | 8.53 ± 7.50 |
| ***P. bilaii*** | TMW 4.2528 | MUM 14.25 | n.f | n.f. |
| ***P. brevicompactum*** | TMW 4.2545 | MUM 14.27 | n.f. | n.f. |
| ***P. burgense*** | TMW 4.2541 | MUM 14.44 | 2.50 ± 2.26 | 21.73 ± 1.48 |
| ***P. citrinum*** | TMW 4.2547 | MUM 14.29 | 0.10 ± 0.17 | 0.00 ± 0.00 |
| ***P. coalescens*** | TMW 4.2537 | MUM 14.37 | n.f. | n.f |
| ***P. crustosum*** | TMW 4.2530 | MUM 14.31 | 4.17 ± 3.63 | 17.77 ± 2.51 |
| ***P. crustosum*** | TMW 4.2548 | MUM 14.30 | 6.33 ± 5.17 | 3.50 ± 2.02 |
| ***P. echinulatum*** | TMW 4.2549 | MUM 14.32 | 0.00 ± 0.00 | 1.93 ± 3.35 |
| ***P. glabrum*** | TMW 4.2531 | MUM 14.33 | n.f. | n.f. |
| ***P. griseofulvum*** | TMW 4.2532 | MUM 14.34 | n.f. | n.f. |
| ***P. janczewskii*** | TMW 4.2550 | MUM 14.35 | 17.17 ± 2.32 | 3.60 ± 3.90 |
| ***P. minioluteum*** | TMW 4.2533 | MUM 14.36 | n.f. | n.f. |
| ***P. novae-zelandiae*** | TMW 4.2551 | MUM 14.39 | 0.33 ± 0.58 | 30.50 ± 5.74 |
| ***P. olsonii*** | TMW 4.2538 | MUM 14.40 | 2.60 ± 4.50 | 6.83 ± 4.05 |
| ***P. oxalicum*** | TMW 4.2539 | MUM 14.41 | 18.10 ± 6.42 | 13.57 ± 5.24 |
| ***P. oxalicum*** | TMW 4.2552 | MUM 14.42 | 24.87 ± 2.59 | 30.03 ± 7.06 |
| ***P. oxalicum*** | TMW 4.2553 | MUM 14.43 | 31.77 ± 4.88 | 29.77 ± 0.90 |
| ***P. paneum*** | TMW 4.2542 | MUM 14.47 | n.f. | n.f. |
| ***P. purpurogenum*** | TMW 4.2540 | MUM 02.55 | n.f. | n.f. |
| ***P. raistrickii*** | TMW 4.2554 | MUM 14.45 | n.f. | n.f. |
| ***P. restrictum*** | TMW 4.2555 | MUM 14.46 | n.f. | n.f. |
| ***P. sclerotiorum*** | TMW 4.2535 | MUM 03.39 | n.f. | n.f. |
| ***P. sclerotiorum*** | TMW 4.2556 | MUM 14.48 | 0.00 ± 0.00 | 0.00 |
| ***P. simplicissimum*** | TMW 4.2557 | MUM 14.49 | n.f. | n.f. |
| ***P. spinulosum*** | TMW 4.2543 | MUM 14.51 | n.f. | n.f. |
| ***P. spinulosum*** | TMW 4.2558 | MUM 14.50 | n.f. | n.f. |
| ***P. thomii*** | TMW 4.2559 | MUM 14.52 | n.f. | n.f. |
| ***P. vagum*** | TMW 4.2534 | MUM 14.38 | 15.93 ± 3.62 | 18.50 ± 0.95 |
| ***P. variabile*** | TMW 4.2560 | MUM 14.53 | 4.80 ± 1.15 | 23.30 ± 2.00 |
| ***P. variabile*** | TMW 4.2561 | MUM 14.54 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| ***P. verrucosum*** | TMW 4.2546 | MUM 14.28 | 0.30 ± 0.06 | n.f. |
| ***P. verrucosum*** | TMW 4.2585 | ITEM 3716 | n.f. | n.f. |
| ***P. verrucosum*** | TMW 4.2586 | ITEM 3717 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| ***P. waksmanii*** | TMW 4.2544 | MUM 14.55 | 0.00 ± 0.00 | 0.00 ± 0.00 |

Consequently, the species *Penicillium oxalicum* clearly stood out in this experimental setting from all tested fungi in the ability to induce gushing in sparkling water by the addition of cell-free culture supernatant.

## 3.5 Gushing test with culture supernatants of *Penicillium oxalicum*

As demonstrated in section 3.4.4, *P. oxalicum* isolates were able to induce severe gushing in sparkling water under the tested conditions. In order to analyze the influence of boundary conditions in the gushing test setup several parameters were assessed.

### 3.5.1 Influence of culture conditions and foam fractions on the gushing potential

*P. oxalicum* isolates were grown in Fernbach flasks instead of Erlenmeyer flasks to evaluate the influence of different culture vessels with different geometry on the gushing potential of supernatants. Cultivation as well as preparation of culture supernatants was conducted as usual. Furthermore, different foam fractions as well as the impact of addition of various sample volumes on the result of the gushing test were assessed.

**Figure 19:** Picture of *P. oxalicum* TMW 4.2539 culture growing in different flasks under otherwise identical conditions for 7 days.

Just by glancing, a considerable effect on the cultural characteristics of the fungi grown in Fernbach flasks could be detected when compared to cultures grown in Erlenmeyer flasks. Under these conditions, cultures produced small yellow flakes of substrate mycelium without showing surface mycelia or sporulation. In contrast to this, the same strain appeared to produce voluminous clots of white substrate mycelium with considerable masses of surface mycelium when grown in Erlenmeyer flask in the same medium under otherwise identical conditions (see Figure 19). A comparison of the volume of gushing demonstrated that the spumate of the same fungal isolate (*P. oxalicum* TMW 4.2539 ) obviously induced almost four times stronger gushing when the fungus was grown in a Fernbach flask as compared to cultivation in an Erlenmeyer flask (see Figure 20 A). For further studies, the different fractions obtained by foam fractionation of the Fernbach flask grown cultures of *P. oxalicum* TMW 4.2539 were assessed in gushing tests. The results showed that vigorous gushing could only be induced by the addition of the spumate fraction (see Figure 20 B). The addition of retentate or filtrate to sparkling water did not or to a much lesser extent lead to gushing in the sparkling water model system (difference in weight never exceeding 1 g). Finally, the amount of spumate added in gushing tests was analyzed. Results shown in Figure 20 C demonstrated that there

was a correlation between the addition of different volumes of spumate (ranging between 100 µl and 3000 µl) and the volume of water expelled by gushing. The addition of 3000 µl spumate to sparkling water resulted in a loss of approximately 100 ml water during opening. When 100 µl were added the amount of gushing was almost ten times smaller.

Obviously, culture conditions had an extreme effect on the gushing-inducing potential of culture supernatants. Moreover, it was confirmed that only with the spumate fraction severe gushing could be induced in the sparkling water model system. A simultaneous increase of the volume of spumate and the volume of water expelled by gushing was detected.

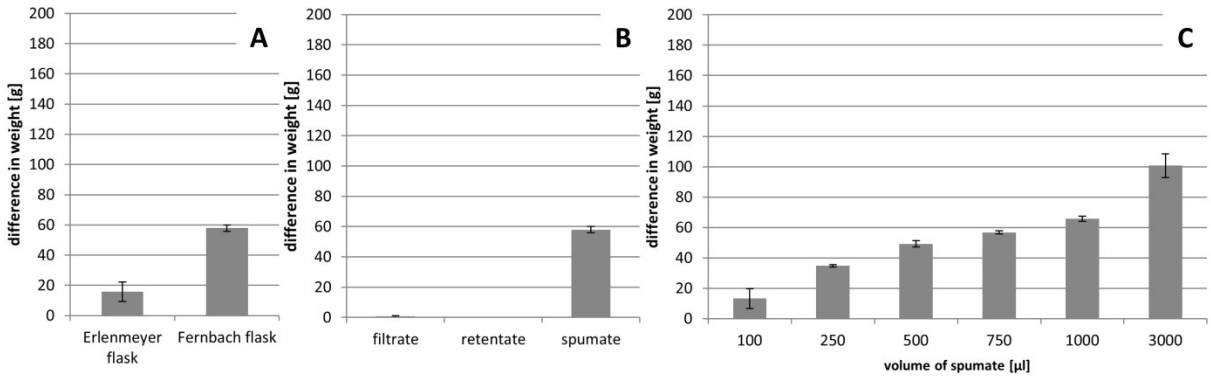


**Figure 20:** Induced gushing with *P. oxalicum* TMW 4.2539 cultures. In gushing tests the influence of culture vessel (A), foam fraction (B) and volume of the sample (C) were assessed.

### 3.5.2 Effect of temperature and pH of the sparkling water on gushing

Besides alteration of the culture conditions, changes in factors relating to the physical properties of the sparkling water model system on the outcome of gushing tests were investigated. By addition of hypochloric acid or sodium hydroxide the pH of sparkling water used for gushing tests was adjusted to different values. The influence of temperature of the sparkling water was tested by incubating the bottles of the gushing test at different temperatures before opening.

Different pH values of the sparkling water (pH range 0.8-10.8) used for gushing tests were investigated. Figure 21 A showed that gushing was not induced when the pH of the water exceeded pH 6. In the pH range of 4.8 to 5.8 no significant difference in the amount of gushing could be determined. A very low pH of 0.8 reduced the gushing amount by about 50 %.

In another series of tests, different temperatures and their influence on gushing were assessed. Results shown in Figure 21 B revealed that the gushing volume and the temperature of the sparkling water at opening of the bottle were positively correlated: The higher the temperature of the liquid, the bigger was the volume loss after opening of a gushing bottle. On the other hand low temperatures reduced or even inhibited gushing. Increase of the temperature from ambient temperature to 60 °C resulted in an approximately three times greater loss of water during opening of the bottle. In contrast, if the temperature was decreased from ambient temperature to 4 °C the amount of gushing was almost a 100-fold smaller.

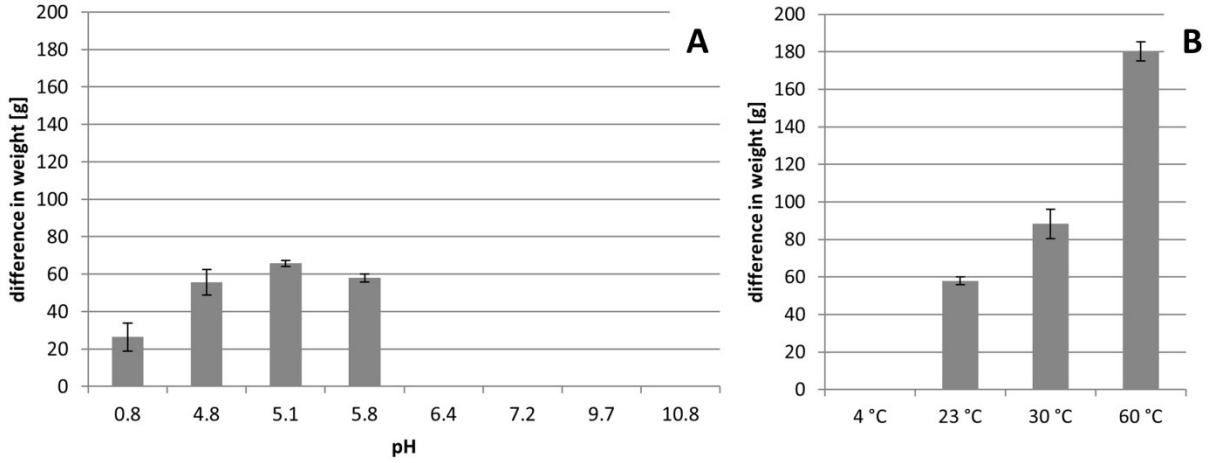


**Figure 21:** Gushing induction with spumate from a culture supernatant of *P. oxalicum* TMW 4.2539 at different pH (A) and temperature (B) of the sparkling water used in gushing tests.

These series of experiments demonstrated that the sparkling water used for gushing tests was of great importance for its outcome. Alterations in pH or temperature had an extreme effect on the volume of water expelled by artificially induced gushing. However, none of the conditions whatsoever led to the occurrence of gushing without the addition of spumate from the culture supernatant of *P. oxalicum* TUM 4.2539.

### 3.5.3 Applicability of sparkling wine in gushing tests

Even though the model system sparkling water was used in general for gushing tests, the application of sparkling wine as the carbonated medium in gushing tests was assessed. Fungal culture supernatant of *P. oxalicum* TMW 4.2539 was added to commercial sparkling wine for gushing tests instead of sparkling water under otherwise identical conditions.

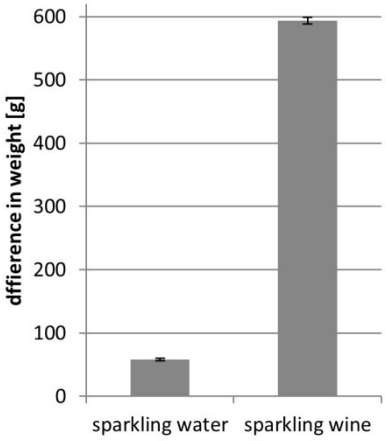


**Figure 22:** Amount of gushing induced with spumate of culture supernatant from *P. oxalicum* TMW 4.2539 in the model system sparkling water and in sparkling wine.

The addition of 1 ml fungal spumate (cultured in a Fernbach flask) led to a loss of liquid of almost 80 % relative to the bottle volume in sparkling wine but only around 20 % in sparkling water. In the case of sparkling wine 593.57 ml ± 5.63 ml were expelled by gushing from a 750 ml bottle. However, as in sparkling water, addition of pure culture medium did not induce any gushing in sparkling wine.

Results showed that the sparkling water model will provide results that can be conferred to the situation in sparkling wine and that it can be used as cheap and easy to use substitute system in further analyses.

## 3.6 Investigation of the exoproteome of *Penicillium oxalicum*

Since all fungal gushing-inducing factors in beer were identified as proteins, (poly)peptides or peptidoglycans (see section 1.2.4) further research to examine the fungal proteins secreted into the surrounding medium were conducted with protein chemical methods. These secreted proteins were referred to as the exoproteome of a fungus. By a diversity of biochemical methods like SDS-PAGE, 2D-GE and RP-HPLC these extracellular proteins were analyzed. An enrichment of the fraction of surface-active proteins from the exoproteome was conducted by foam fractionation.

Cell-free culture supernatant from the available *P. oxalicum* isolates were obtained as described in 2.2.3.4 prior to foam fractionation (see 2.2.3.7). Generated spumate consisted of highly stable foam (see Figure 23 A), whereas the other fractions (retentate, filtrate) did not show the formation of stable foam. SDS-PAGE analysis of all three fractions revealed a strong enrichment of various proteins in the spumate. In contrast, some proteins disappeared in the retentate or were detectable to a lesser extent as in the filtrate. Figure 23 B shows an example of a silver stained polyacrylamide gel from SDS-PAGE analysis of the different foam fractions of *P. oxalicum* TMW 4.2539. Two other analyzed isolates displayed highly similar results. Results showed that the technique of foam fractionation was effective to concentrate proteins which were assumed to have surface-active properties from culture supernatants for further analysis.

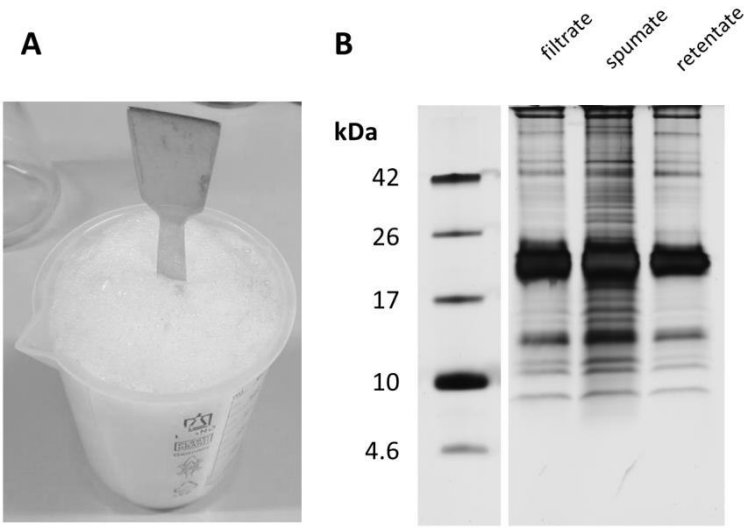


**Figure 23:** Enrichment of surface-active proteins from culture supernatants of *P. oxalicum* TMW 4.2539 by foam fractionation. (A) Picture of spumate generated by foam fractionation. Note that a metal lab spoon stands stable in an upright position (B) Silver stained SDS-PAGE of different fractions obtained by foam fractionation.

In order to obtain a more resolved overview over the proteome of spumate and retentate, protein preparations of both fractions were separated by 2D-GE. Comparison of the gels displayed in Figure 24 shows that the variety of proteins in the spumate (see Figure 24 A) fraction is way higher as compared to the retentate fraction (see Figure 24 B). 2D-GE conducted with spumate of *P. oxalicum*

isolates resulted in silver stained gels with the majority of proteins visible in the low to medium pH-range.

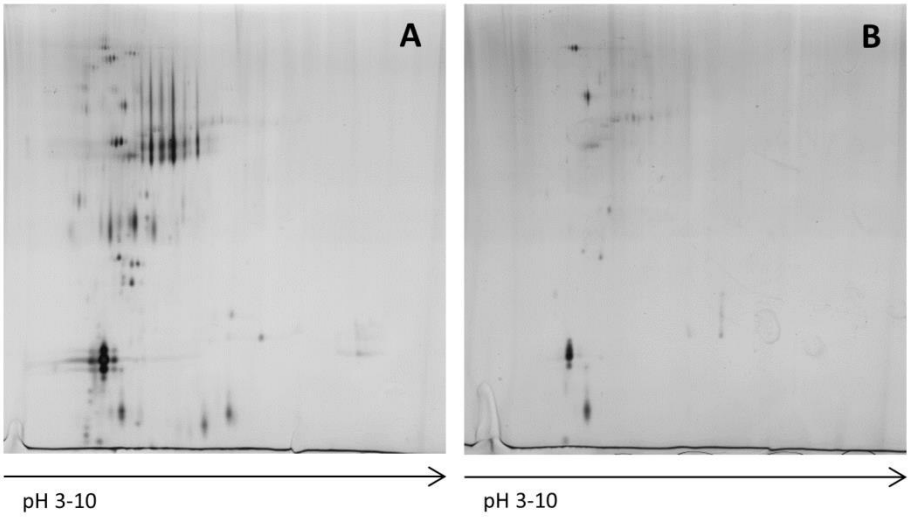


**Figure 24:** 2D-GE of different fractions ((A) spumate, (B) retentate) obtained by foam fractionation of culture supernatants of *P. oxalicum* TMW 4.2539. Proteins were visualized by subsequent silver staining.

On the contrary, the retentate of the corresponding supernatants applied onto 2D-GE displayed just a few scattered spots, also in the low to mid pH-range (Figure 24 B). Just by visual gel mapping many spots could be detected which appeared only in the spumate. According to the IGP strips used for isoelectric focusing the isoelectric point (pI) of almost all proteins of both fractions was detectable in the acidic or neutral pH range. Moreover, many proteins in the spumate fraction occurred in a horizontal row (same molecular mass, slightly different pI) indicating a glycosylation of these proteins. Such glycosylations could not be seen in proteins found in the retentate.

In a next series of experiments, proteins in filtrate, spumate and retentate of *P. oxalicum* culture supernatants of each isolate were separated by RP-HPLC. Comparison of separation patterns showed that there were hardly any qualitative differences detectable between the three fractions. However, some of the protein peaks had a higher intensity in the spumate fraction as compared to filtrate or retentate (Figure 25 A). In particular, two protein fractions which eluted at an acetonitrile content of 40-50 % and a retention time of 21 min and 23 min, respectively, were concentrated in the spumate fraction. Comparing the areas under the peaks of those two proteins in each spumate and retentate with the respective filtrate of the three *P. oxalicum* isolates showed a decrease of at least 20 % in the retentate fraction and an increase between 10 % and over 100 % in the spumate fraction.

Furthermore, absorbance of those proteins at 214 nm was much higher as compared to absorbance at 280 nm (see Figure 25 B). The two prominent protein fractions eluting with an acetonitrile concentration between 40-50 % (retention time 21 min and 23 min) that showed almost no absorbance at 280 nm were collected from the RP-HPLC for further analysis. According to their retention times, fractions were referred to as “RET21” and “RET23”.

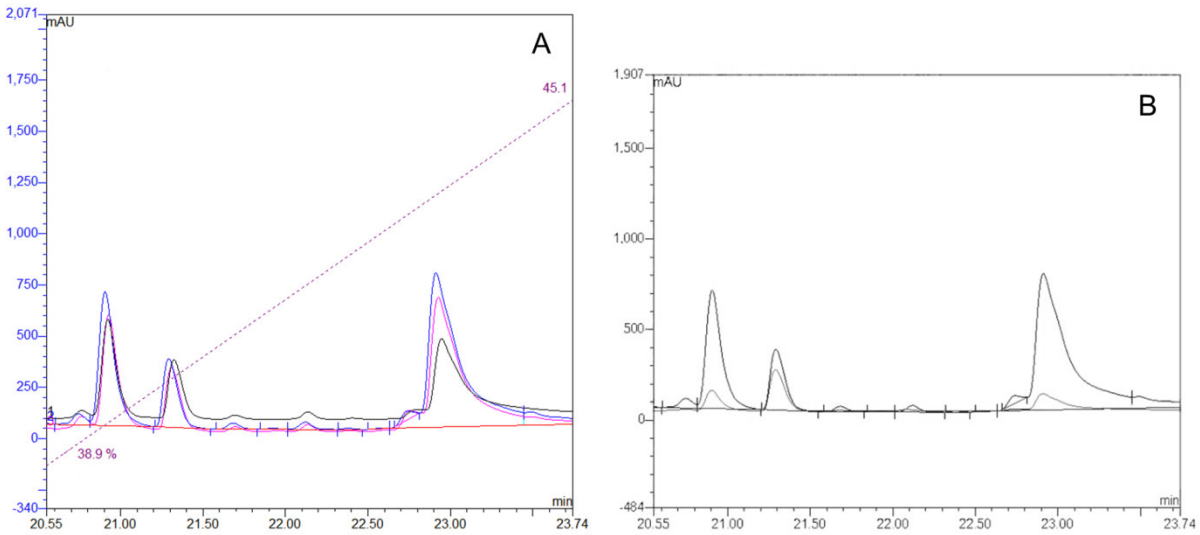


**Figure 25:** Spectra of RP-HPLC of culture supernatant *of P. oxalicum* TMW 4.2539. (A)The absorbance of different fractions from foam fractionation (spumate = blue, filtrate = pink, retentate = black) were compared. The numbers and the dashed line in ruby display the concentration in percentage of acetonitrile. (B) Comparison of absorbance spectra in the spumate fractions at two different wavelengths (light gray=280 nm, dark gray = 214 nm).

The investigation of the exoproteome of the three *P. oxalicum* isolates showed highly similar results among them. Using the technique of foam fractionation several proteins could be enriched in the foamy spumate as was verified by SDS-PAGE and 2D-GE. Moreover, it was demonstrated that most of the fungal proteins had a pI in the acidic or neutral pH. HPLC analysis revealed two proteins with interesting characteristics that were also enriched in the spumate.

## 3.7 Identification of proteins from the exoproteome of *P. oxalicum*

For further investigation of the two protein fractions RET21 and RET23 (detected in 3.6) MALDI-TOF MS and SDS-PAGE were applied. Identification of the proteins was achieved with the help of the protein analysis unit of the Ludwigs-Maximilians-Universität (Munich, Germany).

Fractions RET21 and RET23 were separated during RP-HPLC. The separated fractions were analyzed using MALDI-TOF MS and SDS-PAGE. The obtained MALDI-TOF MS spectra of RET21 and RET23 displayed several peaks in the m/z range between 6,000 and 20,000 (see Figure 26 A). The two analyzed fractions each showed various prominent peaks. For RET21 the two prominent peaks had m/z ratios of ~6,500 and ~13,000 respectively (see Figure 26 A1). From the m/z ratios found for RET21 it could not be decided whether both peaks are different proteins or one and the same. Considering the masses found and the possibility of double ionization, both peaks may well belong to the same protein. The MALDI-TOF MS spectrum of RET23 displayed one prominent peak at m/z ratio of ~17800 (see Figure 26 A2). Again, also in this spectrum a second peak at m/z 8,900 was present suggesting double ionization of the relevant protein. Separation of RP-HPLC fractions RET21 and RET23 by SDS-PAGE with subsequent silver staining showed that the proteins of interest appeared both as single bands at molecular masses of 13 and 20 kDa, respectively (see Figure 26 B).

Both bands were excised from the silver stained SDS-PAGE gel and sent to the Protein Analysis Unit (Ludwig-Maximilians-Universität, Munich, Germany) for identification.

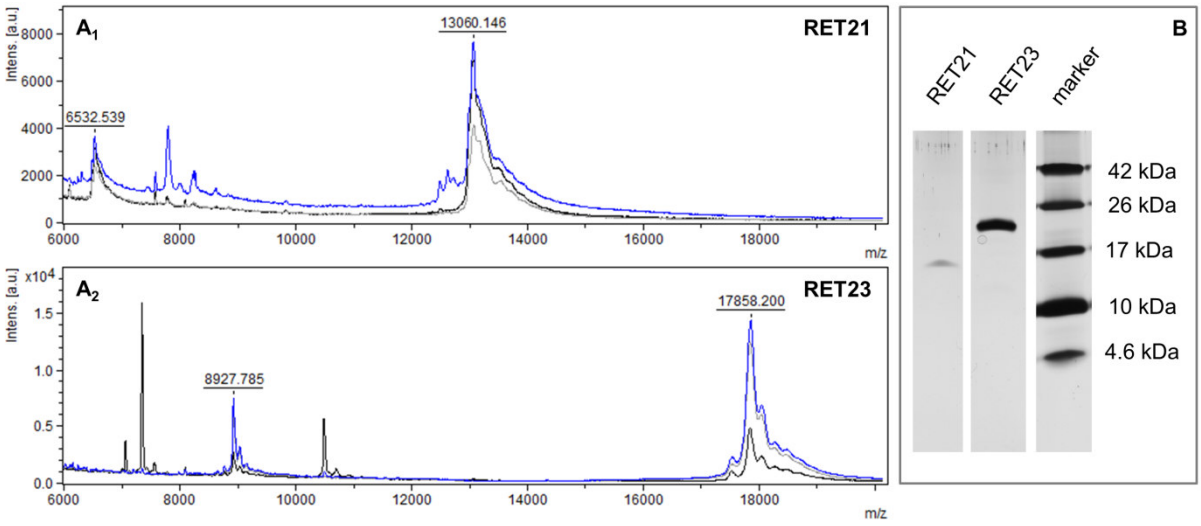


**Figure 26:** Further analyses of RP-HPLC fractions RET 21 and RET23. (A) MALDI-TOF MS spectra in triplicates of RET21 (A1) and RET23 (A2). (B) Silver stained 16 % SDS-PAGE of RP-HPLC fractions RET21 and RET23 after separation via RP-HPLC.

Mascot analysis revealed that both proteins were uncharacterized proteins of *P. oxalicum*. For both proteins high values in sequence coverage as well as a high number of unique peptides were detected (Table 24). The theoretically calculated molecular weight for those proteins matched the protein masses experimentally determined by SDS-PAGE and MALDI-TOF MS (Table 24).

**Table 24**: Identification of proteins from the *P. oxalicum* spumate. Data were obtained by nanoESI-LC-MS/MS followed by Mascot analysis, SDS-PAGE and MALDI-TOF MS. (1) Theoretically calculated mol. mass of proteins w/o signal sequence in kDa. (2) Estimated mol. mass of proteins in kDa visualized via silver staining after SDS-PAGE. (3) Mol. mass of proteins determined by use of MALDI-TOF MS.

| identified protein | unique peptides | sequence coverage [%] | (1) | (2) | (3) | GenBank accession |
|---|---|---|---|---|---|---|
| **uncharacterized protein (RET21)** | 4 | 31 | 13.19 | 13.76 | 13.06 | EPS32147.1 |
| **uncharacterized protein (RET23)** | 9 | 36.5 | 17.97 | 20.11 | 17.86 | EPS29569.1 |

Proteins RET21 and RET23 were identified as proteins S8BBA3 and S7ZH04 in the UniProt database. Both proteins were identical with accessions PDE_07106 and PDE_04519, respectively, in the NCBI database. In both databases the complete amino acid sequence of those proteins had been deposited by others (see Table 33 in the appendix). Consequently the following global sequence features were determined with the ExPASy ProtParam tool. RET23 (PDE_04519) has a theoretical pI of 4.46 (w/o signal sequence) and the theoretical pI of RET21 (PDE_07106) was calculated to be 8.75 (w/o signal sequence). The GRAVY (grand average of hydrophobicity) value was determined to be -0.033 (PDE_04519) and -0.154 (PDE_07106), respectively, indicating RET23 to be more hydrophobic than RET21. The GRAVY value is a measure of hydrophobicity; the higher the value the more hydrophobic the protein. Nonetheless, Kyte & Doolittle plots made with ExPASy ProtScale displayed several hydrophobic parts in both proteins. Moreover, both proteins contain four cysteine

residues. Further, the nucleotide sequence of the genes encoding for proteins PDE_07106 and PDE_04519 had been deposited by others in the NCBI database, too (see Table 34 in the appendix).

The identification of the two proteins of interest was successful. RET21 and RET23 will be referred to as PDE_07106 and PDE_04519, respectively, in the following sections. These results could be confirmed by experimental data obtained by SDS-PAGE and MALDI-TOF MS. Both proteins are from *P. oxalicum* and largely uncharacterized.

## 3.8 Cloning approaches of PDE_07106 and PDE_04519 in *Escherichia coli*

For a further characterization of the two identified proteins of interest (see section 3.7) larger amounts of the pure proteins were needed. Therefore, a cloning approach to express the proteins recombinantly in an expression system like *Escherichia coli* or the yeast *Pichia pastoris* was intended. Depending on the particular approach different vectors were employed.

**Table 25:** Overview over the different constructed inserts of this work. RS = recognition site for restriction enzyme, T = termination sequence, His = 6x His-tag, size is noted after digestion with corresponding restriction enzymes

| insert name | gene | 5' RS | 3' RS | add-ons | size after digest |
|---|---|---|---|---|---|
| **a/ $a_1$** | PDE_07106 | *Pml*I/ *Kpn*I | *Not*I | T | 387 bp/ 385 bp |
| **b/ $b_1$** | PDE_07106 | *Pml*I/ *Kpn*I | *Not*I | His, T | 405 bp/ 403 bp |
| **c/ $c_1$** | PDE_07106 | *Pml*I/ *Kpn*I | *Not*I | | 384 bp/ 382 bp |
| **d/ $d_1$** | PDE_04519 | *Pml*I/ *Kpn*I | *Not*I | T | 528 bp/ 526 bp |
| **e/ $e_1$** | PDE_04519 | *Pml*I/ *Kpn*I | *Not*I | His, T | 546 bp/ 544 bp |
| **f/ $f_1$** | PDE_04519 | *Pml*I/ *Kpn*I | *Not*I | | 525 bp/ 523 bp |
| **$c_2$** | PDE_07106 | *Nco*I | *Sal*I | | 381 bp |
| **$f_2$** | PDE_04519 | *Nco*I | *Sal*I | | 522 bp |

Primers were designed for the amplification of the gene sequences of PDE_07106 and PDE_04519 by PCR using genomic DNA of *P. oxalicum* TMW 4.2539 as template. Simultaneously, the particular primer design included the insertion of recognition sites for specific restriction enzymes at the end of the amplicons (see Table 12). In some cases, the primer design was also used to establish tags or termination sequences. Consequently, various slightly different inserts were constructed as listed in Table 25.

At first, recognition sites for *Pml*I were introduced with the forward primer and recognition sites for *Not*I by use of an appropriate reverse primer. While a digestion with *Not*I resulted in sticky ends, digestion with *Pml*I resulted in blunt ends, respectively, of the restricted fragment. Figure 27 shows the planed cloning procedure schematically for PDE_07106. An analogues approach was used for cloning of PDE_04519.

The amplification of the genes of interest with subsequent restriction digestion is shown for inserts a-f (according to Table 25) in the agarose gel in Figure 28 A. Likewise, the isolation of plasmid DNA from bacteria as well as the digestion with the respective endonucleases was effectively achieved (see Figure 28 B). The DNA fragments a-c had the expected size of ~400 bp, just like the fragments

d-f were displayed with sizes of ~530 bp. The estimated size of the plasmid with ~3600 bp also matched the theoretical size of pPICZαA (= 3593 bp).

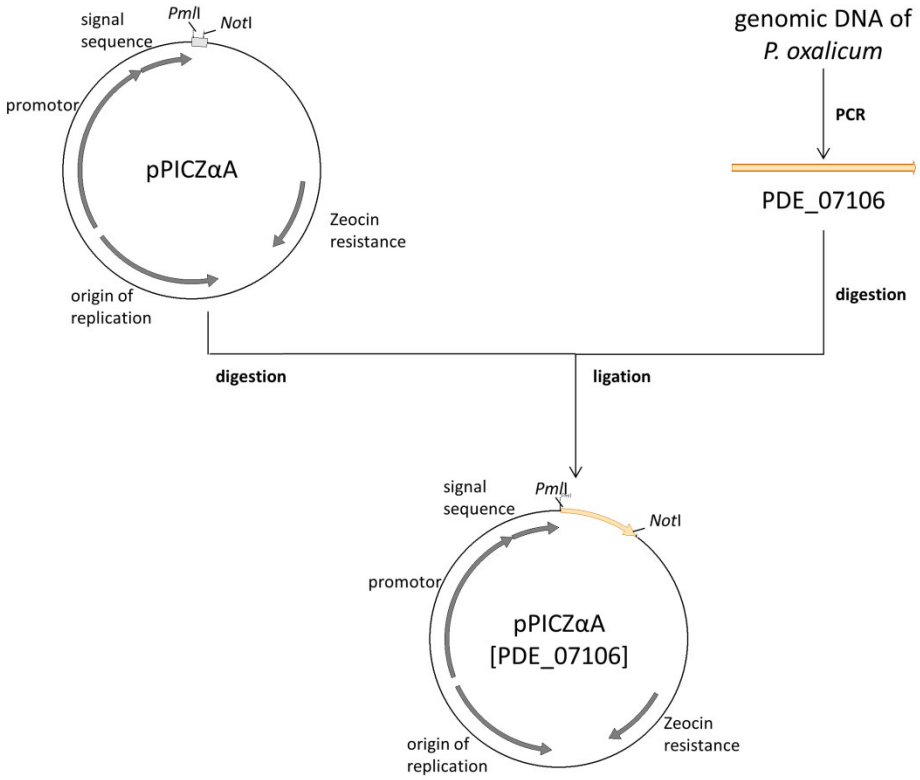


**Figure 27:** Schematic representation of the cloning procedure exemplarily shown with the gene PDE_07106. Most important features of the plasmid pPICZαA are listed as well as the used recognition sites for restriction enzymes *Pml*I and *Not*I. Signal sequence = α-factor, origin of replication = pUC ori, promotor = AOX1.

Before ligation of vector and PCR products, the prepared plasmid DNA was dephosphorylated to avoid self-ligation. After the directed ligation of DNA fragments, chemically competent *E. coli* TOP10 cells were transformed with the reaction mixture and plated onto LB agar plates containing the antibiotic Zeocin for selection. Resulting colonies were picked and screened for the presence of genes of interest by use of colony-PCR.

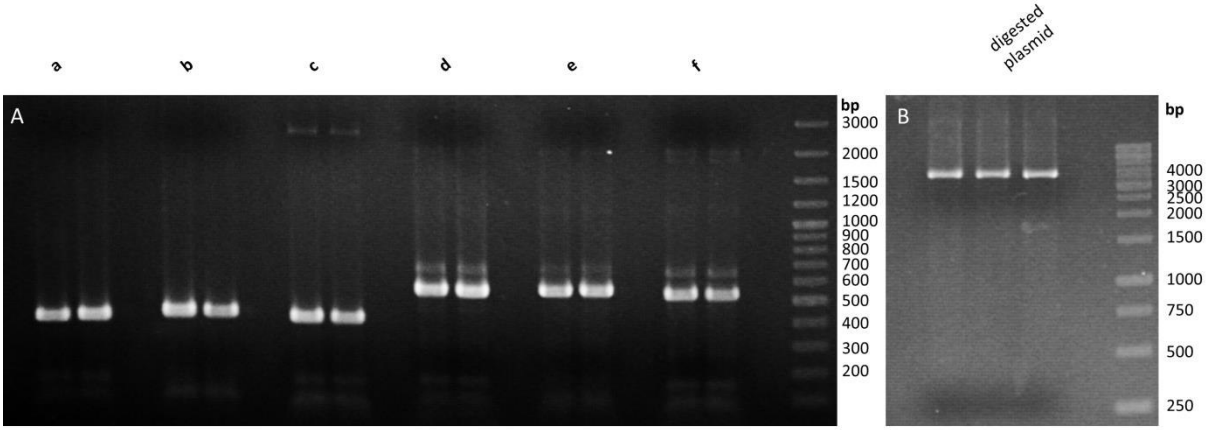


**Figure 28:** Dimidium bromide stained agarose gels. (A) Digested PCR fragments a-f (in technical duplicates) cut with *Pml*I and *Not*I (B) Isolated plasmid pPICZαA (in technical triplicates) digested with *Pml*I and *Not*I.

While the integration of genes of PDE_07106 and PDE_04519, respectively were detected in many Zeozin resistant colonies with colony PCR, the respective clones seemed to have lost their inserted gene when the PCR was repeated after their transfer to a new agar plate for purification. As an example, Figure 29 shows an agarose gel after colony-PCR with bacterial clones picked directly from the transformation plate. For each construct (a-f) PCR of at least one clone resulted in the same PCR fragment as obtained with the positive control using genomic DNA of *P. oxalicum* TMW 4.2539. Colony-PCR conducted under otherwise same conditions with these clones after transfer to a new agar plate resulted in no detectable PCR fragments except for the positive controls.

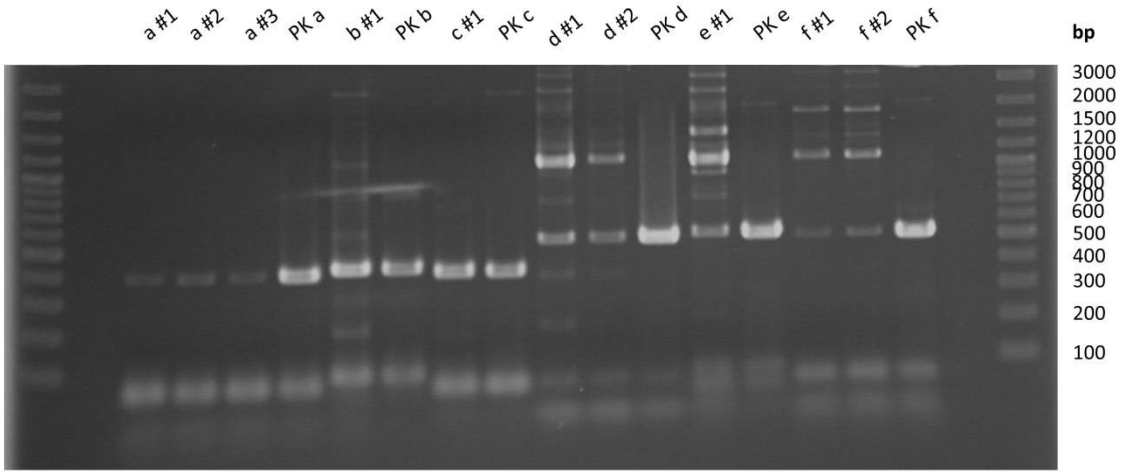


**Figure 29:** Dimidium bromide stained agarose gel. Electrophoresis of colony PCRs made with colonies directly from transformation plates. PK = positive control

To overcome this instability of the constructed vector the insert was modified. By variation of the forward primers a recognition site for *Kpn*I instead of *Pml*I was introduced in the PCR fragments. As a result, a digest with the corresponding enzyme resulted in sticky ends at the 5' end of each insert ($a_1$-$f_1$). However, also vectors constructed with the newly designed inserts showed the same problems of instability. No stable mutants could be generated with neither of the constructs. Finally, a different plasmid was assessed. The bacterial expression vector pBAD/Myc-C was employed instead of the shuttle vector pPICZαA. New inserts were constructed, successfully amplified via PCR and ligated with the pre-digested vector. Although many colonies were detected after transformation of *E. coli* TOP10 with this new vector, the constructed plasmids were instable as described above. Therefore, no stable bacterial mutant could be created with this alternative approach. In order to explain the instability of the inserts in the vectors, the nucleotide sequences of the genes of interest were checked for tandem as well as clustered repeats by use of the programs REPFIND or Tandem Repeats Finder. However, no conspicuous abnormalities could be detected.

The chosen cloning approaches to produce PDE_07106 respectively PDE_04519 in a microbial expression system failed due to inexplicable instability of the constructed vectors already in *E.coli*. Each insert was expelled from the plasmid or the constructed vector was ejected from the bacteria under all tested circumstances. As a consequence, no cloning approaches in *Pichia pastoris* could be conducted.

## 3.9 Establishment of immunochemical methods for the detection of PDE_07106 and PDE_04519

In order to detect the two proteins of interest immunochemical methods were considered. Such methods are characterized by a high sensitivity and specificity due to antigen-antibody interactions. Antigens mimicking the target proteins were designed according to special requirements. These antigenic peptides were used to induce the production of specific antibodies in chicken eggs *in vivo* for subsequent immunochemical assays.

### 3.9.1 Antigen design and antibody production

Antigens used for immunization and subsequent antibody production needed special requirements like solubility, antigenicity or epitope prediction. Therefore, the amino acid sequence of the two proteins of interest was analyzed in detail.

By use of Kyte-Doolitle-Plots drawn with ProtScale hydrophobicity patterns of both proteins were predicted. To ensure maximum solubility, only hydrophilic regions (indicated by values < 0) were considered for the design of the antigen peptides. For the same reason, sequences including charged amino acids were preferentially selected. Another important issue about the antigen design was the accessibility of the antigen sequence in the native protein for the *in vivo* produced antibody. This was achieved by the selection of highly flexible random coil structures. The secondary structure of the amino acid sequence was predicted with YASPIN to identify such areas within the sequence. Further, C-and N-terminal sequences were favored as they were very flexible, too. In contrast, sequences with more than 50 % of hydrophobic amino acids were excluded as such regions are usually buried inside the folded protein. Finally, the length of an epitope is ~7 amino acids long resulting in antigen sequences of at least 8 but not more than 20 amino acids. Under consideration of these facts and after consultation with Davids Biotechnologie GmbH (Regensburg, Germany) one antigen sequence was chosen for each target protein (see Table 26). The entrusted company (Davids Biotechnologie GmbH) produced peptides according to these sequences, which were used for immunization of chicken. After determination of antibody titer, IgY were isolated from eggs and purified via affinity chromatography. These polyclonal antibodies were directed against PDE_07106 or PDE_04519, respectively.

**Table 26:** Antigen sequence with corresponding characteristics (according to Davids Biotechnologie GmbH) used for antibody production.

| target protein | sequence of antigene | solubility | antigenicity | epitope prediction |
|---|---|---|---|---|
| **PDE_07106** | APASGKSMMAQSP | low | good | good |
| **PDE_04519** | EFAYETEGDAAHWTFKGD | medium | good | good |

For the production of specific polyclonal antibodies in chicken eggs antigen sequences were designed. By isolation and purification such antibodies were obtained from eggs of immunized chicken. With these proteins immunochemical methods such as western blot to detect PDE_07106 and PDE_04519 could be developed.

### 3.9.2 Evaluation of produced antibodies by western blot analysis

The antibodies obtained from Davids Biotechnology GmbH (see 3.9.1) were tested for their specific binding against PDE_07106 or PDE_04519, respectively. Therefore, fungal supernatants of *P. oxalicum* containing those proteins were used in western blot analysis.

Fungal supernatants were separated via SDS-PAGE before western blotting was conducted as described in 2.2.1.6. The newly produced antibodies targeting PDE_07106 or PDE_04519 were used as primary antibodies. Results showed that the anti-PDE_07106-antibody had a low specificity. Several bands on the western blot indicated cross-reactions with several different proteins from the supernatant as exemplarily shown for *P. oxalicum* TMW 4.2553 (see Figure 30 A). The lowest band probably displayed the targeted PDE_07106 protein. As a result of the western blot analysis, the anti-PDE_07106-antibody was not considered useful for further studies due to its low specificity.

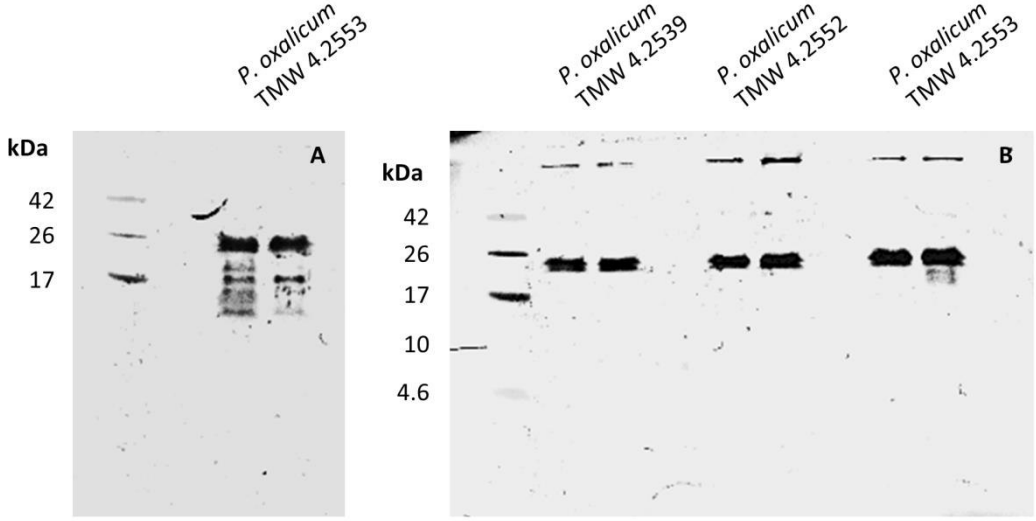


**Figure 30:** Western blot analysis of fungal supernatants of *P. oxalicum*. Primary antibodies targeting (A) PDE_07106 or (B) PDE_04519 were used in technical duplicates.

In contrast to this, only one clear band was detectable on membranes after western blots conducted with the anti-PDE_04519-antibody (see Figure 30 B). The visualized protein had a molecular weight ranging between 17 and 26 kDa. According to Table 24, PDE_04519 displayed a molecular weight of ~20 kDa in SDS-PAGE analysis. Consequently, this antibody was highly specific for its target protein.

The assessment of the produced antibodies revealed that the one targeting PDE_07106 showed several cross reactions. However, the anti-PDE_04519-antibody seemed highly specific for its target protein.

## 3.10 Establishment of a LAMP assay for the detection of *Penicillium oxalicum*

In section 3.5 it was obviously displayed that the spumates made from supernatants of *P. oxalicum* induced gushing when added to sparkling water or sparkling wine. It was therefore assumed that detection of the presence of this fungus in grapes might be a helpful tool in the quality control of grapes. To set up such a tool, a DNA-based method for the detection of *P. oxalicum* on grapes was

developed, optimized and used for the analysis of grape samples. Due to its rapidness, sensitivity and user friendliness, the loop-mediated isothermal amplification (LAMP) technology was applied to set up a species specific detection assay for the fungus.

### 3.10.1 Optimization of the LAMP assay regarding temperature and evaluation of sensitivity and specificity

The LAMP assay for *P. oxalicum* was optimized starting with the conditions described by Denschlag *et al.* (2012) except that neutral red was used as indicator instead of calcein (Tanner *et al.*, 2015) and primers were those designed in this study (see section 2.1.5). The optimal reaction temperature was determined using 100 ng per reaction of genomic DNA of *P. oxalicum* TMW 4.2539 as template. Tubes were incubated in a thermal cycler at temperatures ranging from 55.0-75.4 °C for 1 h. A color change from faint orange to pink indicating a positive reaction was detected in LAMP assays incubated at 55.0-68.5 °C (see Figure 31).

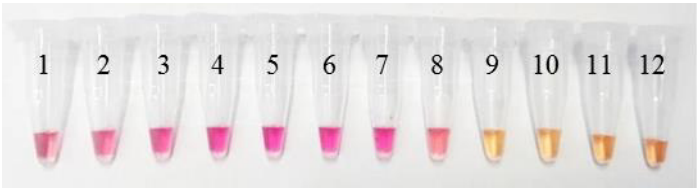


**Figure 31:** Assessment of LAMP assay reaction temperature. LAMP with purified genomic DNA of *P. oxalicum* TMW 4.2539 incubated at 1 = 55 °C, 2 = 55.3 °C, 3 = 56.5 °C, 4 = 58.3 °C, 5 = 60.6 °C, 6 = 63.2 °C, 7 = 65.9 °C, 8 = 68.5 °C, 9 = 71.0 °C, 10 = 73.1 °C, 11 = 74.6 °C, 12 = 75.4 °C for 1 h. Color change of neutral red from faint orange to pink indicates a positive reaction.

Higher temperatures (≥ 71.0 °C) inhibited the LAMP assay completely. Most intense pink coloration occurred in a temperature range between 58.3-65.9 °C. Accordingly, 63 °C was chosen as the standard temperature for isothermal incubation in all further experiments.

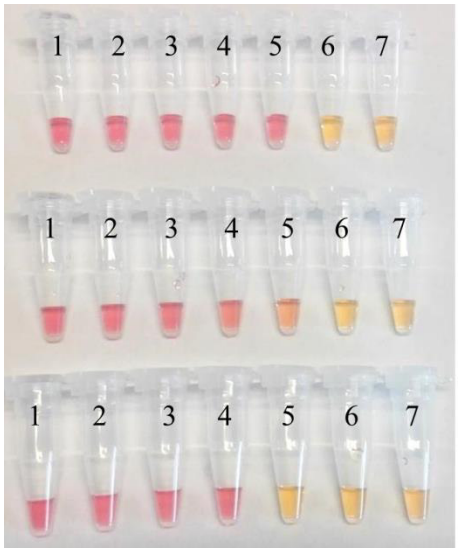


**Figure 32:** Assessment of LAMP assay sensitivity with neutral red as indicator. Purified genomic DNA of three *P. oxalicum* isolates (from top to bottom: TMW 4.2539, TMW 4.2552, TMW 4.2553) was used as template in tenfold serial dilution (1 = 100 ng/reaction (rxn), 2 = 10 ng/rxn, 3 = 1 ng/rxn, 4 = 100 pg/rxn, 5 = 10 pg/rxn, 6 = 1 pg/rxn, 7 = 100 fg/rxn). Color change of neutral red from faint orange to pink indicated a positive reaction.

The sensitivity of the developed LAMP assay was assessed by addition of tenfold serial dilutions of genomic DNA of three *P. oxalicum* isolates (TMW 4.2539, TMW 4.2552, TMW 4.2553) ranging from 100 ng to 100 fg, respectively (see Figure 32). Simultaneously, the incubation time needed to result in a color change of reactions from yellow to pink was recorded. Results revealed that no color change occurred in reactions with DNA concentrations below 10 pg per reaction or with water added as negative control. A DNA concentration of 100 pg per reaction could be detected consistently, whereas 10 pg per reaction were not amplified in all replicates. A positive signal for the set detection limit of 100 pg could be detected within 60 min. A longer incubation time did not improve sensitivity but could lead to false positive reactions. The intensity of a positive signal was not depending on the DNA concentration used.

Calcein was tested as an alternative indicator dye. Here, a positive reaction was represented by a bright green fluorescence under UV light at a wavelength of 365 nm. Under this condition a consistent detection limit was also set to 100 pg per reaction within an incubation time of 60 min. As an example the LAMP results of *P. oxalicum* TMW 4.2553 is displayed in Figure 33.

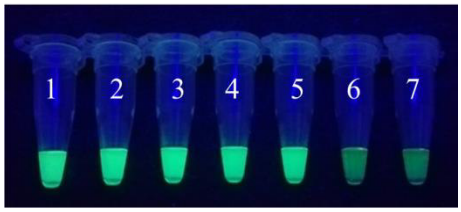


**Figure 33:** Assessment of LAMP assay sensitivity with calcein as indicator dye. Purified genomic DNA of *P. oxalicum* TMW 4.2553 was used as template in tenfold serial dilution (1 = 100 ng/reaction (rxn), 2 = 10 ng/rxn, 3 = 1 ng/rxn, 4 = 100 pg/rxn, 5 = 10 pg/rxn, 6 = 1 pg/rxn, 7 = 100 fg/rxn). Bright green fluorescence of calcein under $UV_{365}$nm light indicates a positive reaction.

The use of a conidial suspension of *P. oxalicum* TMW 4.2539 as an alternative to the analysis of purified DNA was also analyzed in the LAMP assay with regard to sensitivity. Tenfold serial dilutions ranging from $2.5*10^6$-$2.5*10^1$ conidia per reaction were used as template in the LAMP reaction. Results showed that a minimum of $2.5*10^3$ conidia per reaction consistently resulted in a positive LAMP reaction (see Figure 34). Sporadically, the assay was able to detect even a lower number of conidia ($5*10^2$) per reaction.

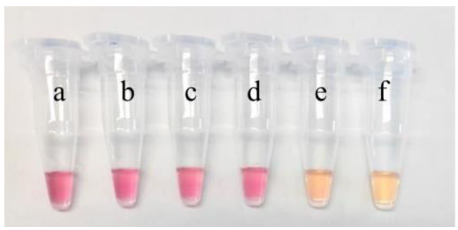


**Figure 34:** Assessment of LAMP assay sensitivity with neutral red as indicator. Conidial suspension of *P. oxalicum* TMW 4.2539 was directly used as template in tenfold serial dilution (a = $2.5*10^6$ conidia/rxn, b = $2.5*10^5$ conidia/rxn, c = $2.5*10^4$ conidia/rxn, d = $2.5*10^3$ conidia/rxn, e = $2.5*10^2$ conidia/rxn, f = $2.5*10^1$ conidia/rxn). Color change to pink indicates a positive reaction.

Assessment of specificity of the LAMP primer set for the detection of *P. oxalicum* was conducted using purified genomic DNA (100 pg per reaction) of 77 microorganisms isolated from or associated

with grapes or wine (see Table 35 in the appendix). Among them were 8 bacterial species, 24 isolates of yeasts representing 14 different species as well as 45 isolates of filamentous fungi covering 36 species. All bacterial and yeast DNA was tested negative with the LAMP assay using both indicators (see Figure 35).

**Figure 35:** Assessment of LAMP assay specificity with neutral red as indicator. Genomic DNA (100 ng/reaction) of different yeasts and bacteria associated with the vineyard were tested for cross reaction. A positive reaction (seen in first row) was only obtained with *P. oxalicum* DNA as positive control. Color change to pink indicates a positive reaction.

Filamentous fungi of the genera *Botrytis*, *Aspergillus* and *Penicillium* were assessed (see Table 27). Whereas the first two genera displayed only negative results in the LAMP assay there were several *Penicillium* isolates provoking a positive reaction. Aside from the DNA of three *P. oxalicum* isolates, also the DNA of *P. burgense* TMW 4.2541, *P. raistrickii* TMW 4.2554 and *P. paneum* TMW 4.2542 consistently resulted in a positive reaction. The result of LAMP assays with DNA of *P. coalescens* TMW 4.2537, *P. variabile* TMW 4.2560 and *P. restrictum* TMW 5.2555 were ambiguous as the color change respectively the fluorescence was only faint, the positive signal occurred only with one of the two indicators or results were not readily reproducible.

Cross reactions of *P. restrictum* TMW 4.2555 and *P. coalescence* TMW 4.2537 with the LAMP reaction were successfully eliminated by increasing the incubation temperature to 65 °C. Since *P. variabile* TMW 4.2560 did not show cross reactivity in the LAMP assay when neutral red was used as indicator, also this cross reaction was not further considered as a problem.

The addition of different volumes (0.00 µl, 0.25 µl, 0.50 µl, 0.75 µl, 1.00 µl, 1.25 µl, 1.50 µl, 1.75 µl) of 100 % formamide to the LAMP reaction was assessed in order to avoid cross reactions of other *Penicillium* isolates. LAMP was incubated at 65 °C so that *P. restrictum* revealed always negative results. DNA of other (cross) reacting *Penicillium* spp. (*P. burgense* TMW 4.2541, *P. paneum* TMW 4.2542, *P. raistrickii* TMW 4.2554, *P. oxalicum* TMW 4.2552, *P. oxalicum* TMW 4.2553) resulted in positive reactions until formamide concentrations reached 3 % (v/v) in the respective reactions.

**Table 27:** Grape-associated filamentous fungi used in this study and their reaction in LAMP assay with different indicators. Fungal DNA resulting in positive LAMP reactions were marked in orange. + = green fluorescence under $UV_{365}$ nm (calcein), pink (neutral red), - = no fluorescence under $UV_{365}$ nm (calcein), faint orange (neutral red)

| filamentous fungi | | | calcein | neutral red |
|---|---|---|---|---|
| ***A. aculeatus*** | TMW 4.2390 | TMW 4.2390 | - | - |
| ***A. carbonarius*** | TMW 4.1512 | M324 | - | - |
| ***A. flavus*** | TMW 4.1829 | TMW 4.1829 | - | - |
| ***A. fumigatus*** | TMW 4.0623 | CBS 113.55 | - | - |
| ***A. japonicus*** | TMW 4.1627 | CBS 114.51 | - | - |
| ***A. japonicus*** | TMW 4.1776 | TMW 4.1776 | - | - |
| ***A. niger*** | TMW 4.1068 | CBS 101.698 | - | - |
| ***A. parasiticus*** | TMW 4.1768 | CBS 126.62 | - | - |
| ***A. ustus*** | TMW 4.1365 | TMW 4.1365 | - | - |
| ***B. cinerea*** | TMW 4.2527 | CBS 121.39 | - | - |
| ***P. adametzioides*** | TMW 4.2529 | MUM 14.26 | - | - |
| ***P. aurantiogriseum*** | TMW 4.2536 | MUM 14.24 | - | - |
| ***P. bilaii*** | TMW 4.2528 | MUM 14.25 | - | - |
| ***P. brevicompactum*** | TMW 4.2545 | MUM 14.27 | - | - |
| ***P. burgense*** | TMW 4.2541 | MUM 14.44 | + | + |
| ***P. citrinum*** | TMW 4.2547 | MUM 14.29 | - | - |
| ***P. coalescens*** | TMW 4.2537 | MUM 14.37 | -/+ | -/+ |
| ***P. crustosum*** | TMW 4.2530 | MUM 14.31 | - | - |
| ***P. crustosum*** | TMW 4.2548 | MUM 14.30 | - | - |
| ***P. echinulatum*** | TMW 4.2549 | MUM 14.32 | - | - |
| ***P. glabrum*** | TMW 4.2531 | MUM 14.33 | - | - |
| ***P. griseofulvum*** | TMW 4.2532 | MUM 14.34 | - | - |
| ***P. janczewskii*** | TMW 4.2550 | MUM 14.35 | - | - |
| ***P. minioluteum*** | TMW 4.2533 | MUM 14.36 | - | - |
| ***P. novae-zelandiae*** | TMW 4.2551 | MUM 14.39 | - | - |
| ***P. olsonii*** | TMW 4.2538 | MUM 14.40 | - | - |
| ***P. oxalicum*** | TMW 4.2539 | MUM 14.41 | + | + |
| ***P. oxalicum*** | TMW 4.2552 | MUM 14.42 | + | + |
| ***P. oxalicum*** | TMW 4.2553 | MUM 14.43 | + | + |
| ***P. paneum*** | TMW 4.2542 | MUM 14.47 | + | + |
| ***P. purpurogenum*** | TMW 4.2540 | MUM 02.55 | - | - |
| ***P. raistrickii*** | TMW 4.2554 | MUM 14.45 | + | + |
| ***P. restrictum*** | TMW 4.2555 | MUM 14.46 | -/+ | -/+ |
| ***P. sclerotiorum*** | TMW 4.2535 | MUM 03.39 | - | - |
| ***P. sclerotiorum*** | TMW 4.2556 | MUM 14.48 | - | - |
| ***P. simplicissimum*** | TMW 4.2557 | MUM 14.49 | - | - |
| ***P. spinulosum*** | TMW 4.2543 | MUM 14.51 | - | - |
| ***P. spinulosum*** | TMW 4.2558 | MUM 14.50 | - | - |
| ***P. thomii*** | TMW 4.2559 | MUM 14.52 | - | - |
| ***P. vagum*** | TMW 4.2534 | MUM 14.38 | - | - |
| ***P. variabile*** | TMW 4.2560 | MUM 14.53 | + | - |
| ***P. variabile*** | TMW 4.2561 | MUM 14.54 | - | - |
| ***P. verrucosum*** | TMW 4.2546 | MUM 14.28 | - | - |
| ***P. verrucosum*** | TMW 4.2585 | ITEM 3716 | - | - |
| ***P. verrucosum*** | TMW 4.2586 | ITEM 3717 | - | - |
| ***P. waksmanii*** | TMW 4.2544 | MUM 14.55 | - | - |

By addition of 1 µl formamide (= 4 % (v/v)), only DNA of *P. oxalicum* still revealed positive reactions in the LAMP assay (see Figure 36). At higher formamide concentrations no *Penicillium* DNA led a positive LAMP reaction.

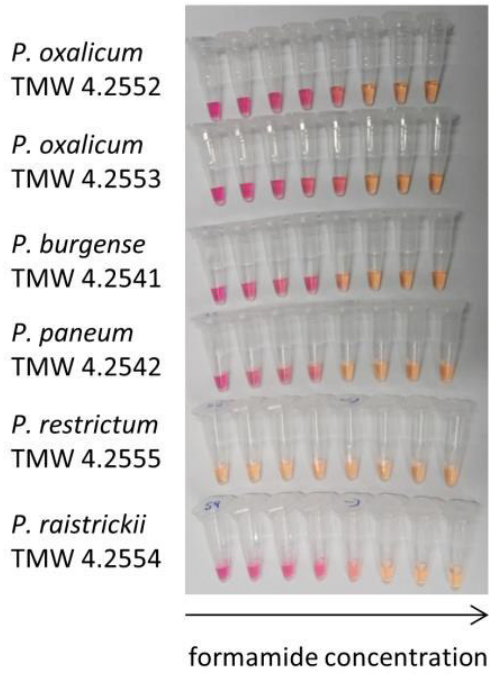


**Figure 36:** Assessment of LAMP assay specificity with neutral red as indicator. Genomic DNA (100 ng/reaction) of selected filamentous fungi were tested for cross reaction under application of formamide in different concentrations (ranging from 0-7 % (v/v)). Color change to pink indicates a positive reaction.

The LAMP assay was characterized by its sensitivity and specificity. Optimization regarding incubation temperature and time as well as the addition of formamide was successfully conducted.

### 3.10.2 Applicability of the LAMP assay for the analysis of grape samples

As the LAMP assay should be applied for on-site measurements, e.g. in the vineyard or at grape delivery, an easy sample preparation protocol for the grapes was set up. Fungal spores and cells were removed from the surface of grapes by washing and subsequent mechanical disruption to set DNA free for analysis. The so processed supernatant was assessed without further treatment using the LAMP assay to detect the presence of *P. oxalicum* in samples.

Individual artificially contaminated grapes were examined over a 22 d period in daily intervals using calcein as indicator dye. Conidial suspensions of three *P. oxalicum* isolates, *P. glabrum* TMW 4.2531, *A. niger* TMW 4.1068 as well as deionized water as negative control were used for infection of grapes. *P. glabrum* TMW 4.2531 and *A. niger* TMW 4.1068 were used for further specificity and applicability testing of the LAMP assay as these fungi occur frequently on grapes in the vineyard. Grapes were analyzed after simple mechanical pre-treatment as described in 2.2.2.8. Positive LAMP signals for *P. oxalicum* were detected at day two and following days post infection in grapes and in the positive control with pure genomic DNA of *P. oxalicum* TMW 4.2539. Uninfected grapes or grapes infected with other filamentous fungi as well as the negative control with water instead of DNA did not show any positive signal in the LAMP assay (see Table 28).

**Table 28:** Results of LAMP assay with artificially infected grapes during an incubation time of 22 d. + = green fluorescence under $UV_{365nm}$, - = no fluorescence under $UV_{365nm}$; TMW 4.2539, TMW 4.2552, TMW 4.2553 = *P. oxalicum*, TMW 4.1068 = *A. niger*, TMW 4.2531 = *P. glabrum*, not infected = grapes "infected" with deionized water, pos. co. = genomic DNA of *P. oxalicum* TMW 4.2539 (100 ng/rxn) as template, neg. co. = deionized water as template

| | TMW 4.2539 | TMW 4.2552 | TMW 4.2553 | TMW 4.1068 | TMW 4.2531 | not infected | pos. co. | neg. co. |
|---|---|---|---|---|---|---|---|---|
| **day 0** | - | - | - | - | - | - | + | - |
| **day 1** | - | + | - | - | - | - | + | - |
| **day 2** | + | + | + | - | - | - | + | - |
| **day 3** | + | + | + | - | - | - | + | - |
| **day 4** | + | + | + | - | - | - | + | - |
| **day 5** | + | + | + | - | - | - | + | - |
| **day 6** | + | + | + | - | - | - | + | - |
| **day 9** | + | + | + | - | - | - | + | - |
| **day 12** | + | + | + | - | - | - | + | - |
| **day 15** | + | + | + | - | - | - | + | - |
| **day 19** | + | + | + | - | - | - | + | - |
| **day 22** | + | + | + | - | - | - | + | - |

The applicability of the LAMP assay for grape samples was tested by an assessment of artificial infected grapes. *P. oxalicum* was detectable in an early stage of infection. In contrast, uninfected grapes or infection of grapes with other filamentous fungi resulted in negative LAMP reactions. Consequently, the developed LAMP assay enables sensitive and specific detection of *P. oxalicum* on grapes with no inhibition of the uninfected grape matrix.

### 3.10.3 Screening of grapes samples of different origins

In order to assess the frequency of *P. oxalicum* on grapes, samples collected from vineyards in different European countries were analyzed. Several bunches of grapes per vineyard were randomly assessed with the LAMP assay to get a preferably informative impression about the occurrence rate of the fungus. Moreover, supernatants of the samples prepared for LAMP analysis were analyzed microbiologically for the occurrence of filamentous fungi in general with a specific view on *P. oxalicum*.

61 grape samples (each consisting of 2-3 bunches of grapes) from different European countries (Germany, Austria, Italy, Spain and Portugal) were tested for the presence of *P. oxalicum* with the developed LAMP assay and neutral red as indicator (see Table 36 in the appendix). Positive reactions indicating the presence of *P. oxalicum* were found in one grape sample from Portugal (see Figure 37) and in two different samples of Italian grapes. Samples collected from vineyards in Spain, Austria, and Germany showed no positive LAMP results indicating they were virtually free from infection by *P. oxalicum*.

By plating of culture supernatants on DRBC agar fungi were isolated by conidial separation (see 2.2.3.1) after several days of growth. Isolates were then incubated for seven days and subsequently

identified microscopically. Results were not quantitatively recorded as some samples were analyzed after several weeks at -20 °C.

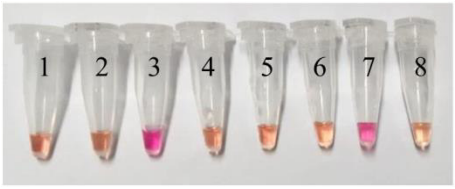


**Figure 37:** Exemplary results of the developed LAMP assay with some Portuguese grape samples. LAMP with crude washing extracts of grape samples from Portugal as template. 1 = variety Cerceal, location Bairrada, bunch 1; 2 = Bical, Bairrada, bunch 2; 3 = Fernão Pires, Bairrada, bunch 2; 4 = Bical, Bairrada, bunch 1; 5 = Trajadura, Vinhos Verde, bunch 1; 6 = Arinto, Bairrada, bunch 3; 7 = positive control; 8 = negative control. Color change of neutral red from faint orange to pink indicates a positive reaction.

A general overview about grape-associated fungi of different countries was prepared from the results obtained by plating surface washings of grape samples. It was noticeable that most of the identified fungal cultures belonged to the genus *Cladosporium*, regardless of their origin. Also many isolates were described as belonging into genera *Alternaria*, *Aspergillus* or *Botrytis* (see Figure 38). In addition, few isolates belonging to genera *Mucor*, *Acremonium* or *Epicoccum* were found.

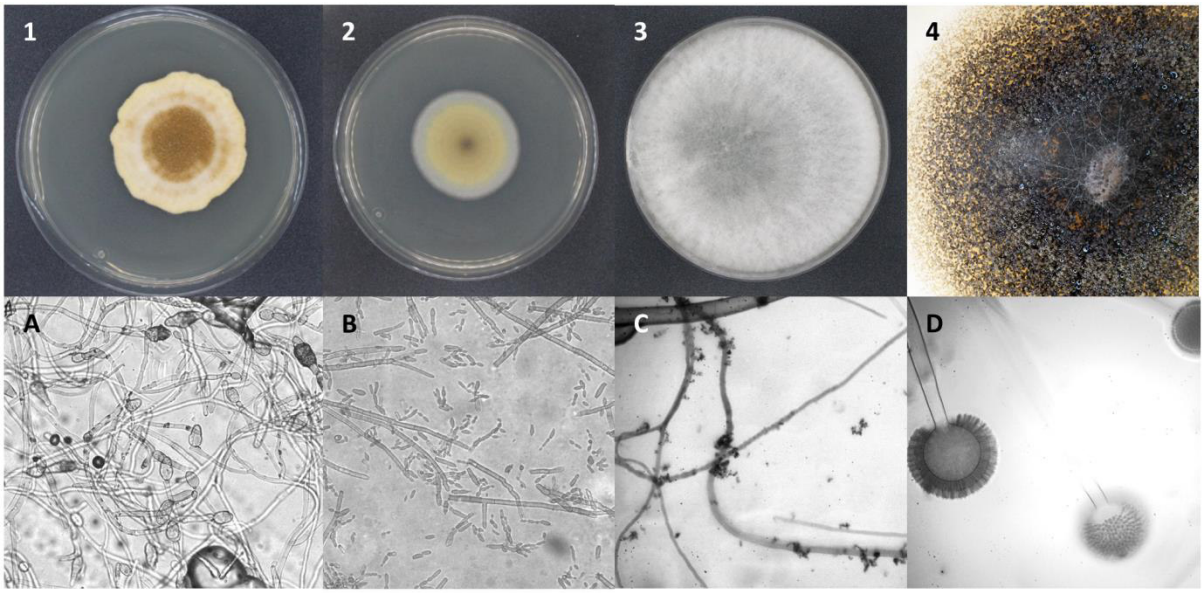


**Figure 38:** Pictures of different filamentous fungi isolated from grapes after 7 days of incubation at ambient temperature. Colonies of (1) *Alternaria* spp., (2) *Cladosporium* spp., (3) *Botrytis cinerea* and (4) *Aspergillus* spp. are illustrated as well as a 40x magnification of mycelium of each corresponding fungus (A-D).

Several different isolates of the genus *Penicillium* were isolated from various grape samples of different origins. They were identified to species level microscopically. Among them were 14 different species but no *Penicillium oxalicum* detectable. Species with corresponding origin are listed in Table 29. Again, it has to be underlined here that this identification only provided a small insight into the diversity of different *Penicillium* species occurring on grapes.

The LAMP assay revealed the presence of *P. oxalicum* in few grape samples from Italy and Portugal. In contrast, this species was not detected in any sample microscopically analyzed. However, a variety

of different filamentous fungi mainly of the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium* and *Penicillium* could be identified.

**Table 29:** *Penicillium* species identified from European grape samples.

| species | origin |
|---|---|
| ***Eupenecillium shearii*** | Portugal |
| ***Penicillium atrovenetum*** | Portugal |
| ***P. brevicompactum*** | Germany, Italy, Portugal |
| ***P. buchwaldii*** | Germany |
| ***P. chrysogenum*** | Germany, Portugal |
| ***P. expansum*** | Austria, Germany, Spain |
| ***P. frequentum*** | Portugal |
| ***P. griseofulvum*** | Germany |
| ***P. lanosum*** | Portugal |
| ***P. spinulosum*** | Portugal |
| ***P. streckii*** | Portugal |
| ***P. thomii*** | Germany, Portugal |
| ***P. verrucosum*** | Germany, Italy |
| ***P. waksmanii*** | Portugal |

## 3.11 Determination of optimum growth conditions of *P. oxalicum*

In order to determine the preferred growth conditions of the three available *Penicillium oxalicum* isolates several growth experiments were conducted in triplicates. As a result, general characteristics about the favored habitat of this species could be deduced.

### 3.11.1 In terms of temperature

As fungal physiology is strongly depending on temperature, this parameter was assessed in growth experiments. *Penicillium oxalicum* isolates (TMW 4.2539, TMW 4.2552, and TMW 4.2553) were grown at different incubation temperatures on ME agar. Photos of the colonies were taken with a digital camera from identical distance daily over a period of 10 d. The size of the fungal colonies was determined as number of pixels with the Photoshop® software package. By comparison of the number of pixels per area, the colony growth over time at different temperatures was evaluated.

Results shown in Figure 39 revealed after 24 h already that *P. oxalicum* grew fastest in a temperature range of 25-30 °C. After 24 h, marginal growth could be detected at these temperatures whereas hardly any growth or no growth at all was visible at 37 °C and at 15-20 °C, respectively. An incubation temperature of 20 °C resulted in first visible growth after 48 h and for those grown at 15 °C after 72 h. During the study, each *Penicillium oxalicum* isolate showed growth in the complete tested temperature range. The different isolates grew highly similar displaying always an optimal temperature of 25 or 30 °C. Growth at 15 °C as well as 37 °C occurred in all isolates, however, it was severely impaired compared to growth under optimum conditions. An incubation temperature of 20 °C led to a delayed but otherwise hardly impaired fungal growth. As an example growth experiments regarding different temperatures of *P. oxalicum* TMW 4.2539 were displayed in Figure 39.

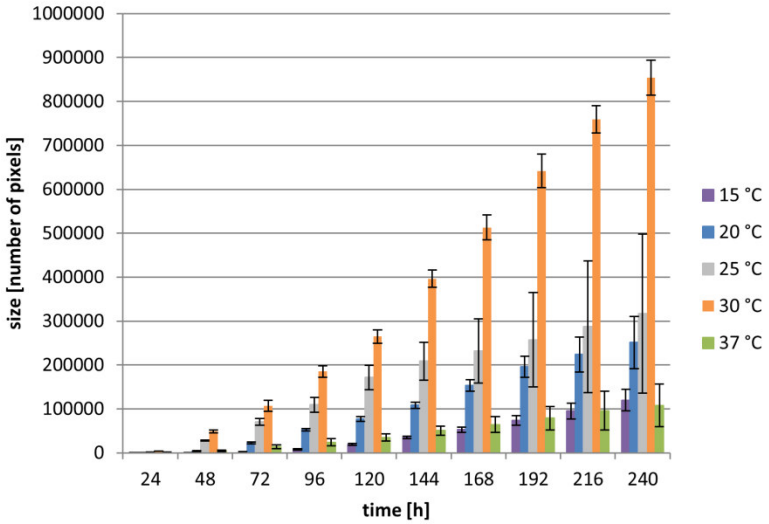


**Figure 39:** Influence of incubation temperature on the growth of *P. oxalicum* TMW 4.2539 on ME agar. Average colony size (number of pixels) over 10 d was determined in biological triplicates.

Obviously, all tested *P. oxalicum* isolates preferred incubation temperatures of 25-30 °C. Nevertheless, growth occurred in the whole temperature range tested (15-37 °C).

### 3.11.2 In terms of pH

As another important parameter influencing fungal physiology, different pH values were tested by use of ME agar with pH 5, 6 or 7. Aside from using ambient temperature for incubation in all treatments, the procedure was the same as described in 3.11.1. However, in order to cover a broader pH range (pH 1-10), growth experiments were performed also in liquid cultures because agar will not solidify at < pH 5. The chapter was closed by an evaluation of pH of various grape samples as they serve as potential habitat of *P. oxalicum*.

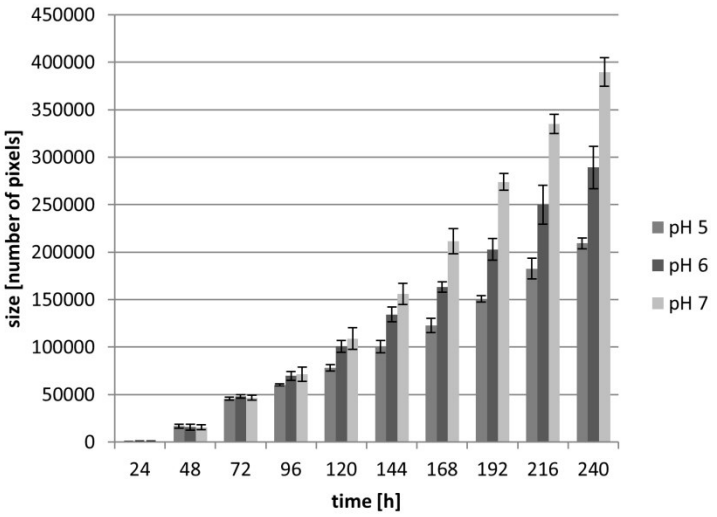


**Figure 40:** Influence of pH on the growth of *P. oxalicum* TMW 4.2539 on ME agar. Average colony size (number of pixels) was determined over 10 d in biological triplicates.

After 24 h tiny fungal colonies of all tested *P. oxalicum* isolates were visible. In the next days, colonies increased in diameter but no differences as a result of different pH values were detectable until day 5. From then on it became more and more obvious that the fungi preferred pH 7 over 5. Growth of all three isolates of *P. oxalicum* responded highly similarly to pH conditions on agar plates. Therefore, only the results of *P. oxalicum* TMW 4.2539 are displayed in Figure 40 exemplarily.

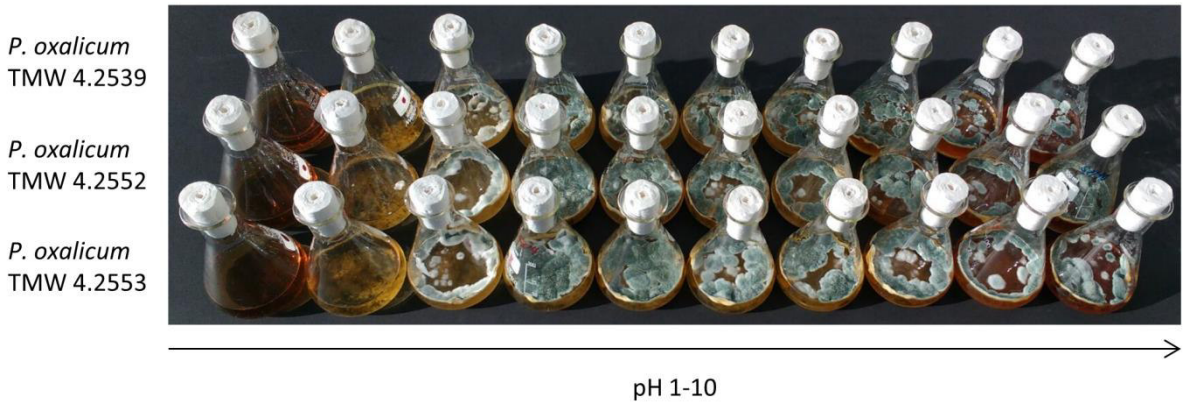


**Figure 41:** Picture of *P. oxalicum* isolates grown in ME broth adjusted to pH 1-10 after 8 days of incubation.

30 ml liquid ME broth adjusted to pH 1 to 10 in increments of 1 were prepared and inoculated each with $10^5$ conidia of three different *P. oxalicum* isolates, respectively. After 8 days of incubation at ambient temperature growth of the *P. oxalicum* isolates was assessed by weighing of the freeze-dried mycelium. No significant differences between fungi grown at pH 4-10 were determined. No growth of neither of the three isolates of *P. oxalicum* was detected in ME broth at pH 1. In contrast, in medium with pH 2 minimal growth was visible by formation of substrate mycelium. Growth increased with production of surface mycelium in broth adjusted to pH 3 but was still slightly impaired when compared to optimal growth conditions at pH ≥ 4. No differences nor anomalies in the growth behavior of *P. oxalicum* grown in ME broth could be seen in the range of pH 4-10 (see Figure 41).

The pH of 48 grape samples from Austria, Italy, Germany and Spain (see Table 36 in the appendix) was determined in technical triplicates of each bunch. The pH was always in the acidic range spanning from 2.78-4.22 (see Figure 42). The pH of grapes tested ranged predominantly from 3.06-3.85. Grape samples with a pH below 3 or above 4 had a rare frequency. No big differences could be detected between grapes of different countries. Also the grape variety as well as the grape color did not influence the pH of the samples significantly.

The experiments in this chapter showed that *P. oxalicum* preferred neutral over acidic pH when grown on agar plates. Also in liquid cultures growth was impaired at pH ≤ 3. Results were confirmed when cultures were grown in ME broth adjusted to pH 4-10. However, most of the evaluated grape samples displayed a pH between 3 and 4.

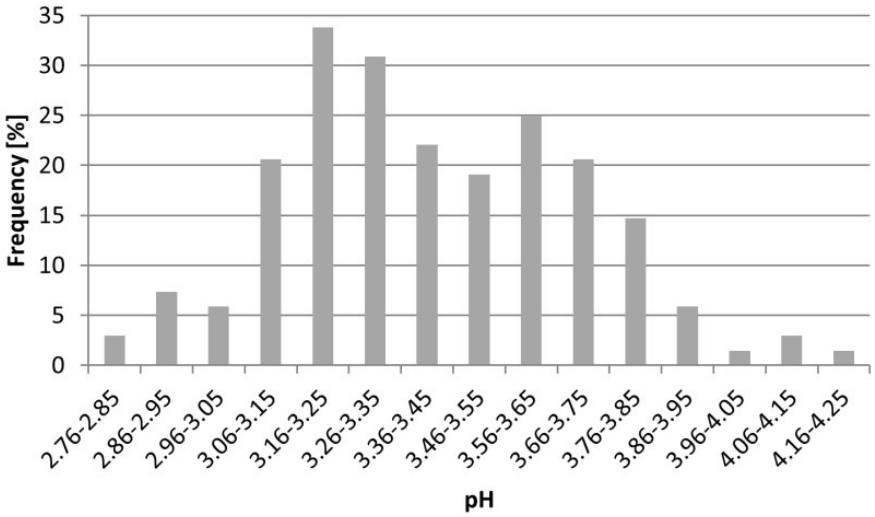


**Figure 42:** Measurement of pH of several grape samples. The frequency of distinct small pH ranges was displayed.

# 4 Discussion

By the results of the current study the initial hypotheses (see section 1.3) can be refined to the following theses:

- The analysis of red (sparkling) wine proteins requires special purification treatment. The use of a special preparation technique developed in this work is essential.
- Various fungal proteins are present in gushing sparkling wine.
- The proteome of wine depends more on external factors like grape variety, vintage or fermentation condition than on the attributes gushing or non-gushing.
- Filamentous fungi isolated from grapes produce and secrete surface-active proteins *in vitro*.
- *Penicillium oxalicum* is one of the key species producing gushing-inducing proteins.
- Artificial gushing in sparkling water or sparkling wine is induced by addition of culture supernatants of *P. oxalicum*.
- Surface-active proteins can be identified by comparative proteomics of spumate and retentate of culture supernatants.
- Identified surface-active proteins of *P. oxalicum* are involved in the initiation of gushing in sparkling wine.
- Primary gushing in sparkling wine can occur when base wines are used for the sparkling wine production that have been made from *P. oxalicum* infected grapes.
- The presence of *P. oxalicum* on grapes can be monitored by the developed LAMP assay.
- *P. oxalicum* has a preference for Mediterranean regions. Nevertheless, it can appear all over Europe due to its broad temperature tolerance.
- *P. oxalicum* can also be involved in the gushing of cider. Due to its broad pH range it can infect apples as well.
- An imbalance between inducing and stabilizing factors in the system “sparkling wine” leads to gushing.

In the subsequent chapters of the discussion these theses are supported and explained in detail.

## 4.1 Review of the novel protein preparation method

The analysis of red (sparkling) wine proteins was almost completely neglected in research so far. Most studies as reviewed by Moreno-Arribas *et al.* (2002) were conducted with white or rosé wine. Probably, the much higher content of polyphenols in red wine compared to white wine (Minussi *et al.*, 2003; Paixão *et al.*, 2007; Rice-Evans & Miller, 1996) can be blamed for this fact. Analysis of the protein composition in red wine by SDS-PAGE was so far only attempted by Wigand *et al.* (2009) and D'Amato *et al.* (2010). SDS-PAGE is a well know method for the analysis of proteins but this technique is susceptible for interfering substances such as polyphenols. The mentioned authors used PVP(P) to minimize the concentration of phenolic compounds in their samples. In the current thesis the use of these substances resulted in silver stained polyacrylamide gels with high background that were unfit for a detailed analysis of single proteins. A comparison of gels obtained with PVP(P) preparation (D'Amato *et al.*, 2010; Wigand *et al.*, 2009) or the newly developed method indicates that PVP(P) has the disadvantage of reducing the protein content of samples dramatically. In contrast, the proposed novel preparation method revealed a diverse pattern of clearly resolved protein bands instead of a

homogenous smear. MALDI-TOF MS can be used as a further powerful tool for the characterization of different biomolecules in wine. However, also with this method, many research groups are mainly working with white wine (Chambery *et al.*, 2009; Szilágyi *et al.*, 1996), which may again be due to the aforementioned high content of polyphenols in red wine. To the best of the authors knowledge only Carpentieri *et al.* (2007) and Nunes-Miranda *et al.* (2012) performed direct MALDI-TOF MS experiments with red wine. However, these authors did not attempt to look at the protein composition of wines but rather at pigments or volatile compounds. As the developed preparation technique for red wine proteins is also effective for subsequent MALDI-TOF MS analysis, future research may well include more analyses of the red (sparkling) wine proteome.

## 4.2 Protein composition in (sparkling) wine

Proteins in (sparkling) wine are derived from the grape itself, as well as from yeasts, bacteria or fungal infection. It can be assumed that these proteins of different origin may affect each other by molecular interaction.

The two proteins of *Vitis vinifera* identified in section 3.2 were found to contain a barwin and a thaumatin domain, respectively. In the review of Singh *et al.* (2013) several plant derived antifungal proteins were described. Among them are pathogenesis-related (PR) proteins of family 4 and 5 carrying as a common feature the mentioned domains, respectively. Despite their name, PR proteins are also produced in ripe berries due to an accumulation of sugars (Tattersall *et al.*, 1997). Therefore it can be assumed that those potent inhibitors of pathogenic fungi are naturally present in grapes used for the production of non-gushing sparkling wine even without fungal infections. As in gushing samples those proteins are strongly reduced but coincidently fungal enzymes are detectable (see Table 22), it seems reasonable to conclude that a fungal infection of grapes used for sparkling wine production leads to degradation of PR proteins. The degradation of grape proteins in sparkling wine, especially PR proteins, upon fungal infection of grapes was previously reported by Cilindre *et al.* (2007). Similar results were detected for grape juice as Girbau *et al.* (2004) reported a decrease for concentrations of different PR proteins in grape juice following *Botrytis cinerea* infection. Marchal *et al.* (1998) reported a protease secreted by *Botrytis cinerea* which caused the degradation of wine proteins in general. This enzyme was still active at low pH and temperature as well as in the presence of ethanol or sulfur dioxide (Marchal *et al.*, 2006). Moreover, also a partial degradation of a yeast derived protein (YJU1) in wine made from *Botrytis* infected grapes is known from literature (Cilindre *et al.*, 2008). This cell wall protein termed YJU1 or CWP1 from *S. cerevisiae* was repeatedly detected in sparkling wine without characterizing it in more detail (Cilindre *et al.*, 2008; Kwon, 2004; Mostert & Divol, 2014; Palmisano *et al.*, 2010; Wigand *et al.*, 2009). A general reduction of protein concentrations in synthetic wine as a result of *B. cinerea* infection was correlated with impaired foaming characteristics (Marchal *et al.*, 2006). Although a reduction of the protein content is generally associated with poor foaming characteristics in sparkling wine (Dambrouck *et al.*, 2005; Marchal *et al.*, 2002; Vanrell *et al.*, 2007), hardly any correlation between the induction of gushing and an infection of the grapes with *B. cinerea* was ever made. Only in the study of Marchal *et al.* (2001) one single bottle of sparkling wine made of Chardonnay grapes infected at a level of 40 % with

*B. cinerea* showed gushing behavior. Since infection of grapes with *B. cinerea* is very frequent (see section 1.1.1), the gushing phenomenon would be supposed to occur more often if such a fungal infection alone was a causal agent for gushing. Consequently, *Botrytis* itself may not provoke gushing, but it is possible that it has an indirect effect by degrading such proteins that have a stabilizing effect on the gushing potential of sparkling wine. Such degradation would result in instable foam and an increased gushing risk. However, grapes affected by grey rot due to a *Botrytis cinerea* infection frequently suffer from simultaneous, less visible secondary infection by other filamentous fungi (Ribéreau-Gayon *et al.*, 2006). Among others, *Aspergillus*- and especially *Penicillium*-species have been found to be associated with rotten grapes (La Guerche *et al.*, 2006). Albeit most other authors only detected *Botrytis* derived proteins in (sparkling) wine (Cilindre *et al.*, 2008; Kwon, 2004), results of the current work showed that one protein each derived from *B. cinerea* and *Aspergillus niger* was identified in gushing sparkling wine (see Table 22). The same protein of *Aspergillus* was already found in red wine by D'Amato *et al.* (2010). These authors found several proteins in their samples originating from different filamentous fungi (*Aspergillus* sp., *Botrytis*, *Sclerotina sclerotiorum*). So a multiple infection with *Botrytis* and other filamentous fungi of grapes that are used in sparkling wine production seems common. In contrast to D'Amato *et al.* (2010) and Kwon (2004), no bacterial proteins were identified in sparkling wine in the current study. However, considering that only four proteins have been selected for further analysis here, bacterial proteins may well have been present in the samples but were obviously not characteristic to either gushing or non-gushing sparkling wine.

Besides a microbial infection, the results of the current work showed that especially the grape variety, the mash fermentation as well as the vintage have a great influence on the protein composition of the produced wine. It is a well-known fact that the grape variety has a tremendous effect on the protein content and composition in the produced wine (Andrés-Lacueva *et al.*, 1997; Dizy & Bisson, 1999; Pueyo *et al.*, 1993; Sarmento *et al.*, 2001). Moreover, Dizy and Bisson (1999) stated that there are differences in the protein quantity in regard to the vintage. Wines of different vintages seem to differ in their protein content in general, and sometimes even in the protein composition (Wigand, 2008). Wigand (2008) reported the absence of a distinct PR protein in one of four analyzed wines produced from grapes of consecutive vintages in an otherwise identical process and variety. The same author also determined differences in wines produced by various fermentation techniques (red wine, white wine, Weißherbst). However, wines produced from grapes of different varieties were used in the study of Wigand (2008) making that particular statement questionable. Nevertheless, it seems obvious that different proteins are extracted when not only grape juice but also grape skin and seeds are fermented. However, results of the current study indicate that the used yeast as well as the growing region displayed no or only a little impact on the resulting wine´s proteome. A marginal influence of the growing region on the protein composition of wine found here was confirmed by the data of Wigand (2008) as well as Sarmento *et al.* (2001). In contrast, an influence of yeast on the composition and the timing of release of different nitrogen fractions like proteins, peptides and amino acids was found in previous studies (Martinez-Rodriguez *et al.*, 2002;

Suárez Valles *et al.*, 2005). By use of SDS-PAGE or MALDI-TOF MS no such influence could be seen in the samples analyzed during the current work.

## 4.3 Selection of *Penicillium oxalicum* as main research object

It is well established that *Alternaria* spp., *Aspergillus* spp., *Botrytis* spp., *Cladosporium* spp. and *Penicillium* spp. occur naturally as opportunistic fungi on grapes especially before harvest (Hewitt, 1988; Loureirol *et al.*, 2012). In typical gushing-years, the phenomenon affects 2-2.5 % of the annual production of sparkling wine in Germany (annual production ~450 million 0.7 l bottles) totaling to 8 million bottles (oral communication, Verband Deutscher Sektkellereien e.V.). Therefore, frequently appearing fungi such as the *Dematiaceae* or *Botrytis* spp. were excluded from the selection, since the gushing phenomenon would occur much more frequently if this group of fungi were considered as causal agents. Because of this and due to their relevance in beer gushing research (see Table 3) emphasis was given to species within the *Aspergillus* and *Penicillium* genera. According to the results of preliminary tests the selection was further restricted to *Penicillium* spp. as that genus had the higher potential to influence surface characteristics of culture supernatants (see section 3.4.2). Gushing tests made with culture supernatants during the current study showed that many *Penicillium* spp. can provoke slight till severe gushing. Nonetheless, for several fungal isolates the inter-run repeatability was poor and only the intra-run repeatability within three technical replicates was given. The study of Amaha *et al.* (1973) also revealed that some of their tested fungal strains induced gushing in a non-reproducible manner. Gushing inducing factors seem to be produced only occasionally in such fungi. In contrast to other tested species, *P. vagum* and three *P. oxalicum* isolates were the only species which were able to induce strong gushing in a reproducible manner. Although all four strains were isolated from grapes, *P. vagum* was not found in studies regarding the mycobiota of grapes (see section 1.1.1). This is in sharp contrast to *P. oxalicum,* which was frequently found on grapes and in grape associated sources such as vineyard air or soil (see Table 1) (Sage *et al.*, 2002; Serra *et al.*, 2006a). Consequently, it seemed justified to focus on *P. oxalicum* isolates during further studies.

## 4.4 Gushing inducing potential of *Penicillium oxalicum*

The aim of the current study was to elucidate the potential of grape-associated fungi to induce gushing in sparkling water as a model system for sparkling wine and to identify proteinaceous factors involved in this process. Based on the results of section 3.4, 3.6 and 3.7, it can be suggested that infection of grapes by *P. oxalicum* may contribute to the occurrence of gushing in sparkling wine made from such raw materials by the formation of at least two distinct proteins which were identified in the exoproteome of this fungus during the current study. YCB was chosen as medium for the analysis of the exoproteome of the fungi under study since it consists only of salts, (amino) acids, vitamins and sugars. Therefore, proteins found in supernatants have to be produced by the growing fungal culture. This fact was confirmed by SDS-PAGE of pure medium, where no protein bands were detected. Moreover, in gushing tests the medium was assessed as control. It was shown that gushing is not induced by the uninoculated medium composition itself. A sparkling water model system was used instead of sparkling wine for gushing tests because substances present in sparkling wine, which

could affect the gushing behavior can be excluded under this condition. Stübner *et al.* (2010) described a similar water model system to be more sensitive to gushing than beer. Nonetheless, our results revealed sparkling wine to be more sensitive for gushing as compared to the sparkling water model system. The addition of 1 ml fungal spumate (cultured in a Fernbach flask) resulted in a loss of liquid of almost 80 % of the bottle volume in sparkling wine but only around 20 % in sparkling water. Different external factors – geometry of the culture flask, foam fractionation of culture supernatant, temperature, pH, and sample volume – were assessed in gushing tests for their influence on the gushing amount (see section 3.5). It was shown that the geometry of the culture flask has a noteworthy influence on the appearance of the fungal cultures as well as on the production of gushing-inducing factors. As the surface of the medium is larger in a Fernbach flask compared to an Erlenmeyer flask the liquid is mixed more vigorously when shaken under otherwise same conditions. Therefore the oxygenation capacity is higher in Fernbach flasks which can affect the fungal metabolism. Moreover, as described by Deckers *et al.* (2012) the formation of stabilized nanobubbles coated by hydrophobins in a closed system is enhanced by the application of energy, e.g. via shaking. Therefore, it can be suggested that surface-active molecules self-assemble differently due to the stronger mixing of the liquid culture in a Fernbach flask. Furthermore, it was demonstrated that only fungal spumate obtained by foam fractionation was able to induce gushing in the sparkling water system. With this method, amphiphilic molecules accumulate at the interface between water and nitrogen bubbles. Consequently, these surface-active compounds will be enriched in the spumate fraction (Ostwald & Siehr, 1936; Uraizee & Narsimhan, 1990). It has previously been established that also fungal hydrophobins self-assemble at the gas-water interface (Szilvay *et al.*, 2007; Wösten *et al.*, 1994b). Moreover, foam fractionation was successfully applied to enrich other proteins potentially involved in beer gushing, e.g. fungispumins (Zapf *et al.*, 2007). Results of the current study showed that surface-active molecules from fungal supernatants influencing gushing can also be enriched by this method (see Figure 23). A positive correlation between temperature and the water loss due to gushing was found. Such temperature dependence is also known for beer. Under consideration of gushing as a gas nucleation process, one explanation could be a temperature-dependent growth of bubbles from nuclei. This would lead to a more intense gushing as described in the review of Gardner (1973). According to Henry´s law (Henry, 1803), the solubility of $CO_2$ in water decreases with increasing temperature. As a result, the gas pressure above the liquid needs to increase. Nevertheless, another possibility would be that the activity of the gushing inducing factor is temperature-dependent respectively the gushing inducing effect only occurs if several gushing inducing molecules form some kind of cluster, which is under consideration of the Brownian movement more probable at higher temperatures. Since the correct serving temperature for sparkling wine is below 10 °C, it serves as gushing reducing factor. As shown in section 3.5.2, gushing could not be induced by addition of spumate when the pH of the sparkling water was higher than pH 6. This points towards a dramatic modification of the gushing-influencing molecules at elevated pH. It is assumed here that under conditions of higher pH the structure of these molecules changes into a conformation in which they lose their ability to induce gushing. Many of the hydrophobins, which are known as gushing-inducing molecules in beer have isoelectric points in the lower pH range (pH 4-5)

(Cox *et al.*, 2007; Peñas *et al.*, 1998; Stübner *et al.*, 2010). As a consequence, a higher pH might induce a considerable conformational change. In the current study, out of the two proteins of interest (PDE_07106 and PDE_04519) only PDE_04519 has an isoelectric point in the acidic range (see section 3.7). Considering this, it is therefore more likely that PDE_04519 acts as a gushing inducing factor because of gushing activity of *P. oxalicum* spumate was restricted to lower pH in gushing tests. However, this does not necessarily exclude PDE_07106 or the presence of other, hitherto undetected factors, to influence gushing. Nonetheless, the average pH of sparkling wine is 2.9-3.15 and in extreme cases at pH 2.8 or 3.4 (Troost *et al.*, 1995), therefore providing the necessary environmental conditions for a possible induction of gushing. The correlation between the sample volume and the gushing amount indicates the presence of at least one gushing-inducing factor in the spumate of *P. oxalicum* and a concentration dependent mode of action.

## 4.5 Supposed gushing-inducing factors of *P. oxalicum*

Gushing-inducing factors produced by diverse filamentous fungi have been found in several studies of gushing in beer (see Table 3). Most authors who dealt with gushing inducing factors characterized them as (poly)peptides or peptidoglycans (Amaha *et al.*, 1973; Kitabatake & Amaha, 1977; Kitabatake *et al.*, 1980) without establishing their identity. Consequently, further experiments to isolate and identify the gushing-inducing factor from *P. oxalicum* culture supernatants in this study were conducted with a focus on protein chemical analysis. RP-HPLC analysis revealed two proteins eluting at an acetonitrile content in the range of 40-50 % with almost no absorbance at a wavelength of 280 nm. The findings of Sarlin et al. (2005 and 2012) showed that hydrophobins elute in this range of acetonitrile concentration. Furthermore, the gushing factor described for beer gushing by Amaha *et al.* (1973) was described as lacking both tyrosine and tryptophan in the amino acid composition. Consequently, absorbance of such a protein at 280 nm would be relatively low as compared to a protein containing these aromatic amino acids. From the amino acid sequence of PDE_07106 and PDE_04519 it is known that both contain these aromatic amino acids (6/3 or 8/2 residues of Tyr/Trp). Nevertheless, the low absorbance can be explained by the low amount of tyrosine and tryptophan or by the presence of other substances interfering with the protein's absorbance at this wavelength. In contrast to hydrophobins (Schuren & Wessels, 1990), PDE_07106 and PDE_04519 do not contain eight cysteine residues but only four. Moreover, hydrophobins are known as relatively small proteins (~10 kDa) whereas PDE_07106 and PDE_04519 (w/o signal sequence) have a considerably higher molecular weight of ~13 kDa and ~20 kDa, respectively. Nevertheless, they are also relatively hydrophobic and display other similar features to hydrophobins as mentioned before in this section. Considering their structural characteristics we suggest that both proteins belong to protein classes hitherto not described as surface-active agents, which might be involved in the gushing mechanism. However, it can be assumed that gushing of sparkling wine follows a similar mechanism as beer gushing (see section 1.2.3) with different classes of fungal proteins involved. According to the mechanism of Deckers *et al.* (2012), gushing is connected to the existence of "nanobombs", which are defined as gas filled nanobubbles covered by a hydrophobin film. The hydrophobin layer stabilizes the growth of the gas bubble upon pressure release during opening of the bottle. According to the theory, nanobubbles expand explosively thereby providing sufficient energy for the transit of

carbon dioxide from the soluble to the gas state in the close vicinity a bubble. The theory, according to which the stabilization of gas bubbles can be explained by the accumulation of amphiphilic molecules at the gas-water interface was supported by experiments in which culture supernatants of *P. oxalicum* isolates that can also induce gushing in a sparkling water model system (see section 3.6) were able to create stable foam during foam fractionation (see Figure 23 A). Surface-active molecules secreted by the fungi accumulate at the liquid/gas interface whereas foam fractionation of pure YCB-medium did not lead to the formation of any foam. Therefore, it can be assumed that PDE_04519 and possibly also PDE_07106 are protein molecules which are able to stabilize gas bubbles in a hydrophobin-like fashion.

## 4.6 LAMP as detection method for *P. oxalicum* already in the vineyard

*P. oxalicum* is known as an opportunistic plant pathogen, mycotoxin producer and according to this thesis as an inducer of gushing in sparkling wine. To date, no on-site test is available for the rapid, robust and specific detection of this fungus. In order to improve this situation a DNA amplification assay on the basis of the loop-mediated isothermal amplification technology was developed, optimized and applied during the current study. The primers used in this study target the gene coding for the surface-active protein PDE_07106 in *P. oxalicum*. This protein was identified in connection with foam stabilization and possible gushing induction in section 3.6 and 3.7. Therefore, the application of the PDE_07106 coding sequence as target for a molecular detection assay appears to be a logical step for screening of samples in regard to a potential gushing inducing activity.

The LAMP assay was functional when incubated within a broad temperature range of 55.0-68.5 °C (see Figure 31). Francois *et al.* (2011) also reported high robustness of their LAMP reaction to detect typhogenic serotypes of *Salmonella enterica* strains within a temperature interval of 10 °C and a range of 2 pH units for the buffering of the mastermix. Robustness against variable buffer and incubation conditions is an important feature of assays which are supposed to run with minimally processed samples under highly variable conditions as in on-site settings. . For the current assay, an incubation temperature of 63 °C was found to be optimum. This temperature lies within the temperature optimum of the *Bst* DNA polymerase which is between 60-65 °C according to the manufacturer. Even though the most intense coloration of reactions occurred in a broad temperature range of 58.3-65.9 °C, too low incubation temperatures should be avoided in LAMP reactions since they favor non-specific binding of primers to DNA and will result in unspecific results of the assay. Calcein or neutral red were used as alternative indirect indicators for the amplification of DNA during the LAMP reaction. Both dyes follow a different mechanism for the color change as reactions switch from negative to positive. In the case of calcein, the complexation of pyrophosphate anions ($P_4O_7^{4-}$) with manganese cations ($Mn^{2+}$) leads to the recovery of fluorescence of the previously quenched calcein fluorophore. Without DNA amplification no pyrophosphate is produced leaving manganese to quench the calcein fluorescence. In contrast, neutral red as a pH indicator displays a positive reaction by a pH dependent color change from yellow-orange (pH 8) to pink (pH 6.8) which allows differentiation of positive and negative reactions by visible detection under day light conditions. During DNA amplification protons are released causing an acidification of the reaction

mixture. In order to allow for such acidification, buffering of the master mix had to be adjusted by increasing the concentration of the MOPS-buffer used in LAMP reactions. With both indicators an in-tube detection via the naked eye was possible directly following the LAMP reaction without any further manipulation needed, except the necessity of a UV lamp for visual detection of calcein fluorescence. Most importantly, cross contaminations due to opening of the reaction tubes for time-consuming AGE was avoided. Regardless of this effect some reaction vessels were opened following LAMP reactions to employ AGE and subsequent dimidium bromide staining for the confirmation of DNA amplification in LAMP reactions. Results obtained during direct signal detection by AGE were in accordance with results obtained by indirect visual detection either using neutral red or calcein as marker dyes. With the developed LAMP assay a minimum of 10 pg of purified genomic *P. oxalicum* DNA per reaction could be detected (see Figure 32 and Figure 33). According to the genome size of *P. oxalicum* found in the NCBI database of ~30 Mb (*Penicillium oxalicum* 114-2 genome size: 30.18 Mb) the minimum detected amount of DNA is equivalent to a copy number of ~300. Consequently, a positive reaction requires the presence of around 300 nuclei from mycelial cells or fungal spores. The minimum detected amount for conidia after direct addition to the LAMP master mix before incubation, was only marginally higher. 500-2500 conidia were sufficient to result in a positive LAMP signal (see Figure 34). Intensity of color or fluorescence was independent from the initial concentration of target DNA as long as the mentioned detection limits are exceeded.

Proper binding of primers to their six target sequences is one requirement for a functional LAMP assay, coincidentally ensuring high specificity. Therefore, different microorganisms commonly prevailing on grapes or in vineyard environments according to the literature were assessed for cross reactions in the *P. oxalicum* specific LAMP assay. Results showed that the LAMP assay for *P. oxalicum* is highly specific for its target species. Nonetheless, one isolate each of *P. burgense*, *P. paneum,* and *P. raistrickii* led to a positive result in the LAMP assay. A comparison of the amino acid sequence of PDE_07106 of *P. oxalicum* with the annotated proteome of the three mentioned species revealed no significant similarities. However, less than 80 proteins for each of these three species have been annotated (status October 2016) so that the occurrence of a cross-reaction with closely related but yet un-annotated proteins could explain the phenomenon. As *P. burgense*, *P. paneum,* and *P. raistrickii* are rarer in vineyards compared to *P. oxalicum* (see section 1.1.1), false positive reactions can be supposed to occur only rarely when samples of grapes are analyzed with the assay. Nevertheless, a further optimization regarding the specificity of the assay was necessary. In consideration of other DNA amplification methods (Sarkar *et al.*, 1990; Wu *et al.*, 1991), this was achieved by the addition of formamide and an increase of incubation temperature to 65 °C.

***P. oxalicum* was detected at least two days post infection in artificially infected grapes (see**

Table 28). Consequently, the detection was possible before the development of visually detectable mold symptoms. It has been demonstrated that simple washing of samples and using the crude washing as target in the LAMP assay is sufficient to provide the minimum DNA concentrations needed for detection of the fungus. This is a maximum simplification of sample processing as compared to other methods which need time-consuming and expensive DNA extraction steps

previous to analysis. Such rapid and simple sample processing together with LAMP based detection greatly promotes early assessment of samples for *P. oxalicum.* Therefore, it will be highly beneficial for vine growers as well as wine and champagne producers because it enables informed decisions about further processing of grapes and grape derived products. Furthermore, the LAMP assay seems not affected by substances originating from grapes as the uninfected grapes led to no positive signal in the reaction within the investigation period of 22 days. A high tolerance against inhibitory substances has previously been reported for LAMP in other studies (Kaneko *et al.*, 2007; Nixon *et al.*, 2014). LAMP assays for the detection of another fungal species (*B. cinerea*) on grapes were already described by Tomlinson *et al.* (2010) and Duan *et al.* (2014). Albeit these assays require more expensive and cumbersome DNA extraction, a higher sensitivity is reached with purified DNA than with crude extracts.

The developed LAMP assay was used to assess the presence of *P. oxalicum* in 61 grape samples (each consisting of 2-3 bunches of grapes) harvested in autumn 2016 from vineyards in Austria, Germany, Italy, Portugal and Spain. *P. oxalicum* could be detected only in three of the samples, which had been harvested in Italy (2) and Portugal (1). According to different European mycological studies about the mycobiota on grapes, *P. oxalicum* occurred occasionally in Portugal, France, Spain, Italy and Slovakia (see Table 1). No data concerning the occurrence of *P. oxalicum* in Germany or Austria are available from the literature. Considering an optimal growth temperature of 25-30 °C for pure cultures of the fungus (Mislivec & Tuite, 1970b; O'Neill *et al.*, 1991) it is more likely that it will have a preference for Mediterranean regions rather than Middle- or North European regions. The natural habitat of *P. oxalicum* in connection with experiments conducted in this study will be discussed in detail in section 4.7.

Even if no inhibition from uninfected grapes has been detected during the experiment, the possibility of false negative results must also be taken into account. Luo *et al.* (2014) reported an inhibition of a LAMP assay for the detection of *Aspergillus flavus* due to a crude sample washing from peanuts and other non-grape samples. According to their study a circumvention of this problem was achieved by diluting the samples prior to LAMP analysis. However, an internal control to distinguish true negative from false negative results would be advisable. Unfortunately, so far no convenient internal control for a LAMP assay has been described due to the fact that two different amplification products cannot be differentiated in a LAMP reaction (Rodríguez *et al.*, 2015). Besides inhibitors from the food matrix, also mycotoxins, causing mutations in the target DNA and therefore affecting the primer hybridization, could be a problem for DNA amplification methods (Paterson & Lima, 2014). Furthermore, it should be considered that LAMP indicates only the presence of fungal DNA and does not give any information about the viability of the detected organism. Nonetheless, the results of the current study showed the great potential of the developed LAMP assay for the detection of *P. oxalicum* on-site.

## 4.7 Natural habitat of *P. oxalicum*

This study demonstrated that *P. oxalicum* isolates grew within the tested temperature range of 15-37 °C. Depending on each isolate the optimal growth temperature was detected between 25-30 °C.

This is in line with previous results: Mislivec and Tuite (1970b) reported growth of *P. oxalicum* between 8-35 °C with an optimum of 30 °C. O'Neill *et al.* (1991) tested a growth range of 15-30 °C for *P. oxalicum* with entirely positive results. Like in our study, they determined an optimal growth temperature of 25-30 °C depending on isolate. Similar results were obtained by Kwon *et al.* (2008) as they evaluated growth within a range of 5-30 °C with a growth optimum at a temperature of 25 °C. Consequently, it is hardly surprising that *P. oxalicum* can be found on grapes of temperate, subtropical or tropical regions (see Table 1).

Apart from grapes, *P. oxalicum* is known to occur on several different plants e.g. tomato, cucumber, apple or corn (Borecka, 1977; Elhariry *et al.*, 2011; Jarvis *et al.*, 1990; Kwon *et al.*, 2008; Mislivec & Tuite, 1970a; O'Neill *et al.*, 1991; Samson *et al.*, 2010; Umemoto *et al.*, 2009). Interestingly, the pH of the mentioned fruits differs in more than 3 pH units. While cucumbers have an almost neutral pH, tomato fruits are more acidic, but not nearly as acidic as grapes (see Figure 42, Manteau *et al.* (2003)). As results in the current study have shown, *P. oxalicum* can grow in liquid media within a wide pH range of pH 4-10 without any impairment (see Figure 41). At pH 3 growth is only a little poorer and even at pH 2 marginal growth was present. Therefore, it is self-evident that *P. oxalicum* is able to infect a wide variety of hosts providing environment with different pH levels. Nevertheless, growth experiments with *P. oxalicum* isolates on solid media displayed a clear preference for pH 7 compared to pH 5. This might explain why *P. oxalicum* is present in several vineyards, though, only in rather small numbers (Bau *et al.*, 2005; Felšöciová *et al.*, 2015b; Sage *et al.*, 2002; Serra *et al.*, 2006a). However, to survive such a broad pH range the fungus needs the ability to adapt itself adequately. There are other fungi like *B. cinerea* or *A. nidulans*, which can live in a broad pH range (pH 2-7 respectively pH 2.5-9), too. To overcome this hurdle they produce different sets of enzymes and other proteins according to the ambient pH (Caddick *et al.*, 1986; Manteau *et al.*, 2003). Probably, also the protein pattern of *P. oxalicum* would change when analyzed after cultivation in media with different pH. Moreover, it might be taken into account that a double infection of grapes with two filamentous fungi is common (see section 3.10.3 and section 4.2). Prusky and Yakoby (2003) reported in their review the ability of some pathogenic fungi to alter the host's pH. Some fungi like e.g. *Alternaria alternata* or *Aspergillus fumigatus* achieve an alkalization of their environment by ammonia secretion. Others, like *B. cinerea* or several *Penicillium* species, acidify their surrounding by accumulation of organic acids or efflux of protons (Prusky *et al.*, 2004; Verhoeff *et al.*, 1988). Therefore, always the complete fungal community on a fruit must be considered in future studies. Perhaps, some interaction between *P. oxalicum* and other filamentous fungi in regard to pH adjustment take place.

## 4.8 Future perspective for alternative cloning approaches

The production of stable bacterial mutants carrying the two genes of interest, PFE_04519 and PDE_07106, has proven to be a difficult task.

Repetitive sequences are known to cause unstable inserts (Temple *et al.*, 2006) but were not found in the particular sequences. Furthermore, known causes for difficulty in cloning display: DNA rich in AT, DNA with strong secondary structure, DNA containing poly(dT) sequences or DNA, which is toxic to

the transformed bacteria (Godiska *et al.*, 2005; Moran, 2003; Ryoiti & Michio, 1994; Temple *et al.*, 2006). Therefore, Godiska *et al.* (2009) suggests the use of the linear cloning vector pJAZZ as it provides otherwise unclonable templates a possibility to remain stable inside the cell. Nevertheless, also circular vectors like pSMART™ were designed for problematic, unstable inserts (Godiska *et al.*, 2005). Besides different vectors, also a change in regard to the antibiotic for selection must be considered.

The gene for ampicillin resistance encodes for β-lactamase. This enzyme is secreted by bacteria to degrade ampicillin. Problematically, non-transformed bacterial colonies can consequently grow as satellite colonies on agar plates next to transformed colonies producing the antibiotic. Further, the correctly transformed clones often grow considerably slower than non-transformed bacteria or bacteria with a partial deleted plasmid. So, the selection with ampicillin in liquid cultures is even more difficult as the antibiotic is not spatially limited. The other tested antibiotic for selection was Zeocin™. According to the manufacturer Zeocin™ is sensitive to light, heat, extreme pH and higher salt concentrations resulting in a per se fragile antibiotic. Moreover, there is evidence that Zeocin™ is not completely inhibited despite the stable expression of its resistance gene (Trastoy *et al.*, 2005). Consequently, it is severely difficult to adjust a useful concentration of this antibiotic for selection of transformants. For future cloning attempts the usage of non-secreted, common antibiotics like kanamycin or chloramphenicol is highly recommended.

Also variations in the growth temperature of transformed *E. coli* may be evaluated as lower temperatures favor the production of recombinant proteins (Schein & Noteborn, 1988). Likewise different *E. coli* strains should be tested. According to different manufacturers recombinase A deficient strains like the used TOP10 or DH5α as well as JM109 have actually a higher stability to keep an insert. In contrast, strains with the recombinase A repair system like K12 or BL21 show improved growth.

## 4.9 Final assessment of gushing in sparkling wine

As it is the case for beer, the gushing of sparkling wine seems of multifactorial nature. Therefore, it is impossible to select or define THE cause for gushing in carbonated beverages. As demonstrated by Dachs and Nitschke (1977), several factors contribute together to the induction of gushing in beer whereas one factor alone did not lead to this economical problem.

In sparkling wine, different macromolecules originating from vine, yeast, bacteria or filamentous fungi were found to be affecting each other mutually (Fleet, 2003). The gushing potential is regulated by different molecules – fatty acids, precipitated salts of acids or, with particular focus on the current work, proteins. In beer gushing research, saturated fatty acids showed a low potential to induce gushing while unsaturated fatty acids were identified as gushing suppressants (Aydin *et al.*, 2014; Carrington *et al.*, 1972; Christian *et al.*, 2011; Zepf, 1998). For gushing in sparkling wine the influence of lipids such as fatty acids was discussed by some authors (Bach, 2001; Rankine, 1977) but was not further investigated. However, this group of surface-active molecules also merits investigation in the future.

Furthermore, precipitations of organic acids are known to induce gushing in several beverages. For beer it is known that calcium oxalate crystals can induce gushing (Zepf & Geiger, 2000). Besides this, in sparkling wine also the calcium salts of tartaric or mucic acid (= galactaric acid) can form crystalline precipitations (Kielhöfer & Würdig, 1961; Schanderl, 1964). Calcium mucate precipitation is often found in wine made of botrytized grapes whereas it is absent in healthy grapes (Würdig *et al.*, 1966). Therefore, in sparkling wine a fungal infection of the raw material might cause a degradation of a sugar polymer into its gushing-provoking organic acids. Consequently, a closer look on crystalline precipitations in gushing sparkling wine should be considered.

Surface-active proteins are known for their influence on gushing in beer as extensively discussed in section 1.2.3 and 1.2.4. Consequently, the investigation of this molecule group in regard to gushing in sparkling wine is reasonable. Albeit no hydrophobins could be detected within this study in gushing sparkling wine, two surface-active molecules in gushing-inducing culture supernatants of *Penicillium oxalicum* were identified (see section 3.7). Besides fungal infection, also the selected yeast strain appears to have an impact on the concentration of gushing-influencing molecules. Bach *et al.* (2001) were able to experimentally prevent overfoaming by addition of yeast mannoproteins to gushing sparkling wine. Consequently, it seems like that there are gushing-inducing and gushing-suppressing molecules in sparkling wine. Probably only their relation is decisive for the gushing in sparkling wine just like in the beer gushing mechanism proposed by Niessen *et al.* (2006).

As in beer, a fungal infection of grapes seems to influence the gushing risk in sparkling wine dramatically. This work showed that many *Penicillium* isolates were able to alter the wettability of a hydrophobic surface due to the secretion of surface-active molecules and some of them were able to induce gushing in a sparkling water model system. *Penicillium oxalicum* was prominent for the induction of consistent, severe gushing in sparkling water and in sparkling wine after addition of culture supernatants. As already described in section 4.2, an infection of grapes with *Botrytis cinerea* is known for degradation of foam stabilizing proteins, thus, an elevated gushing risk. Several studies (La Guerche *et al.*, 2005; La Guerche *et al.*, 2007; Morales *et al.*, 2013) showed an interaction of *Botrytis cinerea* strains with *Penicillium expansum* on grapes resulting in alteration of their metabolism regarding geosmin or patulin production as well as growth. Consequently, one can easily hypothesize that a double infection with *Botrytis cinerea* and some *Penicillium* species (e.g. *Penicillium oxalicum*) could be one reason for primary gushing in sparkling wine: *Botrytis* degrades foam stabilizing and potentially gushing-suppressing proteins, while *Penicillium* secretes gushing-inducing molecules. Due to such an imbalance in the composition of gushing-influencing molecules the phenomenon might be initiated.

It stands to reason that an imbalance between inducing and stabilizing factors in the system “sparkling wine” lead to gushing. Therefore, a strictly hygienic production process (see section 1.2.6), a conscientious handling of fining agents (see section 1.2.7) and, in particular, the selection of sound grapes for vinification might already minimize most of the gushing problems encountered in sparkling wine.

# 5 Summary

Gushing is a phenomenon known to the beverage industry worldwide. It is described as the spontaneous overfoaming of a carbonated beverage upon opening of a bottle, despite correct handling. Besides economic losses, gushing causes damage to the image of affected companies. In contrast to gushing of beer, the reason for its development in sparkling wine is widely unexplored. Nonetheless, also sparkling wine producers have been affected by this unwanted phenomenon. Therefore, the aim of this thesis was to identify proteins involved in the gushing phenomenon in sparkling wine. The working hypotheses were based on the assumption that proteins e.g. of filamentous fungi influence primary gushing in sparkling wine similar to the situation in beer.

For protein analytics a new preparation method for red wine proteins was developed as an effective tool for the removal of non-protein contaminants from samples. Application of this method prior to SDS-PAGE analysis resulted in more distinguishable protein bands and a reduction in background noise. In MALDI-TOF MS analysis the proposed protocol strongly improved signal-to-noise ratios and increased signal intensities. The developed method enabled the comparison of different beverages made of grapes in regard to their protein composition. Development of this new protocol during this thesis provides an important new tool for protein preparation, which allows for highly resolved proteome analysis of red (sparkling) wines and other grape products.

Comparison of non-gushing and gushing sparkling wine samples prepared with this technique did not reveal the presence of a universal gushing-factor: Neither by SDS-PAGE nor MALDI-TOF MS analysis conspicuous anomalies could be detected in the analyzed proteomes. The protein patterns of different wines varied widely more or less regardless of their foaming behavior. Therefore, by application of the before mentioned methods the influence of external factors on the wine's protein composition were assessed. Especially the grape variety as well as the fermentation conditions and vintage year displayed a high impact on the wine's proteome. Nevertheless, two fungal proteins could be identified with nanoESI-LC-MS/MS in gushing sparkling wine.

Consequently, various filamentous fungi isolated from grape or wine were analyzed. A screening of different grape-associated fungi showed their influence on the surface activity of fungal culture supernatants and probably also on gushing of sparkling wine. As assessed in gushing tests, *P. oxalicum* stood out as the most promising candidate to affect the gushing potential. It was demonstrated that gushing can be induced artificially by addition of *P. oxalicum* spumate depending on temperature, pH and sample volume. Two surface-active, small, hydrophobic proteins of this fungus (PDE_07106, PDE_04519) were isolated, identified, and partially characterized. Results suggest that they have prominent characteristics to be involved in the mechanism of gushing. Unfortunately, recombinant production of these proteins failed, which implies an unfinished detailed characterization. As a result their connection to the induction of gushing could not be elucidated in detail. Nonetheless, infection of grapes by *P. oxalicum* is suggested as one possible reason for the occurrence of primary gushing in sparkling wine.

On the basis of these results, a DNA-based diagnostic method (LAMP assay) was established and optimized in order to enable an early detection of *P. oxalicum* on grape samples. Therefore, the decision making in an industrial setting concerning the further processing of grapes might be facilitated. This loop-mediated isothermal amplification (LAMP) assay had a detection limit of 100 pg purified genomic *P. oxalicum* DNA per reaction. 77 grape-associated microorganisms were used for specificity testing. In addition, an efficient assessment of crude washing extracts from artificially infected grape samples was demonstrated. Screening of several grape samples from different European countries revealed the (rare) presence of *P. oxalicum* on grapes harvested in Italy and Portugal. Due to its ability to grow in a temperature range of 8-37 °C, with an optimal growth temperature between 25-30 °C, it is supposed to be common in temperate, subtropical or tropical regions. The developed LAMP assay is suggested as a promising tool for analysis in the food or beverage industry.

It can be stated that sparkling wine processing companies should always use healthy grapes for vinification in order to minimize the gushing risk. On the one hand it is known that an infection with *Botrytis cinerea* or profuse fining reduce foam stabilizing proteins in sparkling wine. On the other hand this thesis showed that an infection with filamentous fungi such as *Penicillium oxalicum* could introduce gushing-triggering proteins into the sparkling wine.

# 6 Zusammenfassung

Das Phänomen Gushing ist in der Getränkeindustrie weltweit bekannt. Es wird als das spontane Überschäumen eines kohlensäurehaltigen Getränks beim Öffnen einer Flasche trotz sachgemäßer Handhabung beschrieben. Neben den wirtschaftlichen Verlusten, verursacht Gushing auch einen Imageschaden bei den betroffenen Unternehmen. Im Gegensatz zum Gushing in Bier ist der Grund für dessen Auftreten im Sekt weitgehend unerforscht. Dennoch sind auch Sekthersteller von diesem unerwünschten Phänomen nicht gefeit. So war es das Ziel dieser Arbeit Proteine zu identifizieren, die an dem Gushingphänomen in Schaumwein beteiligt sind. Die Arbeitshypothese beruhte auf der Annahme, dass Proteine, beispielsweise von filamentösen Pilzen, primäres Gushing im Sekt genauso wie im Bier beeinflussen können.

Zur Proteinanalytik von Rotweinproteinen wurde eine neue Aufreinigungsmethode entwickelt, um Nicht-Protein-Verunreinigungen aus den zu untersuchenden Proben effektiv entfernen zu können. Die Anwendung dieses Verfahrens vor der SDS-PAGE-Analyse führte zu mehr unterscheidbaren Proteinbanden und einem verringertem Hintergrundrauschen. In der MALDI-TOF-MS-Analyse verbesserte das vorgeschlagene Verfahren die Signal-Rausch-Verhältnisse stark und erhöhte die Signalintensitäten. Die entwickelte Methode ermöglicht einen Vergleich von verschiedenen aus Trauben hergestellten Getränken in Bezug auf ihre Proteinzusammensetzung. Durch die Entwicklung dieses neuen Protokolls im Rahmen dieser Doktorarbeit wird ein wichtiges neues Werkzeug für die Proteinaufbereitung bereit gestellt, das eine hochaufgelöste Proteomanalyse von roten (Schaum)Weinen und anderen Traubenprodukten ermöglicht.

Der Vergleich von nicht-gushenden und gushenden Sektproben, die mit dieser Technik aufgereingt wurden, ließ keinen universellen Gushingfaktor erkennen: Weder durch SDS-PAGE noch MALDI-TOF MS-Analysen konnten auffällige Anomalien nachgewiesen werden. Die Proteinmuster verschiedener Weine variierten stark, mehr oder weniger unabhängig von ihrem Schäumungsverhalten. Daher wurde, nach der Anwendung der vorhergenannten Methode, der Einfluss von externen Faktoren auf die Proteinzusammensetzung des Weins beurteilt. Vor allem die Rebsorte sowie die Fermentationsbedingungen und der Erntejahrgang haben einen großen Einfluss auf die Proteinzusammensetzung des Weins. Jedoch konnten auch zwei Pilzproteine mit nanoESI-LC-MS/MS im gushendem Sekt identifiziert werden.

Folglich wurden verschiedene filamentöse Pilze, die von Trauben oder aus Wein isoliert wurden analysiert. Ein großes Screening von verschiedenen Trauben-assoziierten Pilzen machte ihren Einfluss auf die Oberflächenaktivität von Pilzkulturüberständen, und vermutlich auch das Gushing von Schaumwein, deutlich. Anhand von Gushing-Tests wurde gezeigt, dass *P. oxalicum* der vielversprechendste Kandidat ist, um das Gushingpotenzial zu beeinflussen. Es wurde dargelegt, dass Gushing künstlich durch Zugabe von Spumat von *P. oxalicum* in Abhängigkeit von Temperatur, pH und Probenvolumen induziert werden kann. Zwei oberflächenaktive, kleine, hydrophobe Proteine dieses Pilzes (PDE_07106, PDE_04519) wurden isoliert, identifiziert und teilweise charakterisiert. Die Ergebnisse deuten darauf hin, dass sie auffällige Eigenschaften haben, um an dem Gushing-

Mechanismus beteiligt zu sein. Leider ist die rekombinante Produktion dieser Proteine fehlgeschlagen und somit ihre genauere Charakterisierung. Infolgedessen konnte auch ihr Zusammenhang in der Induktion von Gushing nicht im Detail aufgeklärt werden. Trotzdem wird als ein möglicher Grund für das Auftreten von primären Gushing in Sekt eine Infektion der Trauben mit *P. oxalicum* vorgeschlagen.

Auf Basis dieser Ergebnisse wurde ein DNS-basiertes diagnostisches Verfahren (LAMP-Assay) etabliert und optimiert, um eine frühzeitige Erkennung von *P. oxalicum* auf Traubenproben zu ermöglichen. So könnte in einem industriellen Rahmen der Entscheidungsprozess für die Weiterverarbeitung von Trauben erleichtert werden. Dieser Schleifen-vermittelte isotherme Amplifikations-Assay (LAMP-Assay) hatte eine Nachweisgrenze von 100 pg Pilz-DNS pro Reaktion. Weiterhin wurden 77 Trauben-assoziierte Mikroorganismen für Spezifitätstests herangezogen. Darüber hinaus wurde gezeigt, dass auch Extrakte von nur grob gewaschenen künstlich infizierten Traubenproben für den Assay verwendet werden konnten. Das Screening von mehreren Traubenproben verschiedener europäischer Länder, bewies das (seltene) Vorkommen von *P. oxalicum* auf Trauben aus Italien und Portugal. Aufgrund seiner Fähigkeit, in einem Temperaturbereich von 8-37 °C zu wachsen, mit einer optimalen Wachstumstemperatur zwischen 25-30 °C, ist dieser Pilz in gemäßigten, subtropischen und auch tropischen Regionen heimisch. Der entwickelte LAMP-Assay wird als vielversprechendes Analysewerkzeug in der Lebensmittel- oder Getränkeindustrie vorgeschlagen.

Daher ist festzustellen, dass Schaumweinhersteller immer gesunde Trauben zur Vinifizierung verwenden sollten, um das Gushingrisiko zu minimieren. Auf der einen Seite ist bekannt, dass eine Infektion mit *Botrytis cinerea* oder auch übermäßiges Schönen schaumstabilisierende Proteine im Sekt verringern. Auf der anderen Seite zeigt diese Arbeit, dass eine Infektion mit filamentösen Pilzen wie beispielsweise *Penicillium oxalicum* Gushing-auslösende Proteine in den Schaumwein einbringen könnte.

# 7 List of Abbreviations

| | |
|---|---|
| **®** | registered trademark |
| **°C** | degree Celsius |
| **µ** | micro |
| **2D-GE** | two dimensional gel electrophoresis |
| ***A*.** | *Aspergillus* |
| **AAB** | acetic acid bacteria |
| **ACS** | American Chemical Society |
| **AfpA** | Alkaline foam protein A |
| **AG** | German *Aktiengesellschaft* (share corporation) |
| **AGE** | agarose gel electrophoresis |
| **AP** | alkaline phosphatase |
| ***B*.** | *Botrytis* |
| **bp** | base pair |
| **c** | centi |
| **CBS** | Centraalbureau voor Schimmelcultures |
| **$ClO_2$** | chlorine dioxide |
| **CMC** | critical micelle concentration |
| **$CO_2$** | carbon dioxide |
| **CTAC** | hexadecyltrimethylammonium chloride |
| **d** | days |
| **Da** | Dalton |
| **$dH_2O$** | deionized water |
| **DNA** | deoxyribonucleic acid |
| ***E. coli*** | *Escherichia Coli* |
| **e. V.** | German *eingetragener Verein* (registered association) |
| **e.g.** | Latin e*xempli gratia* (for example) |
| **ESI** | electrospray ionization |
| **et al.** | Latin *et alii* (and others) |
| **etc.** | Latin *et cetera* (and other things) |
| **f** | femto |
| ***F*.** | *Fusarium* |
| **for** | forward |
| **g** | gram or gravitational constant |
| **GmbH** | German *Gesellschaft mit beschränkter Haftung* (limited liability company) |
| **h** | hour |
| **HPLC** | high performance liquid chromatography |
| **IgG/Y** | immunoglobulin G/Y |
| **Inc.** | incorporation |
| **ISO** | international organization for standardization |
| **k** | kilo |
| **KGaA** | German *Kommanditgesellschaft auf Aktien* (master limited partnership) |
| **l** | liter |
| **LAB** | lactic acid bacteria |
| **LAMP** | loop-mediated isothermal amplification |
| **LC** | liquid chromatography |
| **m** | milli or meter |
| **M** | molarity |
| **m/z** | mass-to-charge ratio |
| **MALDI** | matrix-assisted laser desorption/ionization |
| **mcs** | multiple cloning site |
| **min** | minute |
| **MLF** | malolactic fermentation |
| **MS** | mass spectrometry |
| **n** | nano |
| **n. d.** | not determined |
| **n. f.** | not foamable |
| **NCBI** | National Center for Biotechnology Information |
| **nsLTP** | non-specific lipid transfer protein |
| **$O_3$** | ozone |
| **p** | pico |
| ***P*.** | *Penicllium* |
| **p.a.** | Latin *pro analysis* (for analysis) |
| **PAGE** | polyacrylamide gel electrophoresis |
| **PCR** | polymerase chain reaction |
| **Ph. Eur.** | Pharmacopoea Europaea |
| **pI** | isoelectric point |
| **ppm** | parts per million |
| **PR** | pathogenesis-related |
| **Reag.** | reagent |
| **rev** | reverse |
| **RNA** | ribonucleic acid |
| **RP** | reversed phase |
| **rpm** | rounds per minute |
| **RS** | restriction site |
| **rxn** | reaction |
| **SDS** | sodium dodecyl sulfate |
| **sec** | second |
| **sMF** | static magnetic field |

| | |
|---|---|
| **sp.** | Species |
| **spp.** | *species pluralis* |
| ***T.*** | *Trichoderma* |
| **$T_m$** | melting temperature |
| **™** | trademark |
| **TMW** | Technische Mikrobiologie Weihenstephan (strain collection) |
| **TOF** | time-of-flight |
| **Trp** | tryptophan |
| **Tyr** | tyrosine |
| **U** | unit for enzyme activity |
| **USP** | United States Pharmacopoeia |
| **UV** | ultraviolet |
| **v/v** | volume per volume |
| **VMP** | varying-permeability model |
| **w/o** | without |
| **w/v** | weight per volume |
| **YCB** | yeast carbon base |
| **Z4** | serpin protein Z4 |

# 8 List of Figures

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# 10 References

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# 11 Appendix

**Table 30:** Filamentous fungi used in this study isolated from grape (products). Besides the species name with corresponding ID of TMW information about origin, substrate and original ID is provided. TMW = Technische Mikrobiologie Weihenstephan, Freising, Germany; ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; MUM = Micoteca da Universidade do Minho, Braga, Portugal.

| species | TMW ID | origin | substrate | original ID |
|---|---|---|---|---|
| ***Aspergillus aculeatus*** | 4.2565 | Italy | grapes | ITEM 4185 |
| ***A. aculeatus*** | 4.2566 | Italy | grapes | ITEM 4494 |
| ***A. alliaceus*** | 4.2572 | Portugal | grapes | ITEM 4548 |
| ***A. awamori*** | 4.2570 | Portugal | grapes | ITEM 4541 |
| ***A. awamori*** | 4.2571 | Portugal | grapes | ITEM 4551 |
| ***A. brasiliensis*** | 4.2523 | Portugal | grapes | ITEM 4539 |
| ***A. brasiliensis*** | 4.2524 | Portugal | grapes | ITEM 4540 |
| ***A. carbonarius*** | 4.2519 | Italy/Apulia | grapes | ITEM 4722 |
| ***A. carbonarius*** | 4.2520 | Italy/Apulia | grapes | ITEM 4724 |
| ***A. foetidus*** | 4.2573 | Italy | grapes | ITEM 4710 |
| ***A. foetidus*** | 4.2574 | Argentina | grapes | ITEM 11843 |
| ***A. homomorphus*** | 4.2575 | Argentina | grapes | ITEM 11870 |
| ***A. ibericus*** | 4.2525 | Portugal | grapes | ITEM 6601 |
| ***A. ibericus*** | 4.2526 | Portugal | grapes | ITEM 6602 |
| ***A. japonicus*** | 4.2564 | Italy | grapes | ITEM 4158 |
| ***A. niger*** | 4.2562 | Italy | white table grapes | ITEM 3713 |
| ***A. niger*** | 4.2563 | Italy | grapes | ITEM 3753 |
| ***A. ochraceus*** | 4.2569 | Italy | grapes | ITEM 4211 |
| ***A. tubingiensis*** | 4.2567 | Italy | grapes | ITEM 4210 |
| ***A. tubingiensis*** | 4.2568 | Italy | grapes | ITEM 4496 |
| ***A. uvarum*** | 4.2521 | Italy/Apulia | grapes | ITEM 4727 |
| ***A. uvarum*** | 4.2522 | Italy | grapes | ITEM 4691 |
| ***Penicillium adametzioides*** | 4.2529 | Portugal/Madeira | grapes | MUM 14.26 |
| ***P.aurantiogriseum*** | 4.2536 | Portugal/Vinhos Verdes | grapes | MUM 14.24 |
| ***P. bilaii*** | 4.2528 | Portugal/Douro | grapes | MUM 14.25 |
| ***P. brevicompactum*** | 4.2545 | Portugal/Vinhos Verdes | grapes | MUM 14.27 |
| ***P. burgense*** | 4.2541 | Portugal/Vinhos Verdes | red grape must | MUM 14.44 |
| ***P. citrinum*** | 4.2547 | Portugal/Vinhos Verdes | grapes | MUM 14.29 |
| ***P. coalescens*** | 4.2537 | Portugal/Vinhos Verdes | grapes | MUM 14.37 |
| ***P. crustosum*** | 4.2530 | Portugal/Vinhos Verdes | grapes | MUM 14.31 |
| ***P. crustosum*** | 4.2548 | Portugal/Vinhos Verdes | red grape must | MUM 14.30 |
| ***P. echinulatum*** | 4.2549 | Portugal/Douro | grapes | MUM 14.32 |
| ***P. glabrum*** | 4.2531 | Portugal/Vinhos Verdes | grapes | MUM 14.33 |
| ***P. griseofulvum*** | 4.2532 | Portugal/Vinhos Verdes | grapes | MUM 14.34 |
| ***P. janczewskii*** | 4.2550 | Portugal/Douro | grapes | MUM 14.35 |
| ***P. minioluteum*** | 4.2533 | Portugal/Vinhos Verdes | grapes | MUM 14.36 |
| ***P. novae-zeelandiae*** | 4.2551 | Portugal/Douro | grapes | MUM 14.39 |

| species | TMW ID | origin | substrate | original ID |
|---|---|---|---|---|
| ***P. olsonii*** | 4.2538 | Portugal/Vinhos Verdes | grapes | MUM 14.40 |
| ***P. oxalicum*** | 4.2539 | Portugal/Vinhos Verdes | grapes | MUM 14.41 |
| ***P. oxalicum*** | 4.2552 | Portugal/Vinhos Verdes | grape stem | MUM 14.42 |
| ***P. oxalicum*** | 4.2553 | Portugal/Ribatejo | grapes | MUM 14.43 |
| ***P. paneum*** | 4.2542 | Portugal/Alentejo | grapes | MUM 14.47 |
| ***P. purpurogenum*** | 4.2540 | Portugal/Douro | grapes | MUM 02.55 |
| ***P. raistrickii*** | 4.2554 | Portugal/Ribatejo | grapes | MUM 14.45 |
| ***P. restrictum*** | 4.2555 | Portugal/Alentejo | grapes | MUM 14.46 |
| ***P. sclerotiorum*** | 4.2535 | Portugal/Madeira | grapes | MUM 03.39 |
| ***P. sclerotiorum*** | 4.2556 | Portugal/Ribatejo | grapes | MUM 14.48 |
| ***P. simplicissimum*** | 4.2557 | Portugal/Vinhos Verdes | grapes | MUM 14.49 |
| ***P. spinulosum*** | 4.2543 | Portugal/Ribatejo | grapes | MUM 14.51 |
| ***P. spinulosum*** | 4.2558 | Portugal/Vinhos Verdes | grape must | MUM 14.50 |
| ***P. thomii*** | 4.2559 | Portugal/Vinhos Verdes | grapes | MUM 14.52 |
| ***P. vagum*** | 4.2534 | Portugal | grapes | MUM 14.38 |
| ***P. variabile*** | 4.2560 | Portugal/Vinhos Verdes | white grape must | MUM 14.53 |
| ***P. variabile*** | 4.2561 | Portugal/Vinhos Verdes | Red grape must | MUM 14.54 |
| ***P. verrucosum*** | 4.2546 | Portugal/Douro | unmatured Grapes | MUM 14.28 |
| ***P. verrucosum*** | 4.2585 | Italy | white table grapes | ITEM 3716 |
| ***P. verrucosum*** | 4.2586 | Italy | white table grapes | ITEM 3717 |
| ***P. waksmanii*** | 4.2544 | Portugal/Alentejo | grapes | MUM 14.55 |

**Table 31:** Results of the protease activity screening of grape-associated filamentous fungi. The diameter of the clearance zone over the drilled whole was measured after 4.5 h and 24 h. As negative control pure YCB medium was used. TMW = Technische Mikrobiologie Weihenstephan, Freising, Germany

| species | TMW ID | Ø [cm] | | | |
|---|---|---|---|---|---|
| | | 4.5 h | 24 h | Δ 24 h – 4.5 h | Δ 4.5 h - NK |
| ***Aspergillus aculeatus*** | 4.2565 | 1.4 | 1.4 | 0.0 | 0.7 |
| ***A. aculeatus*** | 4.2566 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***A. alliaceus*** | 4.2572 | 0.9 | 0.9 | 0.0 | 0.2 |
| ***A. awamori*** | 4.2570 | 1.3 | 1.3 | 0.0 | 0.6 |
| ***A. awamori*** | 4.2571 | 1.0 | 1.1 | 0.1 | 0.3 |
| ***A. brasiliensis*** | 4.2523 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***A. brasiliensis*** | 4.2524 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***A. carbonarius*** | 4.2519 | 1.3 | 1.3 | 0.0 | 0.6 |
| ***A. carbonarius*** | 4.2520 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***A. foetidus*** | 4.2573 | 1.6 | 1.7 | 0.1 | 0.9 |
| ***A. foetidus*** | 4.2574 | 1.2 | 1.3 | 0.1 | 0.5 |
| ***A. homomorphus*** | 4.2575 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***A. ibericus*** | 4.2525 | 1.2 | 1.2 | 0.0 | 0.5 |
| ***A. ibericus*** | 4.2526 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***A. japonicus*** | 4.2564 | 1.3 | 1.4 | 0.1 | 0.6 |

| species | TMW ID | Ø [cm] | | | |
|---|---|---|---|---|---|
| | | 4.5 h | 24 h | Δ 24 h – 4.5 h | Δ 4.5 h - NK |
| ***A. niger*** | 4.2562 | 1.3 | 1.3 | 0.0 | 0.6 |
| ***A. niger*** | 4.2563 | 1.3 | 1.3 | 0.0 | 0.6 |
| ***A. ochraceus*** | 4.2569 | 0.9 | 0.9 | 0.0 | 0.2 |
| ***A. tubingiensis*** | 4.2567 | 1.4 | 1.5 | 0.1 | 0.7 |
| ***A. tubingiensis*** | 4.2568 | 1.5 | 1.5 | 0.0 | 0.8 |
| ***A. uvarum*** | 4.2521 | 1.5 | 1.5 | 0.0 | 0.8 |
| ***A. uvarum*** | 4.2522 | 1.5 | 1.5 | 0.0 | 0.8 |
| ***Penicillium adametzioides*** | 4.2529 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P.aurantiogriseum*** | 4.2536 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. bilaii*** | 4.2528 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. brevicompactum*** | 4.2545 | 0.9 | 1.0 | 0.1 | 0.2 |
| ***P. burgense*** | 4.2541 | 1.0 | 1.1 | 0.0 | 0.3 |
| ***P. citrinum*** | 4.2547 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. coalescens*** | 4.2537 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. crustosum*** | 4.2530 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. crustosum*** | 4.2548 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***P. echinulatum*** | 4.2549 | 1.2 | 1.3 | 0.1 | 0.5 |
| ***P. glabrum*** | 4.2531 | 0.7 | 0.7 | 0.0 | 0.0 |
| ***P. griseofulvum*** | 4.2532 | 0.8 | 0.8 | 0.0 | 0.1 |
| ***P. janczewskii*** | 4.2550 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. minioluteum*** | 4.2533 | 0.7 | 0.8 | 0.1 | 0.0 |
| ***P. novae-zeelandiae*** | 4.2551 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. olsonii*** | 4.2538 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***P. oxalicum*** | 4.2539 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***P. oxalicum*** | 4.2552 | 1.2 | 1.2 | 0.0 | 0.5 |
| ***P. oxalicum*** | 4.2553 | 1.0 | 1.1 | 0.1 | 0.3 |
| ***P. paneum*** | 4.2542 | 0.8 | 0.8 | 0.0 | 0.1 |
| ***P. purpurogenum*** | 4.2540 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. raistrickii*** | 4.2554 | 1.3 | 1.3 | 0.0 | 0.6 |
| ***P. restrictum*** | 4.2555 | 1.0 | 1.1 | 0.1 | 0.3 |
| ***P. sclerotiorum*** | 4.2535 | 0.7 | 0.7 | 0.0 | 0.0 |
| ***P. sclerotiorum*** | 4.2556 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. simplicissimum*** | 4.2557 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. spinulosum*** | 4.2543 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***P. spinulosum*** | 4.2558 | 1.0 | 1.0 | 0.0 | 0.3 |
| ***P. thomii*** | 4.2559 | 0.8 | 0.9 | 0.1 | 0.1 |
| ***P. vagum*** | 4.2534 | 0.8 | 0.8 | 0.0 | 0.1 |
| ***P. variabile*** | 4.2560 | 0.9 | 1.0 | 0.1 | 0.2 |
| ***P. variabile*** | 4.2561 | 0.8 | 1.0 | 0.2 | 0.1 |
| ***P. verrucosum*** | 4.2546 | 0.9 | 1.0 | 0.1 | 0.2 |
| ***P. verrucosum*** | 4.2585 | 1.1 | 1.1 | 0.0 | 0.4 |
| ***P. verrucosum*** | 4.2586 | 0.9 | 1.0 | 0.1 | 0.3 |
| ***P. waksmanii*** | 4.2544 | 0.9 | 0.9 | 0.0 | 0.2 |
| ***negative control*** | | 0.7 | 0.7 | 0.0 | 0.0 |

**Table 32:** Results of the surface activity screening of grape-associated filamentous fungi over an time interval of 12 days. As negative control pure YCB medium was used. + = 'positive', medium forms a stable liquid film over the complete surface of the Parafilm strip, - = 'negative', media drains off immediately and completely, 0 = 'indifferent', single drops of medium adhere to the strip. TMW = Technische Mikrobiologie Weihenstephan, Freising, Germany

| species | TMW ID | 4 d | 6 d | 8 d | 10 d | 12 d |
|---|---|---|---|---|---|---|
| ***Aspergillus aculeatus*** | 4.2565 | - | 0 | 0 | 0 | + |
| ***A. aculeatus*** | 4.2566 | - | - | - | - | - |
| ***A. alliaceus*** | 4.2572 | 0 | 0 | + | 0 | + |
| ***A. awamori*** | 4.2570 | - | - | - | 0 | - |
| ***A. awamori*** | 4.2571 | - | - | - | - | - |
| ***A. brasiliensis*** | 4.2523 | - | - | - | + | - |
| ***A. brasiliensis*** | 4.2524 | - | - | 0 | + | + |
| ***A. carbonarius*** | 4.2519 | - | - | - | - | 0 |
| ***A. carbonarius*** | 4.2520 | - | - | - | - | - |
| ***A. foetidus*** | 4.2573 | - | - | 0 | 0 | 0 |
| ***A. foetidus*** | 4.2574 | - | - | - | 0 | - |
| ***A. homomorphus*** | 4.2575 | + | + | + | + | + |
| ***A. ibericus*** | 4.2525 | - | - | - | 0 | - |
| ***A. ibericus*** | 4.2526 | - | - | - | - | 0 |
| ***A. japonicus*** | 4.2564 | - | + | + | 0 | + |
| ***A. niger*** | 4.2562 | - | - | - | - | - |
| ***A. niger*** | 4.2563 | - | - | - | - | - |
| ***A. ochraceus*** | 4.2569 | + | + | + | + | + |
| ***A. tubingiensis*** | 4.2567 | - | 0 | 0 | 0 | 0 |
| ***A. tubingiensis*** | 4.2568 | - | - | - | - | - |
| ***A. uvarum*** | 4.2521 | - | - | - | - | 0 |
| ***A. uvarum*** | 4.2522 | - | - | - | 0 | 0 |
| ***Penicillium adametzioides*** | 4.2529 | - | - | - | - | - |
| ***P.aurantiogriseum*** | 4.2536 | - | - | 0 | 0 | + |
| ***P. bilaii*** | 4.2528 | - | - | - | - | 0 |
| ***P. brevicompactum*** | 4.2545 | - | - | - | - | - |
| ***P. burgense*** | 4.2541 | + | + | + | + | + |
| ***P. citrinum*** | 4.2547 | - | - | - | 0 | - |
| ***P. coalescens*** | 4.2537 | - | 0 | + | + | + |
| ***P. crustosum*** | 4.2530 | - | 0 | - | 0 | 0 |
| ***P. crustosum*** | 4.2548 | - | - | 0 | 0 | - |
| ***P. echinulatum*** | 4.2549 | - | - | - | - | 0 |
| ***P. glabrum*** | 4.2531 | - | - | - | - | - |
| ***P. griseofulvum*** | 4.2532 | - | - | + | + | + |
| ***P. janczewskii*** | 4.2550 | - | + | + | + | + |
| ***P. minioluteum*** | 4.2533 | + | + | + | + | + |
| ***P. novae-zeelandiae*** | 4.2551 | - | 0 | + | + | + |
| ***P. olsonii*** | 4.2538 | - | - | - | - | - |
| ***P. oxalicum*** | 4.2539 | + | + | + | + | + |
| ***P. oxalicum*** | 4.2552 | + | + | + | + | + |
| ***P. oxalicum*** | 4.2553 | + | + | + | + | + |
| ***P. paneum*** | 4.2542 | - | - | - | - | - |
| ***P. purpurogenum*** | 4.2540 | - | 0 | - | - | - |

| species | TMW ID | 4 d | 6 d | 8 d | 10 d | 12 d |
|---|---|---|---|---|---|---|
| ***P. raistrickii*** | 4.2554 | - | - | - | - | - |
| ***P. restrictum*** | 4.2555 | - | - | + | 0 | + |
| ***P. sclerotiorum*** | 4.2535 | - | - | + | - | + |
| ***P. sclerotiorum*** | 4.2556 | + | + | + | + | + |
| ***P. simplicissimum*** | 4.2557 | - | - | + | + | + |
| ***P. spinulosum*** | 4.2543 | - | - | - | - | 0 |
| ***P. spinulosum*** | 4.2558 | - | - | 0 | + | + |
| ***P. thomii*** | 4.2559 | - | - | - | - | - |
| ***P. vagum*** | 4.2534 | - | + | + | + | + |
| ***P. variabile*** | 4.2560 | - | - | - | + | + |
| ***P. variabile*** | 4.2561 | - | + | + | + | 0 |
| ***P. verrucosum*** | 4.2546 | - | 0 | + | + | + |
| ***P. verrucosum*** | 4.2585 | - | 0 | + | + | + |
| ***P. verrucosum*** | 4.2586 | - | - | + | 0 | 0 |
| ***P. waksmanii*** | 4.2544 | + | + | + | + | + |
| ***negative control*** | | - | - | - | - | - |

**Table 33:** Protein sequence of *P. oxalicum* (strain 114-2 from NCBI database) proteins PDE_07106 and PDE_04519. Predicted signal peptide marked in bold.

| protein | amino acid sequence |
|---|---|
| **PDE_07106** | **MQFTQVLLATLLAVPAALA**APASGKSMMAQSPQWTIQNMKRVCAANDSSCTWTFGIYPGS GNATPCTLVVTGQKASQSNGGPVNCGDYTVTSGWSGQFGPGNGFTTLAVVNNKSRQIAYPA YTDKQVEGGNVVKPDQSYPPYALP |
| **PDE_04519** | **MKTFIASLALPLLAAA**APTSQAPAANPSFGVVAIRSGSGIQYASLNAAGQKFYLGGTTTSYCPSE TVQNCPPGDQTIIAPGGNALDVEVPGGQQIYVDPTGAVSFTQAHSAAMPQGSIVGEFAYETE GDAAHWTFKGDGLLACPTTDNRYQVFAPIENLKVPSGNKDDCLGFSARAFTYSGSTPAWQYI |

**Table 34:** Nucleotide sequence of *P. oxalicum* (strain 114-2 from NCBI database) genes encoding for proteins PDE_07106 and PDE_04519. Nucleotide sequence of predicted signal peptide marked in bold.

| gene encoding for | nucleotide sequence |
|---|---|
| **PDE_07106** | **ATGCAGTTCACTCAAGTCCTCCTGGCTACCCTCCTGGCCGTCCCCGCGGCCCTCGCT**GCTCC TGCCAGCGGCAAGTCCATGATGGCTCAGAGCCCCCAGTGGACCATTCAGAACATGAAGCG CGTTTGCGCCGCCAACGACTCCTCCTGCACCTGGACCTTTGGCATCTACCCCGGCTCCGGCA ACGCCACCCCTTGCACACTCGTCGTGACCGGCCAAAAGGCCTCCCAGTCCAACGGCGGACC CGTCAACTGCGGTGACTACACCGTCACCTCAGGCTGGAGCGGTCAATTCGGTCCCGGCAAT GGCTTCACCACCTTGGCCGTGGTCAACAACAAGAGCCGCCAGATCGCCTACCCTGCTTACA CCGACAAGCAGGTCGAGGGCGGCAATGTTGTCAAGCCTGACCAGAGCTACCCTCCCTACG CTCTCCCTTAG |
| **PDE_04519** | **ATGAAGACCTTCATTGCTTCCCTCGCCCTCCCTCTGCTGGCCGCCGCT**GCGCCCACCAGCCA GGCCCCGGCCGCCAACCCATCCTTTGGCGTGGTCGCCATCCGCTCCGGCTCCGGCATCCAG TATGCCTCCTTGAACGCTGCCGGCCAGAAGTTCTACCTCGGTGGCACCACCACCTCCTACTG CCCCAGTGAGACTGTCCAGAACTGCCCTCCCGGCGACCAGACCATCATCGCTCCCGGTGGC AACGCCCTGGACGTCGAAGTCCCCGGCGGCCAGCAGATCTACGTCGATCCCACTGGTGCC GTGAGCTTCACCCAGGCCCACTCCGCCGCCATGCCCCAGGGCTCCATCGTCGGTGAATTCG CCTACGAGACCGAGGGCGATGCCGCTCACTGGACCTTCAAGGGTGACGGTCTCCTGGCCT GCCCGACCACCGACAACCGCTACCAGGTCTTTGCCCCCATTGAGAACCTGAAGGTTCCCTC GGGCAACAAGGACGACTGCCTGGGCTTCTCGGCCCGTGCTTTCACTTACTCCGGTAGCACC CCTGCCTGGCAGTACATCTAA |

**Table 35:** Microorganisms used for specificity testing of the developed LAMP assay with different indicators. Microorganisms were isolated from or associated with grapes (see references). + = green fluorescence under $UV_{365}$nm (calcein), pink (neutral red), - = no fluorescence under $UV_{365}$nm (calcein), faint orange (neutral red); TMW = Technische Mikrobiologie Weihenstephan, Freising, Germany; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, Germany; BLQ = Forschungsinstitut für Brau- und Lebensmittelqualität, Freising, Germany; CBS = Centraalbureau voor Schummelcultures, Utrecht, Netherlands; MUM = Micoteca da Universidade do Minho, Braga, Portugal; ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy.

| species | original ID | TMW ID | substrate | results in LAMP | |
|---|---|---|---|---|---|
| | | | | calcein | neutral red |
| **bacteria** | | | | | |
| ***Lactobacillus (Lb.) backii*** | not stated | TMW 1.2004 | brewery[1] | - | - |
| ***Lb. brevis*** | not stated | TMW 1.1370 | honey fermentation[1] | - | - |
| ***Lb. lindneri*** | not stated | TMW 1.1433 | brewery[1] | - | - |
| ***Lb. paracasei subsp. paracasei*** | F19 | TMW 1.1434 | human colon[1] | - | - |
| ***Lb. paracollinoides*** | DSM 20197 | TMW 1.1979 | beer[1] | - | - |
| ***Lb. plantarum*** | TMW 1.277 | TMW 1.277 | palmwine[1] | - | - |
| ***Pediococcus (Pc.) clausenii*** | not stated | TMW 2.60 | brewery[1] | - | - |
| ***Pc. damnosus*** | not stated | TMW 2.1641 | brewery[1] | - | - |
| **yeasts** | | | | | |
| ***Aureobasidium (Au.) pullulans*** | TMW 3.0393 | TMW 3.0393 | grape leaf | - | - |
| ***Au. pullulans*** | TMW 3.0400 | TMW 3.0400 | grape leaf | - | - |
| ***Bulleromyces albus*** | TMW 3.0524 | TMW 3.0524 | grape leaf | - | - |
| ***Candida (C.) lambica*** | TMW 3.0645 | TMW 3.0645 | bird cherry blossom[2] | - | - |
| ***C. lambica*** | TMW 3.0646 | TMW 3.0646 | bird cherry blossom[2] | - | - |
| ***C. pulcherrima*** | TMW 3.0643 | TMW 3.0643 | bird cherry blossom[2] | - | - |
| ***C. pulcherrima*** | TMW 3.0644 | TMW 3.0644 | bird cherry blossom[2] | - | - |
| ***C. sake*** | TMW 3.0710 | TMW 3.0710 | white grape juice | - | - |
| ***Cryptococcus (Cr.) heimayensis*** | TMW 3.0525 | TMW 3.0525 | grape leaf | - | - |
| ***Cr. heimaeyensis*** | TMW 3.0526 | TMW 3.0526 | grape leaf | - | - |
| ***Cr. hungaricus*** | TMW 3.0528 | TMW 3.0528 | grape leaf | - | - |
| ***Cr. tephrensis*** | TMW 3.397 | TMW 3.397 | grape leaf | - | - |
| ***Cr. victoriae*** | TMW 3.0389 | TMW 3.0389 | grape leaf | - | - |
| ***Cr. victoriae*** | TMW 3.0390 | TMW 3.0390 | grape leaf | - | - |
| ***Cr. victoriae*** | TMW 3.0398 | TMW 3.0398 | grape leaf | - | - |
| ***Cr. victoriae*** | TMW 3.0563 | TMW 3.0563 | grapes | - | - |
| ***Cr. victoriae*** | TMW 3.0564 | TMW 3.0564 | grapes | - | - |
| ***Cr. wieringae*** | TMW 3.0391 | TMW 3.0391 | grape leaf | - | - |
| ***Hanseniaspora uvarum*** | BLQ H6 | TMW 3.0700 | grapes | - | - |
| ***Saccharomyces cerevisiae*** | TMW 3.0709 | TMW 3.0709 | red grape juice | - | - |
| ***Saccharomycodes ludwigii*** | CBS 820 | TMW 3.0020 | grape must | - | - |

| species | original ID | TMW ID | substrate | results in LAMP | |
|---|---|---|---|---|---|
| | | | | calcein | neutral red |
| ***Sporobolomyces (S.) roseus*** | TMW 3.0394 | TMW 3.0394 | grape leaf | - | - |
| ***S. roseus*** | TMW 3.0395 | TMW 3.0395 | grape leaf | - | - |
| ***Trichosporon spp.*** | TMW 3.0473 | TMW 3.0473 | grapes | - | - |
| **filamentous fungi** | | | | | |
| ***Aspergillus aculeatus*** | TMW 4.2390 | TMW 4.2390 | soil debris[4] | - | - |
| ***A. carbonarius*** | M324 | TMW 4.1512 | coffee[1,3,4,5] | - | - |
| ***A. flavus*** | TMW 4.1829 | TMW 4.1829 | nutmeg[3,5,5] | - | - |
| ***A. fumigatus*** | CBS 113.55 | TMW 4.0623 | human lung[3,4,5] | - | - |
| ***A. japonicus*** | CBS 114.51 | TMW 4.1627 | -[3,5] | - | - |
| ***A. japonicus*** | TMW 4.1776 | TMW 4.1776 | oil tanker[3,5] | - | - |
| ***A. niger*** | CBS 101.698 | TMW 4.1068 | mesocarp finga - coffee bean[1,3] | - | - |
| ***A. parasiticus*** | CBS 126.62 | TMW 4.1768 | peanut[3] | - | - |
| ***A. ustus*** | TMW 4.1365 | TMW 4.1365 | -[4,5] | - | - |
| ***P. adametzioides*** | TMW 4.2529 | MUM 14.26 | grapes | - | - |
| ***P. aurantiogriseum*** | TMW 4.2536 | MUM 14.24 | grapes | - | - |
| ***P. bilaii*** | TMW 4.2528 | MUM 14.25 | grapes | - | - |
| ***P. brevicompactum*** | TMW 4.2545 | MUM 14.27 | grapes | - | - |
| ***P. burgense*** | TMW 4.2541 | MUM 14.44 | red grape must | + | + |
| ***P. citrinum*** | TMW 4.2547 | MUM 14.29 | grapes | - | - |
| ***P. coalescens*** | TMW 4.2537 | MUM 14.37 | grapes | -/+ | -/+ |
| ***P. crustosum*** | TMW 4.2530 | MUM 14.31 | red grape must | - | - |
| ***P. crustosum*** | TMW 4.2548 | MUM 14.30 | grapes | - | - |
| ***P. echinulatum*** | TMW 4.2549 | MUM 14.32 | grapes | - | - |
| ***P. glabrum*** | TMW 4.2531 | MUM 14.33 | grapes | - | - |
| ***P. griseofulvum*** | TMW 4.2532 | MUM 14.34 | grapes | - | - |
| ***P. janczewskii*** | TMW 4.2550 | MUM 14.35 | grapes | - | - |
| ***P. minioluteum*** | TMW 4.2533 | MUM 14.36 | grapes | - | - |
| ***P. novae-zelandiae*** | TMW 4.2551 | MUM 14.39 | grapes | - | - |
| ***P. olsonii*** | TMW 4.2538 | MUM 14.40 | grapes | - | - |
| ***P. oxalicum*** | TMW 4.2539 | MUM 14.41 | grapes | + | + |
| ***P. oxalicum*** | TMW 4.2552 | MUM 14.42 | grape stalk | + | + |
| ***P. oxalicum*** | TMW 4.2553 | MUM 14.43 | grapes | + | + |
| ***P. paneum*** | TMW 4.2542 | MUM 14.47 | grapes | + | + |
| ***P. purpurogenum*** | TMW 4.2540 | MUM 02.55 | grapes | - | - |
| ***P. raistrickii*** | TMW 4.2554 | MUM 14.45 | grapes | + | + |
| ***P. restrictum*** | TMW 4.2555 | MUM 14.46 | grapes | - | -/+ |
| ***P. sclerotiorum*** | TMW 4.2535 | MUM 03.39 | grapes | - | - |
| ***P. sclerotiorum*** | TMW 4.2556 | MUM 14.48 | grapes | - | - |
| ***P. simplicissimum*** | TMW 4.2557 | MUM 14.49 | grapes | - | - |
| ***P. spinulosum*** | TMW 4.2543 | MUM 14.51 | grapes | - | - |
| ***P. spinulosum*** | TMW 4.2558 | MUM 14.50 | grape must | - | - |
| ***P. thomii*** | TMW 4.2559 | MUM 14.52 | grapes | - | - |
| ***P. vagum*** | TMW 4.2534 | MUM 14.38 | grapes | - | - |

| species | original ID | TMW ID | substrate | results in LAMP | |
|---|---|---|---|---|---|
| | | | | calcein | neutral red |
| ***P. variabile*** | TMW 4.2560 | MUM 14.53 | white grape must | + | - |
| ***P. variabile*** | TMW 4.2561 | MUM 14.54 | red grape must | - | - |
| ***P. verrucosum*** | TMW 4.2546 | MUM 14.28 | green grapes | - | - |
| ***P. verrucosum*** | TMW 4.2585 | ITEM 3716 | white table grapes | - | - |
| ***P. verrucosum*** | TMW 4.2586 | ITEM 3717 | white table grapes | - | - |
| ***P. waksmanii*** | TMW 4.2544 | MUM 14.55 | grapes | - | - |

[1] =Barata *et al.* (2012), [2] = Jolly *et al.* (2003), [3] = Bau *et al.* (2005), [4] = Sage *et al.* (2002), [5] = Serra *et al.* (2006a)

**Table 36:** Grape samples used in this study. pH values of 3 bunches per sample were assessed in triplicates. *indicating organic farming, # indication a positive reaction in the LAMP assay for the detection of *P. oxalicum*, n.d. = not determined

| ID | grape variety | origin | country | Ø pH of grape bunches | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 |
| **1** | Falanghina* | Nova Siri | Italy | 3.55 | 3.35 | 3.59 |
| **2** | Fiano (Minutolo) | Nova Siri | Italy | 3.82 | 3.76 | 3.82 |
| **3** | Montonico bianco | Nova Siri | Italy | 2.78 | 2.78 | 3.18 |
| **4** | Moscato Giallo | Nova Siri | Italy | 4.22 | 4.15 | 3.95 |
| **5** | Greco bianco | Nova Siri | Italy | 3.25 | 3.19 | 3.11 |
| **6** | Malvasia bianca | Nova Siri | Italy | 3.32 | 3.84 | 3.24 |
| **7** | Pinot noir | Nova Siri | Italy | 3.14 | 2.98 | 3.13 |
| **8** | Cabernet | Nova Siri | Italy | 3.67 | 3.77 | 3.28 |
| **9** | Aglianico | Venosa | Italy | 3.27 | **3.48**[#] | 3.48 |
| **10** | Syrah | Nova Siri | Italy | 2.88 | 2.90 | 3.04 |
| **11** | Primitivo | Nova Siri | Italy | 2.90 | 3.09 | 3.12 |
| **12** | Petit verdot | Nova Siri | Italy | 4.00 | 3.74 | 3.74 |
| **13** | Castiglione | Nova Siri | Italy | 3.59 | 3.57 | 3.62 |
| **14** | Aleatico | Cerignola | Italy | 4.06 | 3.66 | 3.72 |
| **15** | Fiano* | Cerignola | Italy | 3.66 | 3.34 | **3.37**[#] |
| **16** | Uva di Troia* | Cerignola | Italy | 3.83 | 3.19 | 3.25 |
| **PN** | Pinot noir | Langenlois | Austria | 2.98 | 3.12 | 3.22 |
| **GV** | Green Veltliner | Landersdorf | Austria | 3.25 | 3.27 | 3.66 |
| **Z** | Zweigelt | Landersdorf | Austria | 3.68 | 3.40 | 3.25 |
| **W1** | Pinot blanc | Mußbach | Germany | 3.17 | 3.09 | 3.23 |
| **W2** | Pinot blanc | Eschbach | Germany | 3.01 | 3.28 | 3.41 |
| **W3** | Pinot blanc | Laumersheim | Germany | 3.29 | 3.48 | 3.52 |
| **W4** | Pinot blanc | Bockenheim | Germany | 3.25 | 3.27 | 3.33 |
| **W5** | Pinot blanc | Bad Dürkheim | Germany | 3.22 | 3.47 | 3.46 |
| **R** | Riesling | Deidesheim | Germany | 3.69 | 3.63 | 3.57 |
| **Ri** | Riesling | Weinsberg | Germany | 3.18 | 3.32 | 3.17 |
| **K** | Kerner | Weinsberg | Germany | 3.39 | 3.40 | 3.40 |
| **C** | Chardonnay | Weinsberg | Germany | 3.61 | 3.39 | 3.32 |
| **MT** | Müller-Thurgau | Weinsberg | Germany | 3.77 | 3.59 | 3.16 |
| **A** | Acolon | Weinsberg | Germany | 3.47 | 3.28 | 3.64 |
| **Wei** | Pinot blanc | Weinsberg | Germany | 3.11 | 3.47 | 3.19 |
| **SB** | Sauvignon blanc | Weinsberg | Germany | 3.13 | 3.72 | 3.41 |

| ID | grape variety | origin | country | Ø pH of grape bunches | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 |
| **GT** | Gewürztraminer | Weinsberg | Germany | 3.45 | 3.18 | 3.25 |
| **PN** | Pinot noir | Weinsberg | Germany | 3.29 | 3.23 | 3.40 |
| **GB** | Pinot gris | Weinsberg | Germany | 3.57 | 3.56 | 3.76 |
| **T** | Trollinger | Weinsberg | Germany | 3.47 | 3.63 | 3.36 |
| **L** | Lemberger | Weinsberg | Germany | 3.22 | 3.08 | 3.14 |
| **SV** | Silvaner | Veitshöchheim | Germany | 3.49 | 3.26 | 3.68 |
| **Ries** | Riesling | Veitshöchheim | Germany | 3.52 | 3.34 | 3.65 |
| **WB** | Pinot blanc | Veitshöchheim | Germany | 3.30 | 3.52 | 3.64 |
| **RS** | Riesling | Neuweier | Germany | 3.15 | 3.30 | 2.93 |
| **CD** | Chardonnay | Geisenheim | Germany | 3.59 | 3.69 | 3.37 |
| **W-RS** | Riesling | Geisenheim | Germany | 3.36 | 3.25 | 2.95 |
| **BI-S** | Pinot noir | Geisenheim | Germany | 3.77 | 3.67 | 3.73 |
| **MR** | Pinot Meunier | Geisenheim | Germany | 3.36 | 3.61 | 3.77 |
| **P** | Parellada | Pontons | Spain | 3.86 | 3.57 | 3.18 |
| **X** | Xarello | (not stated) | Spain | 3.22 | 3.31 | 3.26 |
| **TR** | Trepat | Rocafort | Spain | 3.44 | 3.92 | 3.91 |
| **FP** | Fernão Pires | Bairrada | Portugal | n.d. | **n.d.**[#] | n.d. |
| **Ari** | Arinto | Bairrada | Portugal | n.d. | n.d. | n.d. |
| **Rabo** | Rabo de Ovelha | Bairrada | Portugal | n.d. | n.d. | n.d. |
| **Bi** | Bical | Bairrada | Portugal | n.d. | n.d. | n.d. |
| **Cer** | Cerceal | Bairrada | Portugal | n.d. | n.d. | n.d. |
| **Al** | Alvarinho | Vinhos Verde | Portugal | n.d. | n.d. | n.d. |
| **Lou** | Loureiro | Vinhos Verde | Portugal | n.d. | n.d. | n.d. |
| **Tra** | Trajadura | Vinhos Verde | Portugal | n.d. | n.d. | n.d. |
| **Vin** | Vinhão | Vinhos Verde | Portugal | n.d. | n.d. | |
| **Franc** | Touriga franca | Vinhos Verde | Portugal | n.d. | n.d. | |
| **Nac** | Touriga nacional | Vinhos Verde | Portugal | n.d. | n.d. | |
| **Colo** | Colombard | Vinhos Verde | Portugal | n.d. | n.d. | |
| **Ugni** | Ugni blanc | Vinhos Verde | Portugal | n.d. | n.d. | |

# 12 Publications

**Published manuscripts:**

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

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

**Vogt, E. I.**, Kupfer, V. M., Vogel, R. F., & Niessen L. (2017). Überschäumendem Sekt auf der Spur. *Der Deutsche Weinbau*, 11, 29-35.

Kupfer, V. M., **Vogt, E. I.**, Ziegler, T., Vogel, R. F. & Niessen L. (2017). 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(1), 501-509.

Kupfer, V. M., **Vogt, E. I.**, Siebert, A., Meyer, M., Vogel, R. F. & Niessen L. (2017). Foaming characteristics of the gushing modulating protein PAU5 with detailed investigation on the influence of sparkling wine processing. *Food Research International*, 102, 111-118.

**Vogt, E. I.**, Kupfer, V. M., Bechtner, J. D., Frisch, L. M, Vogel, R. F., & Niessen L. (2017) Detection of *Penicillium oxalicum* by use of a loop-mediated isothermal amplification (LAMP) assay. *The Journal of Microbiology, Biotechnology and Food Sciences*, *7*(3), 265-270.

**Presented posters:**

Kupfer, V. M., **Vogt, E. I.**, Vogel, R. F. & Niessen L. (2015). Preparation of red sparkling wine proteins for analysis by SDS-PAGE and MALDI-TOF MS. Presented at In Vino Analytica Sciencia, Trento/Italy.

**Vogt, E. I.**, Kupfer, V. M., Vogel, R. F., & Niessen, L. (2016). Influence of *Penicillium oxalicum* proteins on gushing of sparkling wine. Presented at Workshop International Commission on Food Mycology, Freising/Germany and Macrowine, Changins/Schweiz.

Kupfer, V. M., **Vogt, E. I.**, Ziegler, T., Vogel, R. F. & Niessen L. (2016). Comparative proteomic analysis of wines made from *Botrytis cinerea* infected and healthy grapes reveals interesting parallels to the gushing phenomenon in sparkling wine. Presented at Macrowine, Changins/Schweiz.

**Oral presentations:**

**Vogt, E. I.**, Kupfer, V. M., Vogel, R. F., & Niessen, L. (2015). Einfluss von oberflächenaktiven Proteinen auf das Gushing von Sekt und Schaumwein. Presented at Mitgliederversammlung des Verband Deutscher Sektkellereien, Wachenheim/Germany.

Kupfer, V. M., **Vogt, E. I.**, Vogel, R. F. & Niessen L. (2016). Einsatz bioanalytischer Methoden zur Identifizierung von gushing-relevanten Markerproteinen in Wein & Schaumwein. Presented at Tagung des Forschungsrings Deutscher Weinbau, Bad Kreuznach/Germany.

# 13 Curriculum vitae

## Elisabeth Ingeborg Vogt-Hrabak

born on 23.11.1988
in Munich
unmarried, German

### University and school education

**04.2014 – 01.2018** **Doctorate in Molecular Biotechnology** — **Technische Universität München**
Degree: Dr. rer. nat.

**04.2014 – 10.2017** **Doctoral thesis** — **Technische Universität München, Lehrstuhl für Technische Mikrobiologie**
*Influence of grape-associated filamentous fungi and their exoproteome on the gushing in sparkling wine*

**10.2011 – 12.2013** **Master in Molecular Biotechnology** — **Technische Universität München**
Degree: Master of Science

**05.2013 – 11.2013** **Master's thesis** — **Technische Universität München, Lehrstuhl für Mikrobielle Ökologie**
*The impact of intestinal epithelial cells on strain-specific spore germination and transcription of virulence genes and regulators in enteropathogenic B. cereus*

**10.2008 – 07.2011** **Bachelor in Biochemistry** — **Technische Universität München**
Degree: Bachelor of Science

**04.2011 – 07.2011** **Bachelor's thesis** — **Technische Universität München, Lehrstuhl für Biologische Chemie**
*Cloning and production of different PASylated mouse interferons*

**09.1999 – 06.2008** **A-level** — **Franz-Marc-Gymnasium, Markt Schwaben**
Degree: Abitur

### International experience

**09.2016 – 10.2016** **Research internship** — **Universidade do Minho (Braga/Portugal), Departamento de Engenharia Biológica**
*Evaluation of a newly developed LAMP assay for the detection of Penicillium oxalicum on Portuguese grapes*

**04.2006 – 05.2006** **Student exchange** — **John F. Kennedy Highschool, Seattle, USA**