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Surface-active proteins from *Fusarium* spp. and their role in gushing in carbonated beverages

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Abbreviations

AfpA	Alkaline foam protein A from *Fusarium graminearum*
APS	Ammonium persulfate
atm	Atmosphere
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMGY	Buffered glycerol complex medium
BMM	Buffered minimal methanol medium
BMMY	Buffered methanol complex medium
BSA	Bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CBB-G250	Coomassie Brilliant Blue G-250
DEPC	Diethylpyrocarbonate
DHA	Dehydrated humulinic acid
DMF	*N,N*-Dimethylformamid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FcHyd3p	Class I hydrophobin FcHyd3p from *Fusarium culmorum*
FcHyd5p	Class II hydrophobin FcHyd5p from *Fusarium culmorum*
Hfb2	Class II hydrophobin Hfb2 from *Trichoderma reesei*
HIC	Hydrophobic interaction chromatography
HOTD	Hop oil type dry
*I*p	Isoelectric point
LB	Lysogeny broth
MWCO	Molecular weight cut off
NBT	Nitro blue tetrazolium chloride
nsLTP1	Non-specific lipid transfer protein 1 from *Hordeum vulgare* (barley)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
p_F	Pressure of fluid
p_G	Pressure in the gas bubble
p_L	Laplace pressure
PM	Protein marker
p_R	Reservoir pressure
p_S	Skin pressure

PVDF	Polyvinylidenfluorid
rpm	Rounds per minute
SDS	Sodium dodecyl sulphate
SOC	Super optimal broth with catabolite repression
TCEP	Tris(2-carboxyethyl)phosphine
TEMED	*N,N,N',N'*-tetramethylethylendiamine
TFA	Trifluoroacetic acid
TMW	Technische Mikrobiologie Weihenstephan
Tricin	N-(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine
Tris	Tris(hydroxymethyl)aminomethane
x g	Gravity
YPD	Yeast extract peptone dextrose medium
YPDS	Yeast extract peptone dextrose medium with sorbitol
Γ	Skin compression

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1 Introduction

1.1 Gushing

Gushing is the spontaneous over-foaming of bottled carbonated beverages immediately upon opening without previous agitation and is a very unpleasant experience for customers. From a physical point of view, gushing is the result of an abrupt change of carbon dioxide from a dissolved form into a gaseous form. This phenomenon can be found in beer, champagne, cider, carbonated fruit juices from red berries and in carbonated water. It implicates negative influences on the image of affected companies. Beer however, is the most affected product from those mentioned above (Pellaud, 2002). The documented history of gushing goes back more than a hundred years. In 1909 Kastner described this phenomenon as "Das "Wildwerden" des Malzbieres" (Gushing of malt beer; German, *wildwerden* – to go wild). The problem of gushing should even be older and can be supposed to exist since carbonated beverages are filled and stored in bottles. With the upcoming industrial dimension of beer brewing in the first half of the 20th century the negative economical consequences of gushing were recognized and resulted in intensified research activities. Up to now this research goes on, indicating the trickiness of this problem. A first step towards handling this problem was done in 1963 by Gjertsen *et al.* who divided this unwanted property of beer into two groups by recognizing its origins in technological failure – known as secondary gushing – and in the quality of the raw material more precisely in the use of malt produced from mould infected grain – the primary gushing. This separation showed on the one hand that the causes of gushing are diverse and cannot be overcome by a single solution. On the other hand, it provided the possibility of a cause-oriented research leading to applicable solutions for secondary gushing. Nevertheless, research on solutions for primary gushing is still on its way to identify the gushing-inducing agents and mechanisms. A survey made in 1999 among German breweries showed that 54 % of the participating breweries had problems with gushing for at least one time (Kunert *et al.*, 2000). Further, the fact that 41 % of their sorts of beer were affected points to a considerable economical impact of gushing to the brewing industry.

1.1.1 Primary gushing

Hints for a malt related form of gushing, the primary gushing, can be found already in the year 1923. It was observed that the conversion of two different lots of malt into beer with identical brewing parameters resulted in gushing and non-gushing beer (Windisch, 1923). Helm and Richardt (1938) reported an epidemical occurrence of over-foaming of beer in

Denmark, Norway, the Netherlands, South America and the United States. Several other articles reporting outbreaks of gushing can be found in literature (Beattie, 1951; Nakamura, 1954; Casey, 1996). Since these gushing problems appeared in many breweries of a distinct region at the same time, the explanation could not lie in their processes, but rather in the raw material used. In the following years the reason for this type of gushing was supposed to be related to the presence of microorganisms on barley and wheat kernels. An examination of the common microflora found on barley grains revealed that a set of quality related changes in malt and beer are associated with the presence of these microorganisms. Those changes were demonstrated for wort nitrogen, malt α-amylase, diastatic power of malt and significant changes in beer gas stability. The screening revealed that mainly *Fusarium* isolates were able to cause this beer gas instability, also known as gushing (Prentice and Sloey, 1960; Sloey and Prentice, 1962). Investigations by Niessen *et al.* (1992) further confirmed fungal infections of grain as a source for the occurrence of gushing. In their studies they classified malt or grain samples using the gushing test by Donhauser (Donhauser *et al.*, 1990). Subsequently, the variety of fungal species found on gushing-positive and gushing–negative samples was compared. They identified *F. graminearum* as the major gushing inducer on barley and wheat processed in German breweries. Several other fungi present in these samples were also found to be gushing-inducing, among those *F. avenaceum*, *Microdochium nivale* and *Rhizopus stolonifer*. The relation between the occurrence of these fungi on kernels and gushing was verified by artificial infections of cereals on the field with *Fusarium* spp. as well as with artificial infections during malting with several fungi isolated from grains, including *Fusarium* spp.. In these expermiments *F. culmorum* also was found to hold a strong gushing-inducing potential, whereas other species of *Fusarium* possessed no or a low gushing-inducing potential. Other fungi able to induce gushing are described in literature, such as *Nigrospora* sp. (Kitabatake and Amaha, 1974), *Aspergillus* sp. (Gyllang and Martinson, 1976) *Alternaria* sp., *Stemphylium* sp., *Penicillium* sp., *Rhizopus* sp. (Amaha *et al.*, 1973). After the connection between the epidemical occurrence of gushing and the infections of cereals by fungi had been established, a multitude of consequences and new starting points concerning prospective research approaches were deduced. The phenomenon of gushing advanced from a problem of breweries to a problem of farmers, maltsters, traders and brewers. Research about the favorable conditions for the growth of moulds on grain revealed that warm and humid weather during the flowering seasons of barley and wheat promote infection with *Fusarium* sp. as well as some preceding crops do. In this context maize was found to have a strong negative influence (Bottalico and Perrone, 2002; Dill-Macky and Jones, 2000). The correlation between grain infection and favorable weather conditions leads to an increasing occurrence of infected barley and wheat lots in years with

humid and warm growing seasons and may explain the epidemical outbreaks of gushing. As a consequence, the acquisition of non-affected grains or malts becomes more difficult and more expensive for maltsters and brewers in typical gushing years. Therefore, primary gushing bears strong, negative effects on the image and the economical situation of the brewing industry.

1.1.2 Secondary gushing

Besides malt-related gushing, secondary gushing is caused by technological failure during the process of beer production as a result of introduction or generation of substances, which favor the release of carbon dioxide from the beverage. Substances most often causing secondary gushing were identified to be rough particles, which are able to retain micro gas bubbles attached to their surface. The list of triggers for secondary gushing described in the literature consists of calcium oxalate crystals (Zepf and Geiger, 1999), cleansing agent residues (Dachs and Nitschke, 1977), metal ions (Rudin and Hudson, 1958), residual filter aids (Zarnkow and Back, 2001) or rough surfaces in bottles (Rammert and Pahl, 1992). A major factor for induction of secondary gushing was found to be the presence of calcium oxalate crystals in beer bottles. The amount of such crystals can be decreased by precipitation via addition of calcium before the filtering step and reduction of calcium entry after filtration (Brenner, 1957). A method for the calculation of the necessary dosage of calcium was presented by Zepf and Geiger (1999). The possibility of elimination by directed, technological measures was found to be the common feature to all inducers of this type of gushing. Therefore, secondary gushing in these days plays a minor role in modern brewing technology.

1.2 Physical background for the phenomenon of gushing

1.2.1 Release of carbon dioxide from supersaturated solutions

Spontaneous over-foaming can only be observed in carbon dioxide containing beverages. This role of carbon dioxide in gushing, however, must not be misidentified, since it is not an inducer but the driving force for over-foaming. The higher the level of carbonation in a beverage is, the higher the gushing volume will be, if the beverage is susceptible for gushing. The source of this problem, however, has to be searched in the way how dissolved carbon dioxide is released from the beverage. Generally, CO_2 is released by diffusion of gas molecules from aqueous solution into the gas phase of bubbles or into the

head space of an open bottle of a carbonated beverage. Understanding the origin of those bubbles is a major step towards understanding gushing. According to Jones *et al.* (1999) the theory for the genesis of bubbles differentiates four types of bubble nucleation (Figure 1). Type I describes the classical homogeneous bubble nucleation in a homogeneous solution, which is defined as a solution without any particles or walls. There are no pre-existing gas cavities present and bubbles have to form due to supersaturation. Water, which is saturated with carbon dioxide at 100 atm, shows no bubble formation after a pressure release to 1 atm (Kenrick *et al.*, 1924). This indicates that a supersaturation driven form of gas release is very unlikely for a carbonated beverage, since those beverages are saturated at pressures below 10 atm. Type II represents the classical heterogeneous bubble nucleation. Bubble nucleation from this type takes place in systems containing particles and walls, but still no gas inclusions are present. After supersaturation bubbles may form in cavities of particles or barriers, or on smooth surfaces. Bubble nucleation at already existing bubbles in solution, on surfaces or in cavities is referred to as type III or pseudo-classical nucleation, if the radius of these pre-existing bubbles is less than the necessary radius for bubble growth r_{crit} (explained below). Type III nucleation at pending bubbles is followed by ascension of the bubbles to the surface of the liquid. For bubbles in cavities at the wall of the container type III nucleation can be followed by type IV, as they remain in the system. Pseudo-classical or type III nucleation happens at low levels of supersaturation, however, to overcome r_{crit} energy has to be expended. This is not the case for type IV or non-classical nucleation. Here, bubbles present in the system possess radii > r_{crit} and start growing as soon as the surrounding fluid is made supersaturated. They are a stable source for bubble formation.

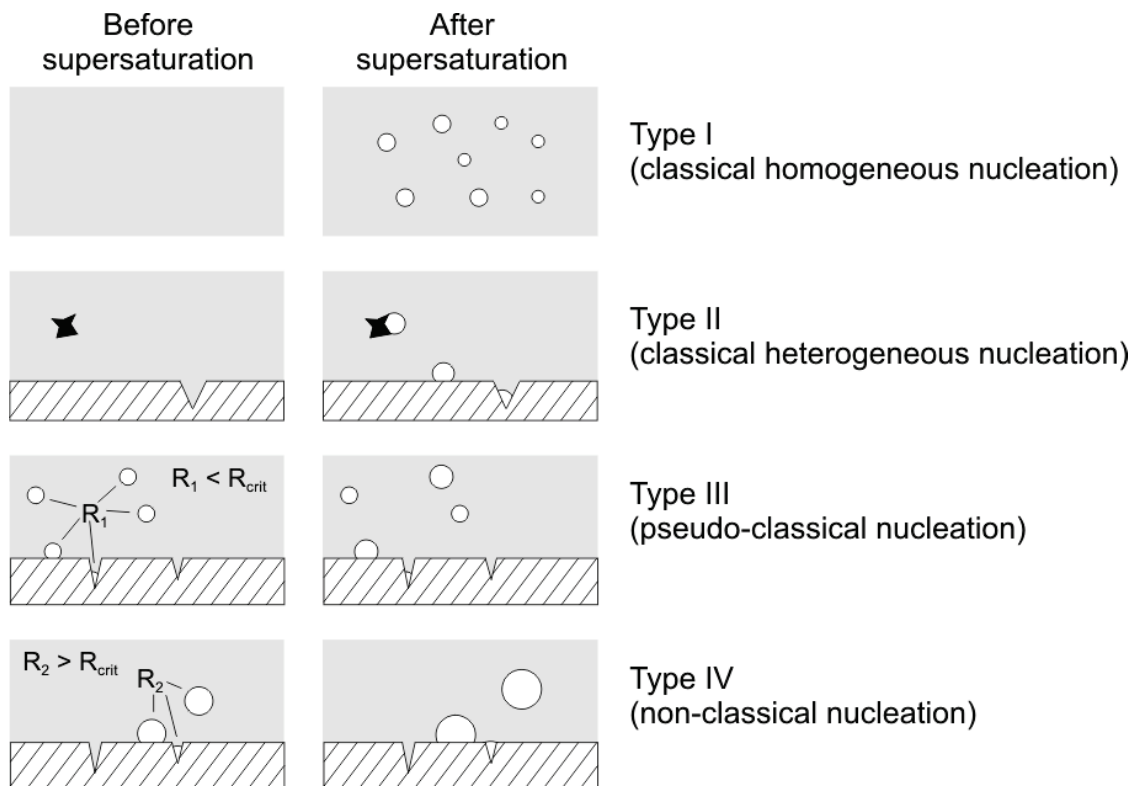

Figure 1: Ways of bubble nucleation according to Jones *et al.* (1999);

1.2.2 Processes upon opening of a carbonated beverage

Carbonated beverages in closed bottles are under pressure, which derives from carbon dioxide formation during fermentation, e.g. in beer and champagne, or from carbonation as applied in production of sparkling water or carbonated lemonades and fruit juices. The amount of carbon dioxide dissolved in the beverage is dependent on the pressure and the temperature of the aqueous system. The higher the pressure is, the more carbon dioxide can be dissolved. Concerning temperature the relation is reversed. The lower the temperature is, the more carbon dioxide can be dissolved in the liquid. As long as a bottle is closed, the dissolved carbon dioxide in the beverage and the gaseous carbon dioxide in the headspace above the liquid are in balance. After pressure release by opening, a thin layer of the liquid at the interface of gas and fluid is in equilibrium with the gas phase. In this layer the supersaturation is decreased instantly by diffusion of carbon dioxide into the air. For the rest of the beverage this balance is not generated immediately. The limiting factor for this is the diffusion of carbon dioxide to the surface. This results in a gradient of increasing CO_2 concentration with advancing distance to the interface maintaining supersaturation for almost the whole beverage. At the degree of supersaturation reached by opening of the bottle, only type III or type IV for bubble nucleation are probable. Meaning that gas release can only take place at pre-existing bubbles.

1.2.3 Stabilized microbubbles in aqueous fluids

Gas bubbles in aqueous liquids are the result of gas pressure (p_G), which pushes surrounding water molecules apart. This force has to overcome the pressure of the fluid (p_F) and the surface tension of the bubble – water interface expressed as Laplace pressure (p_L). The relation between bubble forming and bubble impeding parameters is given by the Laplace equation (Yount, 1982):

$$p_G = p_F + p_L = p_F + \frac{2\sigma}{r_{crit}} \quad (1)$$

From this equation it can be derived that bubbles with a radius > r_{crit} will increase, as the pressure in the bubble outweighs the pressure of the fluid and the surface tension. Those bubbles will grow and leave the system, since they ascend to the surface. For bubbles with radii < r_{crit} the surface tension will dominate, forcing the bubbles to shrink and collapse. Therefore, bubbles can only exist, if their radius is equal to the critical radius (Draeger, 1996). However, those meta-stable bubbles are very improbable, since Fischer (2001) concludes that in pure fluids at thermo dynamical equilibrium no bubbles can even exist. The latter statement, according to which no bubbles can exist, is not valid for beer, since this rule is valid only for pure liquids. Beer, however, contains about 8000 different ingredients, including surface-active compounds. Those surface-active ingredients interfere with bubbles by incorporation into the interface between gaseous phase of the bubble and surrounding aqueous phase. The occupation of the interface by those substances exerts a stabilizing effect on the bubble, as a compression of these molecules upon shrinking of the bubble opposes surface tension. In consequence the availability of bubble skins leads to the presence of a set of stable bubbles at various sizes, which often lose their spherical shape. Johnson and Cooke (1981) found this behavior by their observations made in seawater. They introduced bubbles in a sample of seawater and followed their behavior. Most of the bubbles dissolved completely due to surface tension, but several bubbles with sizes from less than 1 µm up to 13.5 µm remained. They assigned this observation to a diffusion-limiting or mechanical effect caused by compression of the surface films. Bubbles normally dissolve faster with decreasing radius, which is mainly triggered by the increasing surface tension. For example a typical surface tension for a 13.5 µm bubble is around 0.2 atm, for 0.1 µm bubble 3 atm. After application of pressure they found, that some of these bubbles collapsed and left streaks at the positions of the destroyed bubbles. The authors assigned these streaks to the material, which was incorporated in the bubble skin before collapsing. Yount (1982) described the processes in the bubble skin and the presence of stabilized gas nuclei by his "Varying Permeability Model" and proved this theory with experiments in supersaturated gelatine. He extended the Laplace equation by a

term for surface pressure (p_S), whereas skin compression Γ is dependent on substance and thickness of the bubble skin.

$$p_G + p_S = p_G + \frac{2\Gamma}{r} = p_F + \frac{2\sigma}{r} \quad (2)$$

He outlined his model as shown in Figure 2 and described the bubble layout and the pressure course near the bubble interface. Next to the bubble skin there is an adsorbed reservoir of additional surface-active substances. In this layer the pressure reaches it maximum, expressed as surface tension (Laplace pressure) plus system pressure for the outside and surface pressure plus gas pressure for the inside.

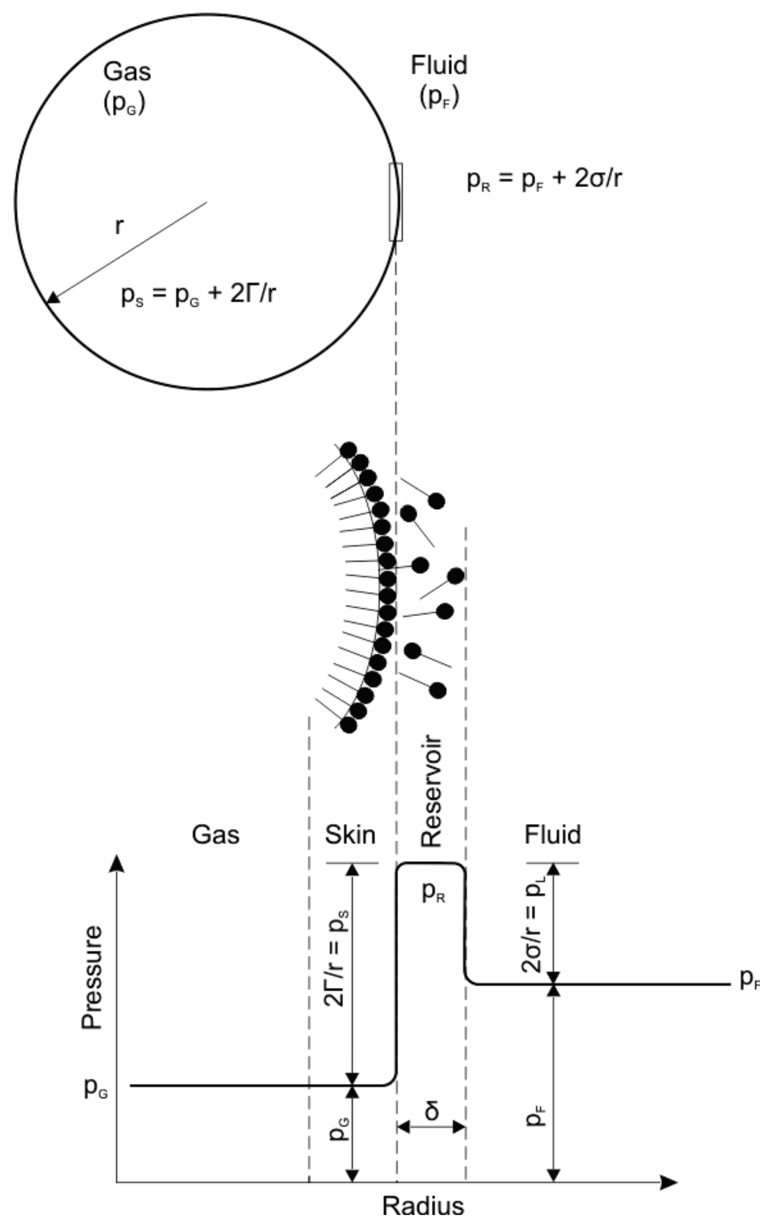

Figure 2: Structure and pressure course of a gas bubble according to Yount (1982);

Next to different pressure conditions between bubble and surrounding liquid, the concentration of carbon dioxide also has to be considered, since it is the driving force for CO_2 diffusion of into the bubble. In supersaturated carbonated beverages the gas concentration in the bulk liquid is higher as compared to the bubbles, generating a concentration gradient. The course of gas concentration in close proximity to a carbon dioxide bubble is shown in Figure 3.

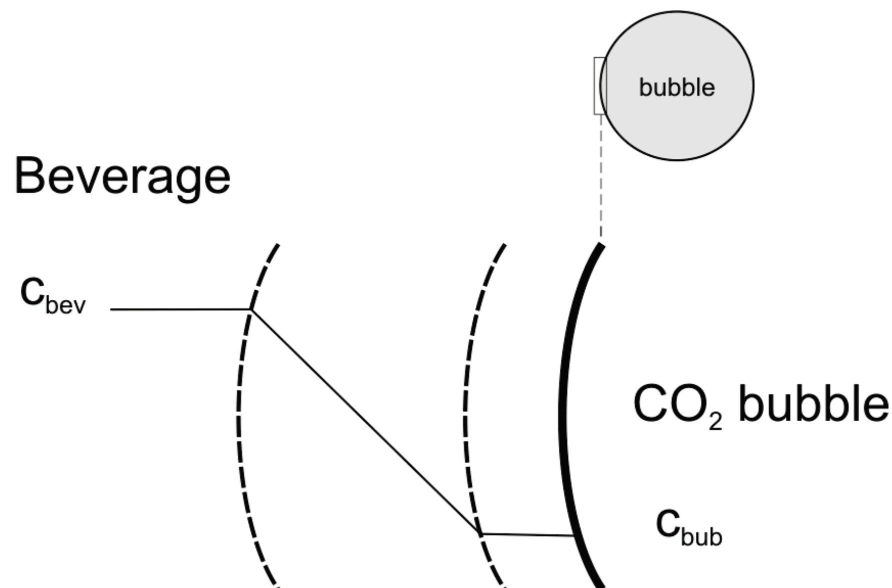

Figure 3: Course of carbon dioxide concentration in the close neighborhood of a gas bubble according to Liger-Belair *et al.* (2008), c_{bev} = carbon dioxide concentration in the bulk liquid of the beverage, c_{bub} = carbon dioxide concentration in the bubble;

1.2.4 Composition of the bubble skin

The composition of the bubble skin is dependent on surface-active molecules present in the surrounding fluid. The availability of those substances in foam positive beverages, e. g. beer, is higher than in beverages, which show no foam formation upon opening and pouring, e. g. sparkling water. A further aspect is the number of different kinds of surface-active compounds, which are able to occupy the interface between bubble and adjacent aqueous phase, and their ability to interact with each other. Depending on the presence of such compounds, two types of bubble skins are conceivable (Figure 4). Single-domain bubble skins consist of a single type of physically and chemically similar surface-active compounds. The amphiphilic molecules are arranged homogeneously throughout the bubble surface. Multi-domain bubble skins are occupied by two or more different types of

surfactants, which are not able to interact with each other. They form domains, each consisting of only a single type of molecules.

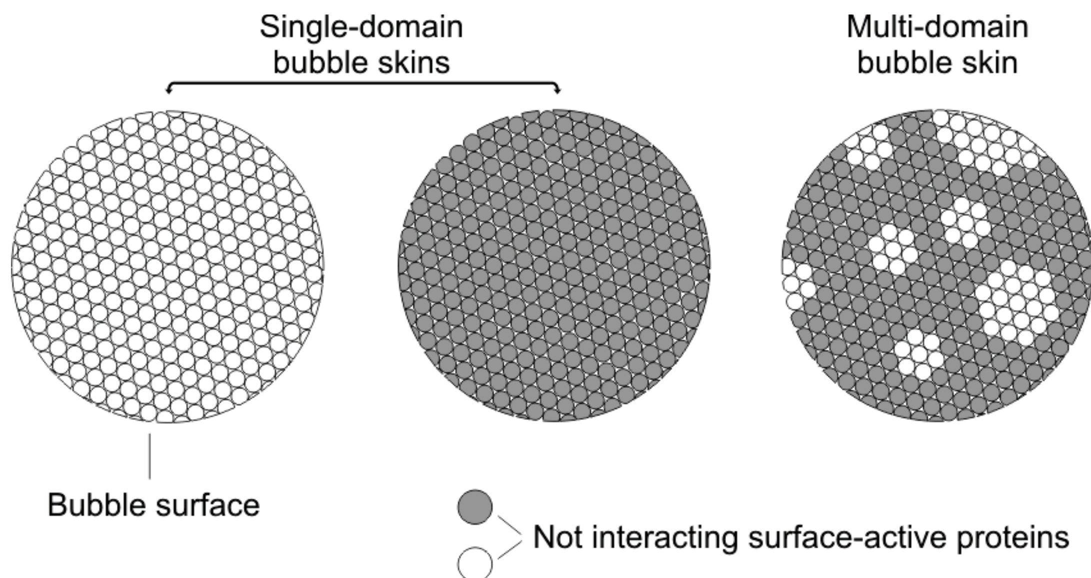

Figure 4: Conceivable compositions of the bubble skin in foam-positive carbonated beverages;

1.2.5 Bubble growth

Once a bubble has overcome the critical radius, it is able to grow and absorb gas from surrounding liquid. The growth of such bubbles is a function of several factors i.e. molecular diffusion rate of gas to the bubble interface, inertia of the fluid, liquid viscosity and surface tension. Among these factors the molecular diffusion most probably has the largest influence for the main growing phase of a bubble. Parameters for the initial growing phase, however, are not yet fully understood (Jones *et al.*, 1999). The velocity of bubble ascension in fluids according to the Stokes velocity is next to the bubble radius dependent on the density and the viscosity of the liquid phase. However, this velocity is proportional to the second power of the radius, which lends a strong impact on the velocity of rise to the bubble size. Another influencing factor is the surface quality of the bubble. Protein loaded bubbles ascend slower than unloaded. This is caused by molecular friction. Loaded bubbles behave like a spherical, solid body, while partially loaded or unloaded surfaces exhibit a higher ascension velocity and tend to deform. Along with the friction comes a shift of surface-active molecules towards the lower part of the bubble ("Marangoni-effect"). This effect leads to a rigidification of the bottom part of the bubble and to a further reduction of the velocity of rise. During growth the bubble surface increases, giving room for more surface-active substances. Experiments in beer showed that growing bubbles are fully

covered with surface-active molecules during the entire process of growth, since they behave like solid particles. This rapid filling up of the bubble skin in beer can be assigned to the high content of surface-active substances in the commodity (Liger-Belair, 2005).

1.2.6 Prerequisites for the generation of gushing

The rules derived from the physical background lead to the assumption of some practical prerequisites for the occurrence of gushing. Gushing will not be observed, if there are no microbubbles present in the beverage before opening, since in this case there are no nucleation sites, at which carbon dioxide can be released from the solution. Therefore, those bubbles can be assumed to have been introduced previously into a beer, which tends to gush. This can happen by an inappropriate design of flow pathways in the manufacturing process. If there process steps exist, in which the beverage is mixed thoroughly with air or carbon dioxide, bubbles can be formed and remain in the product after sealing of the bottle. Another possibility of incorporating bubbles is by shaking or turning of the bottled product. In doing so the gas phase of the head space is led through the liquid several times. Bubbles in beer formed in this stage are immediately covered by surface-active compounds and may thus be prevented from collapsing. From this point of view, the phenomenon that horizontally stored bottles hold a higher gushing tendency than vertically stored bottles is comprehensible. Ilberg (2008) observed that carbonated wort produced from a gushing malt led to heavy foam formation after pressure release when previously shaken. Such wort could not be filled into bottles. In contrast, bottles, which are completely filled with beverage leaving no headspace, will never show gushing (Gjertsen *et al.*, 1963). The sole presence of stabilized microbubbles, however, is not a safe sign for the gushing tendency of beer, as in conventional non-gushing beer about 2500 micro bubbles per milliliter can be found (Curtis and Martindale, 1961). It remains to be elucidated whether the number or the quality of stabilized micro bubbles is the decisive factor for the occurrence of gushing. Fischer (2001) reported that over-foaming in beverages is dependent on the diameter, at which bubbles are stabilized. According to the author, bubbles with radii greater than the critical radius r_{crit} can grow after supersaturation, whereas bubbles with smaller radii are not able to grow due to the dominating forces of surface tension and pressure of the surrounding liquid. This means that in gushing beer the number of bubbles capable of growing must be assumed to be increased as compared to non-gushing beer.

1.3 Factors causing primary gushing

Previous research on triggers of primary gushing mainly focused on the isolation and identification of single compounds. Some studies used pure cultures of fungal species known to be associated with cereal grains, which were fractionated and screened for their gushing behavior. Other studies used wort prepared from gushing malt for the same purpose. Positive samples were further separated and analyzed, resulting in a list of gushing-inducing compounds, which can be found in literature. These substances mainly were of proteinaceous nature and comprised peptides (Amaha *et al.*, 1973; Kitabatake, 1978; Kitabatake *et al.*, 1980), peptidoglycans (Amaha *et al.*, 1973) and highly amphiphilic proteins (Kitabatake and Amaha, 1974). Although many of these findings were made in the seventies of the last century already, primary gushing still is a problem in the beverage industry. One reason for this is that in most approaches primary gushing was not understood as a multi causal problem with several interfering factors. Another reason is that the interaction between plant and fungi was not fully considered in those studies. First investigations in this direction were done by Gjertsen *et al.* (1965) who showed that gushing in laboratory scale only occurred, when living fungal mycelia and germinating cereal grains were brought together. This indicated that an interaction between plant and fungus was necessary as a prerequisite for the formation of gushing. A promising new way to address the mechanisms and inducing agents for primary gushing was an interdisciplinary approach, in which the role of both the plant and fungi was considered. With this approach the behavior of the fungus and the reactions of the affected plant upon infection were studied. It was considered for the first time that not only production of substances during fungal growth but also the degradation of substances during the malting and brewing process as well as the storage of beer may be responsible for the occurrence of gushing (Hippeli and Hecht, 2008). Two groups of proteins, which were discovered and described in the nineties of the last century, came into the focus for an involvement in gushing induction: The *non specific lipid transfer proteins* (nsLTPs) from barley or wheat, and the hydrophobins as a group of proteins secreted by filamentous fungi. Both groups of compounds comprise highly surface-active proteins, which can be found in beer and are therefore potential candidates for an involvement into gushing induction.

1.3.1 Surface-active proteins from fungi

The described gushing factors, which were found in the past, mainly were of proteinaceous character. Since non-amphiphilic proteins are not able to integrate into the bubble skin, methods were developed to isolate surface-active proteins from fungi or cereals. One of those methods is the concentration of surfactants in foams by bubbling air or gas through

an aqueous solution. With this technology, also a new class of fungal surface-active proteins was detected, the fungispumins. However, hydrophobins are an even more important group of proteins possessing an extremely high surface activity. These proteins have been the subject of several studies concerning gushing in the past ten years and will therefore be described in more detail.

1.3.1.1 Hydrophobins

Hydrophobins are small, extremely surface-active proteins, about 10 kDa in size. They play structural roles in the growth of filamentous fungi. This group of proteins was first described by Wessels *et al.* (1991). In their studies the authors found a family of abundantly expressed homologous genes coding for cysteine-rich proteins. These proteins were involved in the formation of aerial structures and fruit bodies of the basidiomycete *Schizophyllum commune*. The authors reported that the hydrophobins were secreted in large amounts by submerged hyphae and could be found as insoluble complexes in the cell walls of aerial structures. These highly stable complexes were detected by the use of radioactive S^{35}, taking advantage of the high content of cysteine residues. Up to now several hydrophobins from basidiomycetes and ascomycetes were identified, but Sc3 from *S. commune* (de Vries et al., 1993; Martin *et al.*, 2000; Stroud *et al.*, 2003; Wessels *et al.*, 1991), Hfb1 and Hfb2 from *Trichoderma reesei* (Askolin *et al.*, 2005; Kisko *et al.*, 2008, 2009; Nakari-Setälä *et al.*, 1997; Sarlin *et al.*, 2005; Torkkeli *et al.*, 2002) may be the best characterized of this group of proteins. A noticeable feature, which all known hydrophobins have in common, is the presence of eight cysteine residues at conserved positions. These residues are arranged in such a way that the second cysteine is always a direct neighbor to the third and the sixth next to the seventh (Linder *et al.*, 2005). These cysteine residues were found to form only intramolecular bridges and therefore are not contributing to the high stability of the observed aggregates (de Vries *et al.* 1993). The remaining cysteine residues are positioned in greater distances to each other. Alignment of amino acid sequences of several known hydrophobins along the conserved cysteine residues led to a division of hydrophobins into two groups by comparing the clustering of their hydrophilic and hydrophobic domains. Class I represents hydrophobins, which are able to form highly insoluble aggregates. Such assemblies can be found in fungal cell walls of submerged mycelia or form in culture media upon shaking. They could not be solubilized in hot 2 % SDS. However, those aggregates were susceptible solubilization in cold 100 % formic acid or cold 100 % trifluoroacetic acid. Class II hydrophobins are also able to form aggregates, but these can be solubilized in water or aqueous alcohol (Wessels, 1994). Studies of the class II hydrophobin Hfb2 revealed that its secondary structure contains a central β-sheet

formed by two β-hairpin loops. These loops cover most of the hydrophobic part of the protein, whereas an α-helix represents the hydrophilic domain. Another fact, which separates hydrophobins from other protein groups is the high content of hydrophobic amino acids at the surface of the folded protein. Normally hydrophobic components are turned inside forming a hydrophobic core, which provides a stabilizing effect for the protein. This stabilization in case of hydrophobins is accomplished by four disulfide bridges, which stretch the whole protein. The presence of many non-polar amino acids at the outside, which form a hydrophobic domain on the otherwise hydrophilic surface, leads to an enormous high surface activity of these proteins. Hydrophobins are among the proteins with the highest amphiphilic character known (Linder *et al.*, 2005). This property is related to a main function, which is ascribed to hydrophobins. They are able to considerably lower the surface tension at interfaces between aqueous solutions and air. In doing so, they enable the fungal mycelium to cross that barrier and to escape from submerged medium into the air. In the spatial dimension of fungal cells, surface tension becomes immensely high as compared to gravity, which can be neglected at this scale. The amphiphilic nature of hydrophobins and the ability to form highly ordered and highly stable aggregates at interfaces can be utilized in technical applications by altering the properties of surfaces, to which they were applied. For example, hydrophobins are applied in the improvement of biocompatibility of implant materials used in medical applications, as anchors in the production of protein-biochips or in the modification of electrodes (Janssen *et al.*, 2002, Janssen *et al.*, 2004, Qin *et al.*, 2007, Bilewicz *et al.*, 2004).

Apart from their application in creating new bio-surfaces, there is a chain of hints, which point to an involvement of hydrophobins in the induction of gushing in beer. Amaha and coworkers identified and produced a gushing factor for beer from a culture of *Nigrospora* sp.. They found that this factor was acidic, had a molecular weight of 16.5 kDa and possessed 16 cysteine residues (Amaha *et al.,* 1973, Kitabatake and Amaha, 1974, 1977). Due to its similar chemical and molecular properties, Hippeli and Elstner (2002) assumed that this factor is an aggregate of two hydrophobin molecules. Weideneder (1992) isolated uncharacterized acidic proteins with a molecular weight of about 10 kDa from wheat, which was strongly contaminated with *F. culmorum*. These proteins were able to induce gushing and have features, which resemble hydrophobins. Further evidence for the involvement of hydrophobins in beer gushing were found by several other authors (Haikara *et al.*, 1999, Kleemola *et al.*, 2001, Sarlin *et al.*, 2005). They observed that the addition of hydrophobins from *T. reesei*, *F. poae* and *Nigrospora* sp. to bottled beer led to gushing (Sarlin *et al.*, 2005). Not only was the resulting gushing volume correlated to the protein amounts added, but also it depended on the source, from which the hydrophobin had been isolated. This means that not all hydrophobins had the same potential to induce gushing. In 2006 Zapf *et*

al. described the production of transformant brewing yeast strains, which produced the class I hydrophobin FcHyd3p or the class II hydrophobin FcHyd5p from *F. culmorum* during wort fermentation. They found that gushing only occurred, if the class II hydrophobin producing yeast was used. The clone transformed with the gene coding for the class I hydrophobin did not lead to a gushing beer.

1.3.1.2 Fungispumins

In search of non-hydrophobin surface-active proteins produced by *F. culmorum,* the new class of fungal proteins, fungispumins, was detected by Zapf *et al.* (2007). They isolated two new surface-active proteins from a liquid culture of *F. culmorum*. For isolation they enriched the fraction of surface-active proteins from cell free culture supernatant using nitrogen, which was led into the supernatant through a glass frit. In the generated foam they found a 17 and 20 kDa protein, which were structurally related to hypothetical proteins XP_384911.1 and XP_388298 (UniProtKB) identified in the genomic sequence of *Gibberella zeae* (anamorph: *F. graminearum*). These proteins were named AfpB (17 kDa protein, GenBank Accession DQ336149) and AfpA (20 kDa protein, GenBank Accession DQ336148). Analysis revealed a strong alkaline character of the proteins with an isoelectric point around 9. AfpA and AfpB showed properties similar to hydrophobins, i.e. their ability to form aggregates, their surface activity and the fact that they were prepared with methods also used for isolation of hydrophobins. However, they were not part of this group of proteins. Gushing experiments with both proteins revealed that they could not be connected with the induction of gushing. However, it was found that AfpA is able to increase the over-foaming volume in gushing beer. AfpB proved to have only a very low influence on such beers. Immunochemical analysis of malts artificially infected with *F. culmorum* and *F. graminearum* confirmed the presence of AfpA in malts, which were tested gushing positive, whereas in gushing negative malts no AfpA was found.

1.3.2 Surface-active proteins from plants

Two major surface-active proteins can be found in beer. Both nsLTP1 and the Z-proteins are highly important for the formation and stability of beer foam after pouring (Sørensen *et al.*, 1993). Although these proteins can be found in any beer made from barley, a hypothesis discussing that nsLTP1 is involved in the formation of gushing was published previously (Hippeli and Elstner, 2002). The authors assumed that elevated levels of nsLTP1 or modifications of this protein are inducers of gushing in beer. An up regulation of

ltp genes due to reactions of barley to fungal infections or adverse growth conditions was described by García-Olmedo *et al.* (1995). This complies with the mentioned hypothesis and also with the epidemical type of occurrence of gushing. However, immunochemical characterization of gushing-positive and gushing-negative beer samples disproved this hypothesis. The results obtained were much in contrast to the assumptions made, because it showed that gushing beer had a lower nsLTP1 content as compared to stable beer. From their findings they set up an alternative hypothesis, which ascribed the occurrence of gushing to the presence of highly surface-active degradation products of nsLTP1 (Hippeli and Hecht, 2008). A proof for the presence of such amphiphilic fragments in gushing beer, however, has not been given yet.

The nsLTP1-protein belongs to the albumin fraction of barley proteins. It has a molecular weight of 9,7 kDa and an isoelectric point of 8.2. Its secondary structure contains four α-helices and it is stabilized by eight cysteine residues forming four disulfide bridges (Heinemann *et al.*, 1996). Its physiological function is not fully clear, but it has been associated with processes of lipid transport in living plant cells but is also involved in the plant´s defense reactions against microbial infections (Breu *et al.*, 1989, Terras *et al.*, 1992). In its native state the protein is hardly susceptible to proteases and shows no surface activity. After denaturation and glycosylation during the brewing process, however, the protein attains foam promoting properties and becomes digestable by proteases (Jégou *et al.* 2001, Leisegang and Stahl, 2005). Therefore, LTP has also been termed a protease inhibitor in the literature (Jones, 2005; Davy *et al.,* 1999).

1.4 Influence of hop compounds on gushing

The reducing effect of hop on gushing is well known since long time. Curtis *et al.* (1961) found that over-foaming of beer can be prevented by the use of hop when applied in sufficient amounts. This effect, however, cannot be generally ascribed to all the compounds present in hops, since some hop components are known to have gushing enhancing properties. It is reported that dehydrated humulinic acid (DHA) is a strong gushing promoter. It can occur during production of iso-extract under alkaline conditions. Further compounds with gushing enhancing properties have been described as being oxidation products of iso α-acids (Laws and McGuinness, 1972) and reduced isohumulones, addition of which to beer was found to be a means of gushing induction (Carrington *et al.*, 1972). Among gushing suppressants or inhibitors, hop oils and unsaturated lipids from hops are described as very effective. In his experiments with gushing inhibitors, Gardener *et al.*

(1973) observed complete gushing inhibition after addition of hop oil to a gushing beer. Next to hop oils also iso-α-acids are known to have gushing inhibiting properties. Despite the possibility of negative as well as positive influences on gushing in beer, it is generally assumed that increasing amounts of hop lead to a reduction of the gushing potential of beer (Hanke *et al.*, 2010).

1.5 Aim of the study

The occurrence of primary gushing has been related to fungal infections of barley or wheat especially with species from *Fusarium* as well as to the presence of fungal surface-active proteins. It is accepted that the causes of this type of gushing are various and that over-foaming in this context is the result of a coincidence of several factors. However, the number and the identity of these factors are not known as well as the circumstances, which lead to this unwanted phenomenon. Therefore, the aim of the present study was, to establish a system that provides the possibility to evaluate the contribution of single surface-active proteins to gushing. It should also enable to assess the influence of process parameter, such as heat treatment, or the influence of other substances present in beer, such as hop compounds or surface-active proteins, on gushing-inducing agents. This should be accomplished by heterologous expression of surface-active proteins from *F. culmorum* and *F. graminearum*, both well known plant pathogens and gushing-inducing fungi.

The genes coding for the class II hydrophobin FcHyd5p, the class I hydrophobin FcHyd3p, both extremely surface-active proteins from *F. culmorum*, and for AfpA, a fungispumin from *F. graminearum* should be transferred into *P. pastoris* as a host for protein production and isolation protocols should be developed. The isolation procedures should provide access to quantities of purified protein sufficient for application in gushing experiments in carbonated beverages. In these tests crucial requirements for a gushing-inducing agent such like surface activity or heat stability as well as the influence of hop components should be investigated. For comparison a second class II hydrophobin, Hfb2 from *T. reesei*, a fungus, which is not known as plant pathogen, should also be heterologously expressed in *P. pastoris* and its influence on carbonated beverages tested.

The second aim of this work was, to study the influence of nsLTP1 from barley on carbonated beverages, since this protein is also involved in the current discussion on gushing-inducing compounds. Therefore an isolation method for native nsLTP1 from barley should be developed, which provides access to quantities sufficient for an application in gushing experiments. After isolation nsLTP1 should be added to carbonated beverages including previous heat treatment to examine its influence on the gas release in these systems.

2 Materials and methods

2.1 Materials

2.1.1 Instrumentation

Equipment	Type	Manufacturer
1D-gel electrophoresis	Mini Protean III Cell	Bio-Rad Laboratories, Hercules, CA, USA
Anion exchange column	HiLoad 16/10 Q Sepharose High Performance	GE Healthcare, Bio-Sciences AB, Uppsala, Sweden
Autoclave	2540 ELV	Systec GmbH, Wettenberg, Germany
Autoclave	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Büchner funnel	Ø 70 mm	Haldenwang, Berlin, Germany
Centrifuge	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
Centrifuge	Lab centrifuge J-6	Beckman, Palo Alto, CA, USA
Centrifuge	Lab centrifuge J-2	Beckman, Palo Alto, CA, USA
Centrifuge	Lab centrifuge Hermle Z 383 K	Hermle Labortechnik, Wehningen, Germany
Clean air safety cabinet	Hera Safe	Heraeus, Hanau, Germany
FPLC	Biologic HR	Bio-Rad Laboratories, Hercules, CA, USA
Fraction collector	Model 2128	Bio-Rad Laboratories, Hercules, CA, USA
Freeze dryer	L05-60	WKF, Modautal, Germany
Hydrophobic interaction chromatography column	HiLoad 16/10 Phenyl Sepharose High Performance	GE Healthcare, Bio-Sciences AB, Uppsala, Sweden
Homogenisator	Miccra D-8	ART Prozess- & Labortechnik, Mühlheim, Germany
Jet pump	Metal work	HAAKE, Berlin/Karlsruhe, Germany
Magnetic stirrer	RCT-Basic	Mettler-Toledo, Gießen, Germany
Mechanical shaker	Certomat	R B. Braun Biotech International, Melsungen, Germany
Microtiter plate-reader	TECAN SPECTRAFluor	TECAN Deutschland GmbH, Crailsheim, Germany
Mill	DFLU disc mill	Bühler, Braunschweig, Germany
pH meter	Knick pH 761 Calimatic	Knick elektronische Geräte, Berlin, Germany

pH-electrode	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany
Pipettes	Pipetman (2 µL, 20 µL, 100 µL, 200 µL and 1000 µL	Gilson-Abimed, Langenfeld, Germany
Rotator	Modified cement mixer (ATIKA 130)	ATIKA, Ahlen, Germany
Scanner	Epson Expression 1600	Epson, Meerbusch, Germany
Semi-dry blotter	HEP-1	peqLab, Erlangen, Germany
Ultrasonic bath	Sonorex	Bandelin, Berlin, Germany
Ultrasonic probe	Sonoplus	Bandelin, Berlin, Germany

2.1.2 Consumables

Product	Type	Manufacturer
Amicon Centrifugal Filter Units	MWCO 5kDa and 30kDa	Millipore, Billerica, MA, USA
Blotting paper	-	Munktell, Bärenstein, Germany
Centrifugation tubes	15 and 50 mL	Sarstedt, Nümbrecht, Germany
Dialysis tubes	MWCO 3500 Da, Ø 16 mm	Serva, Heidelberg, Germany
Gauze bandage	-	DM Drogerie Markt, Karlsruhe, Germany
Hybond™ ECL™ nitrocellulose membrane	-	Amersham pharmacia biotech, AB, Uppsala, Sweden
Immuno-Blot™ PVDF membrane	0.2 µm	Bio-Rad Laboratories, Hercules, CA, USA
Parafilm®	-	Pechiney Plastic Packaging, Menasha, Wi, USA
Proteinmarker (PM)	Unstained SDS PAGE Protein Marker 6.5 - 200 KDa, liquid mix	Serva, Heidelberg, Germany
Proteinmarker (PM2)	PageRuler Prestained Protein Ladder	Fermentas, St. Leon-Rot, Germany
Reaction tubes	200 µL, 1,5 and 2,0 mL	Eppendorf, Hamburg, Germany
Steril filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany

2.1.3 Kits

Kit	Type	Manufacturer
Bio-Rad Protein Assay	Bradford test	Bio-Rad Laboratories, Hercules, CA, USA
E.Z.N.A. Cycle Pure Kit	PCR purification kit	Omega bio-tek, Norcross, GA, USA
EasySelect™ *Pichia* Expression Kit	Expression kit	Invitrogen, Carlsbad, USA

Lumitein™ Protein Gel Stain		Biotium, Hayward, CA, USA
peqGOLD gel extraction kit	DNA isolation kit	peqLab, Erlangen, Germany
peqGOLD plasmid miniprep kit	Plasmid isolation kit	peqLab, Erlangen, Germany
PrestoSpin D	DNA isolation kit	Molzym, Bremen, Germany
RNeasy Plant Mini Kit	RNA isolation kit	Qiagen, Hilden, Germany
YeaStar RNA Kit™	RNA isolation kit	Zymo Research, Orange, CA, USA
YeaStar™ Genomic DNA Kit	DNA isolation kit	Zymo Research, Orange, CA, USA

2.1.4 Organisms

The following list contains the organisms, which were involved in establishing of recombinant yeast. Next to gene donors and acceptors the transformed yeast strains are mentioned. For all organisms, which are stored in the strain collection of the Lehrstuhl für Technische Mikrobiologie, Weihenstephan, their related TMW number is given in the right column.

Organism	**TMW-No.**
Escherichia coli TOP 10	2.580
Fusarium graminearum	4.0157
Hordeum vulgare cv. Marnie	-
Pichia pastoris X33	3.177
Pichia pastoris X33 [pPICZαA-AfpA]	3.217
Pichia pastoris X33 [pPICZαA-FcHyd3]	3.218
Pichia pastoris X33 [pPICZαA-FcHyd5]	3.213
Pichia pastoris X33 [pPICZαA-Hfb2]	3.219
Pichia pastoris X33 [pPICZαA-nsLTP1]	3.214

2.1.5 Enzymes

All restriction enzymes used were FastDigest enzymes. As all genes were transferred using two different restriction sites, restriction enzymes were applied pair wise at 37°C for 30 minutes. Other enzymes were applied as described in the corresponding experiment.

Product	**Manufacturer**
Age1 (BshT1) FastDigest	Fermentas, St. Leon-Rot, Germany

DNAse	Promega, Madison, WI, USA
EcoR1 FastDigest	Fermentas, St. Leon-Rot, Germany
Reverse transcriptase M-MLV RT H(-) Point Mutant	Promega, Madison, WI, USA
Sac1 FastDigest	Fermentas, St. Leon-Rot, Germany
T4 DNA ligase	Fermentas, St. Leon-Rot, Germany
Taq Polymerase	MP Biomedicals, Illkirch, France
Xba1 FastDigest	Fermentas, St. Leon-Rot, Germany

2.1.6 Chemicals

Substance	Purity	Manufacturer
$(NH_4)_2SO_4$	pure, if taken for precipitation, p.a., if used for media	Merck, Darmstadt, Germany
5-Bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP)	-	Gerbu Biotechnik GmbH, Gailberg, Germany
Acetic acid (99-100 %)	HPLC-grade	Mallinckrodt Baker B.V., Deventer, The Netherlands
Acrylamide/Bis 37,5:1	electrophoresis grade	Bio-Rad Laboratories, Munich, Germany
Agar	European Agar	Difco, BD Biosciences, Heidelberg, Germany
Agarose	Biozym LE Agarose	Biozym Scientific GmbH, Hess. Oldendorf, Germany
$AgNO_3$	ultra pure	Merck, Darmstadt, Germany
Ammonium persulfate	electrophoresis grade	Serva, Heidelberg, Germany
Anti-rabbit-IgG antibodies from goat	-	DAKO Cytomation, Hamburg, Germany
Biotin	Labeling grade	Gerbu Biotechnik GmbH, Gailberg, Germany
Bovine serum albumine (BSA) Fraktion V	for biochemical purposes	Merck, Darmstadt, Germany
Bromophenol blue, Na-salt	for electrophoresis	SIGMA–Aldrich, Steinheim, Germany
CAPS	-	SIGMA–Aldrich, Steinheim, Germany
Citric acid	Biochemika Ultra 99,5 %	SIGMA–Aldrich, Steinheim, Germany
Coomassie-Blue	R350 PhastGel™ Blue R	Amersham Pharmacia Biotech AB, Uppsala, Sweden
D(+)-Glucose monohydrate	for microbiology	Merck, Darmstadt, Germany
DL-Lactic acid	Ph. Eur.	Merck, Darmstadt, Germany
DL-Malic acid	Ph. Eur.	Merck, Darmstadt, Germany

DTT	high purity, for molecular biology	Gerbu Biotechnik GmbH, Gailberg, Germany
Ethanol, absolute	HPLC-grade	Mallinckrodt Baker B.V., Deventer, The Netherlands
Ethanol, denatured	99 % denatured with 1 % methyl ethyl ketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
EDTA	For molecular biology	SIGMA–Aldrich, Steinheim, Germany
Formaldehyde, 36,5-37 %	for molecular biology	SIGMA–Aldrich, Steinheim, Germany
Formic acid	98-100 %, p.a.	Merck, Darmstadt, Germany
Fructose	for microbiology	Merck, Darmstadt, Germany
Glycerol, 87 %	p.a.	Gerbu Biotechnik GmbH, Gailberg, Germany
Glycerol, waterfree	BioChemika Ultra 99,5 %	SIGMA–Aldrich, Steinheim, Germany
H_3PO_4, 85 %	p.a.	Merck, Darmstadt, Germany
HCl,	Ph. Eur.	Merck, Darmstadt, Germany
Hop oil type dry	-	Hopsteiner, Mainburg, Germany
Imidazole	for biochemical use	SIGMA–Aldrich, Steinheim, Germany
Isopropanol	-	Mallinckrodt Baker B.V., Deventer, Netherlands
K_2HPO_4 x $3H_2O$	p.a.	Merck, Darmstadt, Germany
KCl	p.a.	Merck, Darmstadt, Germany
KNO_3	p.a.	Merck, Darmstadt, Germany
Maltose	for microbiology	Merck, Darmstadt, Germany
Maltodextrin		FLUKA, Steinheim, Germany
Methanol	HPLC-grade	Mallinckrodt Baker B.V., Deventer, The Netherlands
$MgCl_2$ x 6 H_2O	p.a.	Merck, Darmstadt, Germany
$MgSO_4$ x 7 H_2O	p.a.	Merck, Darmstadt, Germany
N,*N*-Dimethylformamide	LAB	Merck, Darmstadt, Germany
Na_2CO_3	p.a.	Merck, Darmstadt, Germany
$Na_2S_2O_3$ x 5 H_2O	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
NaOH	p.a.	Merck, Darmstadt, Germany
Nitro blue tetrazolium chloride (NBT)		Gerbu Biotechnik GmbH, Gailberg, Germany
Pyruvic acid	p.a.	FLUKA, Steinheim, Germany
Rho 35 %	-	Hopsteiner, Mainburg, Germany
Roti®Blue, 5 x concentrated	Colloidal CBB G-250	Carl Roth GmbH & Co, Karlsruhe, Germany

Sodium dodecyl sulphate (SDS)	research grade	Serva, Heidelberg, Germany
Sucrose	for microbiology	Merck, Darmstadt, Germany
TEMED	p.a.	Merck, Darmstadt, Germany
Tetra 10 %	-	Hopsteiner, Mainburg, Germany
Tricine	p.a.	Merck, Darmstadt, Germany
Trifluoracetic acid	p.a.	Merck, Darmstadt, Germany
Tris	ultra pure	ICN Biomedicals, Inc., Ohio, USA
Tris(2-carboxyethyl)phosphine HCl	-	TCI Europe, Eschborn, Germany
Tris-HCl	p.a.	Merck, Darmstadt, Germany
Tween 20	Ph. Eur.	Merck, Darmstadt, Germany
Water	deionized	Lab equipment
Yeast extract	-	BactoR Difco, BD Biosciences, Heidelberg, Germany
Yeast Nitrogen Base	-	BactoR Difco, BD Biosciences, Heidelberg, Germany
Zinc acetate	99.99%	SIGMA–Aldrich, Steinheim, Germany

2.1.7 Buffers and solutions

2.1.7.1 TAE buffer (50x)

(0.1 M EDTA; 2.0 M Tris; 1.0 M Acetic acid; pH 8.2)

242.2 g	Tris dissolved in 700 mL of deionized water
57.1 mL	Acetic acid (100 %); Add carefully!
200 mL	EDTA (0.5 M, pH 8.0)
pH 8.2	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

For the EDTA stock solution it is necessary to adjust the pH to 8.0 to provide a complete dissolution of EDTA.

2.1.7.2 SDS PAGE according to Schägger and Jagow (1987)

Separating gel

(16 % acrylamide; 1.0 M Tris; pH 8.45)

5.30 mL	Acrylamide/Bis, 30 %, 37.5:1
3.33 mL	Gel buffer
1.26 mL	Deionized water
40 µL	SDS-solution, 25 % (w/v)
7 µL	TEMED
50 µL	APS, 10 % (w/v), freshly prepared

Stacking gel

(4 % acrylamide; 0.74 M Tris; pH 8.45)

0.68 mL	Acrylamide/Bis, 30 %, 37.5:1
1.29 mL	Gel buffer
3.21 mL	Deionized water
16 µL	SDS-solution, 25 % (w/v)
7 µL	TEMED
33µL	APS, 10 % (w/v), freshly prepared

Gel buffer

(3.0 M Tris, pH 8.45)

90.86 g	Tris, dissolved in 250 mL deionized water
pH 8.45	Adjusted by addition of HCl

5x-Cathode buffer

(0.5 M Tris; 0.5 Tricine, 0.5 % SDS, pH 8.25)

60.57 g	Tris
89.58 g	Tricine
20 mL	SDS-solution, 25 % (w/v)
ad 1000 mL	deionized water

pH had not to be adjusted.

5x-Anode buffer

(1 M Tris; pH 8.9)

121.14 g	Tris
pH 8.9	Adjusted by addition of HCl
ad 1000 mL	Deionized water

2x-Incubation buffer

1.0 mL	1 M Tris-HCl, pH 6.8
0.8 g	SDS
2.4 mL	Glycerol (3.0 g)
0.31 g	DTT
20 mg	TCEP, added just before use
1.0 mg	Bromophenol blue
ad 10 mL	Deionized water

2.1.7.3 Solutions for coomassie staining of protein gels

2.1.7.3.1 Colloidal Coomassie staining (Roti®-Blue)

Staining solution

20 mL	Roti®-Blue concentrate (5x)
20 mL	Methanol
59 mL	Deionized water
1 mL	H_3PO_4 (85 %)

Destaining solution

20 mL	Methanol
79 mL	Deionized water
1 mL	H_3PO_4 (85 %)

2.1.7.3.2 Hot Coomassie staining

Staining solution

0.09 g	PlusOne™ Coomassie™ Blue PhastGel™ R-350; (0.03 % (w/v))

15.6 mL	Acetic acid
140.6 mL	Deionized water

Destaining solution

10.0 mL	Acetic acid
90.0 mL	Deionized water

2.1.7.4 Silver staining according to Blum *et al.* (1987)

Fixative

40 mL	Ethanol
10 mL	Acetic acid
50 mL	Deionized water

Wash solution

30 mL	Ethanol
70 mL	Deionized water

Thiosulfate reagent

20 mg	Sodium thiosulfate
100 mL	Deionized water

Silver nitrate reagent

0.2 g	Silver nitrate
100 mL	Deionized water
20 µL	Formaldehyde (37 %) (add immediately prior to use)

Developer

3 g	Sodium carbonate
0.5 mg	Sodium thiosulfate
100 mL	Deionized water
20 µL	Formaldehyde (37 %) (add immediately prior to use)

Stop reagent

0.5 g	Glycin
100 mL	Deionized water

2.1.7.5 Negative (Imidazole-Zn) staining

Sodium carbonate solution

1.0 g	Sodium carbonate
100 mL	Deionized water

Imidazole solution

(0.2 M imidazole, 0.1 % SDS (w/v))

1.36 g	Imidazole
0.1 g	SDS
100 mL	Deionized water

Zinc solution

(0.2 M zinc acetate)

3.67 g	Zinc acetate
100 mL	Deionized water

2.1.7.6 CAPS buffer for semi-dry blotting

(10 mM CAPS, 10 % methanol, pH 11.0)

2.21 g	CAPS in 700 mL deionized water
100 mL	Methanol
pH 11.0	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

2.1.7.7 Buffer for Western Blots

TBS buffer

(20 mM Tris, 50 mM NaCl, pH 7.5)

2.42 g	Tris
2.92 g	NaCl dissolved in 800 mL of deionized water
pH 7.5	Adjusted by addition of HCl
ad 1000 mL	Deionized water

Blocking buffer

(20 mM Tris, 150 mM NaCl, pH 7.5)

2.42 g	Tris
8.76 g	NaCl dissolved in 800 mL deionized water
pH 7.5	Adjusted by addition of HCl
ad 1000 mL	Deionized water

Prior to use 1 g bovine serum album (fraction V) was added per 100 mL.

TBS-T buffer

(20 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.5)

2.42 g	Tris
8.76 g	NaCl dissolved in 800 mL deionized water
pH 7.5	Adjusted by addition of HCl
0.5 g	Tween 20
ad 1000 mL	Deionized water

Developer solution

(100 mM Tris, 100 mM NaCl, 5 mM MgCl2, pH 7.5)

12.1 g	Tris
5.8 g	NaCl dissolved in 800 mL deionized water
1.0 g	$MgCl_2$ x 6 H_2O
pH 7.5	Adjusted by addition of HCl
ad 1000 mL	Deionized water

Stop solution

(10 mM EDTA-Na_2)

3.7 g	EDTA-Na_2 x 2 H_2O
ad 1000 mL	Deionized water

NBT concentrate

75 mg	NBT
1.0 mL	70 % N,N´-dimethyl-formamid

BCIP concentrate

50 mg	BCIP
1.0 mL	70 % N,N´-dimethyl-formamid
1.0 mL	Deionized water

2.1.7.8 Buffer for anion exchange chromatography

Start buffer (IEX)

(20 mM Tris, pH 11.0)

2.42 g	Tris
pH 11.0	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

Elution buffer (IEX)

(20 mM Tris, 1 M NaCl, pH 11.0)

2.42 g	Tris
58.4 g	NaCl
pH 11.0	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

2.1.7.9 Buffer for hydrophobic interaction chromatography

Start buffer (HIC)

(1.0 mM ammonium sulfate, 100 mM Tris, pH 7.0)

132.1 g	Ammonium sulfate
12.1 g	Tris
pH 7.0	Adjusted by addition of HCl
ad 1000 mL	Deionized water

For purification of nsLTP1 1.5 M ammonium sulfate (198.2 g) were used

Elution buffer (HIC)

(100 mM Tris, pH 7.0)

12.1 g	Tris
pH 7.0	Adjusted by addition of HCl
ad 1000 mL	Deionized water

2.1.7.10 Synthetic wort according to Zapf (2006a)

Acid component (2 fold concentrated)

15 mg	DL-malic acid
15 mg	Pyruvic acid
40 mg	Citric acid
20 mg	Acetic acid
52 mg	DL-lactic acid
4 mg	Formic acid
ad 100 mL	Deionized water

Sugar component (2 fold concentrated)

2.3 g	Glucose-monohydrate
0.6 g	Fructose
0.6 g	Sucrose
10.0 g	Maltose-monohydrate
6.0 g	Maltodextrin
ad 100 mL	Deionized water

Desired pH was set before adjusting of volume. After autoclaving both components were brought together and mixed thoroughly.

2.1.7.11 Production of DEPC water

To reduce the risk of RNA degradation by RNAse contaminations from water, DEPC water was used for all RNA work. DEPC water was made with diethylpyrocarbonate 0.1 % (v/v) in deionized water. After stirring at room temperature for 48 h, solution was autoclaved at 121°C for 20 minutes.

2.2 Media and culture conditions

In all media sugar components were dissolved in a proportionate volume of deionized water and autoclaved separately to avoid formation of Maillard products. After cooling down to room temperature sugar- and protein components were brought together and mixed thoroughly.

2.2.1 YPD (Yeast extract peptone dextrose medium)

10 g	Yeast extract
20 g	Peptone from casein
20 g	Glucose-monohydrate
ad 1000 mL	Deionized water

pH was not adjusted. For transformation experiments with *P. pastoris* 182.2 g/l sorbitol were added (YPDS medium). If plates were made 15 g/l agar were added before autoclaving. For selection of yeast cells containing Zeocin resistance gene 1 mL of Zeocin (100 mg/mL) was added to the medium after cooling down below 55°C.

2.2.2 Low salt LB (Low salt lysogeny broth; Bertani, 2004)

5 g	Yeast extract
10 g	Tryptone
5 g	NaCl
pH 7.5	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

Low salt content was needed to assure the effect of the salt sensitive antibiotic Zeocin. For selection of *E. coli* cells containing Zeocin resistance gene 250 µL of Zeocin (100 mg/mL) were added to the medium after cooling down under 55°C.

2.2.3 SOC (Super optimal broth)

5 g	Yeast extract
20 g	Peptone from casein
0.58 g	NaCl
0.186 g	KCl
2.5 g	$MgSO_4$ x 7 H_2O
2 g	$MgCl_2$ x 6 H_2O
pH 7.0	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

2.2.4 BMGY / BMMY (Buffer glycerol/methanol complex medium)

10 g	Yeast extract
20 g	Peptone from casein
100 mL	1 M potassium phosphate buffer, pH 6,0
100 mL	YNB solution (13,4 % (w/v))
2 mL	Biotin solution (4x 10^{-5} % (w/v))
100 mL	Glycerol (10 % (v/v))
100 mL	Methanol (5 % (v/v)), only for BMMY
ad 1000 mL	Deionized water

Yeast extract and peptone were dissolved in 700 mL deionized water and autoclaved. After cooling down to room temperature remaining components were filter sterilized and well mixed with protein components. pH was set by the potassium phosphate buffer.

2.2.5 BMM (Buffered minimal methanol medium)

100 mL	1 M potassium phosphate buffer, pH 6,0
100 mL	YNB solution (13,4 % (w/v))
2 mL	Biotin solution (4x 10^{-5} % (w/v))
100 mL	Methanol (5 % (v/v)), only for BMMY
ad 1000 mL	Deionized water

700 mL deionized water were autoclaved. After cooling down to room temperature remaining components were filter sterilized and well mixed with the autoclaved water. pH was set by the potassium phosphate buffer.

2.2.6 Malt extract medium

20 g	Malt extract
2 g	Soy peptone
pH 6,5	Adjusted by addition of NaOH
ad 1000 mL	Deionized water

If plates were made 15 g/L agar were added before autoclaving.

2.2.7 Culture conditions for *E. coli*, *P pastoris* and *F. graminearum*

E. coli cells were cultivated in LB-medium at 37°C and 180 rpm shaking overnight. *P. pastoris* strains were cultivated at 30°C and 180 rpm shaking. If the yeast cells were prepared for electroporation, cultivation was performed in YPD-medium. For production of heterologous proteins *P. pastoris* strains were pre-cultivated in the complex medium BMGY overnight. After removal of the culture medium, cells were transferred into the protein free minimal medium BMM and cultivation was continued for 4 days at 30°C and 180 rpm shaking. Cultivation of *F. graminearum* was performed in malt-extract medium at 28°C and 120 rpm shaking for 3 days.

2.2.8 Back up of transformed strains

All established and confirmed transgenic strains were backed up as cryo-cultures at -80°C. For *E. coli* strains cells were cultivated in 10 mL LB-medium overnight. After removal of the culture medium by centrifugation at 2500 x g for 5 minutes cells were resuspended in 2 mL of fresh LB-medium. 2 tubes containing 1 mL of sterile glycerol were each completed with 1 mL of cell suspension. Tubes were immediately closed, inverted several times and frozen in liquid nitrogen. Frozen tubes were stored at -80°C. For *P. pastoris* strains cells were cultivated in 10 mL YPD-medium overnight. After removal of the culture medium by centrifugation at 2500 x g for 5 minutes cells were resuspended in 2 mL of fresh YPD-medium. 2 tubes containing 1 mL of sterile glycerol were each completed with 1 mL of cell suspension. Tubes were closed, inverted several times and cooled down in a methanol bath from room temperature to -80°C. Frozen tubes were stored at -80°C

2.3 Methods of molecular genetics

2.3.1 Primers

Table 1: Primers used in the study;

Primer	**Sequence**	**Calculated annealing temperature**
Oligo(dT)15	TTTTTTTTTTTTTTT (Promega, Madison, WI, USA)	-
AOX1-primer_f	5'-GACTGGTTCCAATTGACAAGC-3'	55°C
AOX1-primer_r	5'-GCAAATGGCATTCTGACATCC-3'	55°C
EcoR1-LTP1-f	5'-CCGGAATTCCTCAACTGCGGCCAGGTTG-3'	62°C
LTP1-Age1-r	5'-TATACCGGTTCAGTAAATCCTGGAGCAGTC-3'	62°C
EcoR1-AfpA-f	5'-CCGGAATTCGCCCCCGAGCCCAAGAC-3'	60°C
AfpA-Xba1-r	5'-GCGTCTAGATCAGTTGCTGTACTGGGAGT-3'	60°C
EcoR1-HFB2-f	5'-CCGGAATTCGCTGTTTGTCCAACTGGTTT-3'	62°C
HFB2-Xba1-r	5'-CGGTCTAGATTAGAAAGTACCGATAGCCTT-3'	62°C
EcoR1-FcHyd3-f	5'-CCGGAATTCGCTCCACATGGTTC-3'	60°C
FcHyd3-Age1-r	5'-TATACCGGTTCATTACAACAATTTAA-3'	60°C

2.3.2 Plasmids

Three different plasmids were used in the study. Plasmid 052549pPCR-Script was obtained from Geneart AG (Regensburg, Germany) containing the *fchyd3* gene from *F. culmorum*. Plasmid 1002685_HFB2_pMA also was obtained from Geneart AG and contained the *hfb2* gene from *T. reesei*. Both plasmids carried the optimized nucleotide sequences of the corresponding genes according to the codon usage of *S. cerevisiae*. For selection of positive transformation events the *ampr* gene coding for ampicillin resistance was present on the plasmid. Plasmid pPICZαA was part of the EasySelect^{TM} *Pichia* Expression Kit (Invitrogen, Carlsbad, USA) and used as vector for integration of genes into the genome of *P. pastoris* X33. This plasmid contained the secretion signal sequence from the *S. cerevisiae* α factor and the s*h ble* gene conferring resistance against the antibiotic Zeocin. Maps of all plasmids can be found in the appendix section.

Plasmid	**Source**
pPICZαA	EasySelect^{TM} Pichia Expression Kit, Invitrogen, Carlsbad, USA
052549pPCR-Script	Geneart AG, Regensburg, Germany
1002685_HFB2_pMA	Geneart AG, Regensburg, Germany

2.3.3 Conditions for polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify genes for further processing, for controls and to introduce restriction sites at the 3'- and 5'- of the nucleotide sequence. Amplification was performed using 0.25 µl Taq-polymerase, 2.5 µl ready to use 10fold incubation mix with $MgCl_2$, 0.5 µl dNTPs mix (10 mM each) as included in the Taq CORE Kit (MP Biomedicals, Illkirch, France), 0.25 µL each forward and reverse primer (100 pmol/µL, eurofins mwg operon, Ebersberg, Germany) as well as filter sterilized and UV treated, deionized water and DNA to make a total reaction volume of 25 µL. Mixtures were incubated in a Primus 96 plus thermal cycler (mwg biotech ag, Ebersberg, Germany). Reaction was lead by the following temperature profile: Initial denaturing of DNA at 95°C for 4 minutes followed by 35 cycles of denaturing at 95°C for 45 seconds, 45 seconds for annealing at temperatures as mentioned above in Table 1, 72°C for elongation for 1 minutes. Final elongation time was 5 minutes.

2.3.4 Agarose gel electrophoresis

Separation of DNA or DNA fragments from PCR was performed using agarose gel electrophoresis. 1 % (w/w) agarose (Biozym Scientific GmbH, Hess. Oldendorf, Germany) was prepared in 1x TAE buffer and boiled in a micro wave oven until all agarose particles were dissolved and the original volume was reconstituted with deionized water. For an improved separation of DNA-fragments smaller than 500 kDa 1.5 % (w/w) of agarose was used. For gel casting the hot agarose solution was cooled to 58°C, poured into a sealed gel tray and combs were inserted into the provided comb slots. After solidification of the gel combs and sealing of the gel tray were removed and the tray was placed into the electrophoresis chamber, which was filled with 1x TAE buffer up to 1 cm above the gel surface. DNA separation was performed by mixing DNA-samples 5:1 with loading dye (5x) and filled into sample slots. Size marker standards were applied for evaluation of DNA fragment length in adjacent sample pockets. Separation was performed at 5 V/cm field strength for 1-1.5 h. Current was set to 200 mA, to provide enough mA for the adjusted voltage. A separation run was stopped, when the blue bromophenol front reached a point 2 cm before the end of the gel to avoid loss of DNA by running out of the gel. Visualization of DNA bands was accomplished by using dimidium bromide. After incubation for 20 minutes, gels were washed in deionized water to reduce background staining and evaluated in an UV-chamber at 320 nm. Documentation of gels was performed using a video camera in combination with Intas® GDS equipment and software. For separation of PCR products from the gel, recovery of DNA was performed by cutting off bands from the

gel and subsequent extraction of DNA using peqGOLD gel extraction kit according to the manufacturers recommendations (peqLab, Erlangen, Germany).

2.3.5 Nucleotide sequence analysis

All *P. pastoris* clones and plasmids from *E. coli* subclones were checked for carrying the correct insert by nucleotide sequence analysis. As heterologous genes were integrated into the yeast genome, analysis was performed by isolation of DNA from an overnight culture using YeaStar Genomic DNA Kit (Zymo Research, Orange, USA) and amplification of the corresponding gene by PCR using primer pair AOX1-primer_f and AOX1-primer_r (see Table 1 for sequences). Plasmids from *E. coli* strains were isolated from an overnight culture using peqGOLD plasmid miniprep kit (peqLab, Erlangen, Germany). After the concentration of the purified PCR product was measured photometrical against deionized water using a Nanodrop photometer (Thermo Scientific, Wilmington, USA), concentration was set to 10 – 50 ng/µL in a final volume of 30 µL by dilution with DNAse free, deionized water. Primers were diluted to 10 pmol/µL in a final volume of 30 µL. For sequencing samples were submitted to GATC Biotech (Konstanz, Germany). Sequence evaluation was performed using Microsoft Word and the online tool clustalw (http://www.ebi.ac.uk/ Tools/clustalw2/index.html).

2.3.6 Extraction of RNA

RNA of *F. graminearum* was isolated after incubation in malt extract medium for 3 days at 28°C and 120 rpm. Medium was removed by filtration using a Büchner funnel with filter inlay. Pieces of agar, which were used for inoculation, were taken away from the filter and discarded. Excess water was removed by soft pressing of mycelial cells wrapped in the filter paper. Mycelial cells were transferred in 15 mL Sarstedt-tubes and frozen in liquid nitrogen. Disruption of fungal cells was performed in presence of liquid nitrogen using a mortar and pestle. Isolation of total RNA was performed according to the RNeasy Plant Mini Kit manual (Qiagen, Hilden, Germany). RNA isolation from *P. pastoris* X33 [pPICZαA-FcHyd3] was performed using YeaStar™ RNA Kit (Zymo Research, Irvine, Canada). Prior to isolation the yeast strain was cultivated in BMGY medium overnight and then cells were transferred into a Fernbach flask with BMM medium containing methanol for induction of gene expression. After incubation overnight 5 mL of yeast culture were used for RNA isolation. During all RNA isolation procedures RNAse activity was prevented by wearing gloves and sufficient freezing of the cell material. This was achieved by addition of liquid

nitrogen and freezing of any equipment used. All RNA samples were eluted using DEPC water and immediately stored at -80°C until further processing.

2.3.7 Reverse transcription of mRNA into cDNA

Transcription was performed according to Hew Ferstl (2008). 20 µL of RNA were treated with 5 µL DNase (Promega, Madison, WI, USA) and incubated at 37°C for 30 minutes. DNAse reaction was stopped by addition of 4 µL stop solution (Promega) and incubation at 65°C for 10 minutes. 10 µL of RNA were transcribed into cDNA using 3 µL Oligo(dT) 15 primer (Promega), 2 µL dNTPs (10 mM each, Qbiogene, Illkirch, France), 4 µL RT Buffer (Promega), 0.5 µL M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) and 1.5 µL DEPC water. Transcription was performed at 25°C for 5 minutes followed by incubation at 42°C for 1 hour. cDNA samples were stored at -20°C. All samples were also prepared without DNAse and/or reverse transcriptase as a control for DNA contamination. Residual DNA was detected by PCR.

2.3.8 Production of chemically competent *E. coli* TOP 10 cells

10 mL of fresh LB medium were inoculated from an overnight culture of *E. coli* TOP 10. After growth to logarithmic phase, which corresponds to an optical density of 0.5 at 590 nm, cells were harvested by centrifugation at 5000 x g for 5 minutes at 4°C. The pellet was carefully resuspended in 5 mL of 4°C cold 100 mM $CaCl_2$ solution and incubated for 30 minutes at 0°C. Cells again were harvested at 4°C by centrifugation at 5000 x g for 5 minutes. The obtained pellet was resuspended in 1 mL of 4°C cold 100 mM $CaCl_2$ solution and completed by addition of 175 µL of cold glycerol 87 % (v/v). Cell suspension was aliquoted in 0.1 mL portions, incubated overnight at 0°C and finally stored at -80°C.

2.3.9 Heat shock transformation of chemically competent *E. coli* TOP 10 cells

Aliquots of 100 µL of chemically competent *E. coli* were thawed on ice for several minutes and mixed with 5 µL plasmid. Cells were subjected to a heat shock at 42°C for 40 seconds after incubation on ice for 20 minutes and again chilled on ice for 5 minutes after the heat treatment. Subsequently cells were regenerated in 1 mL SOC medium and incubated for 1 hour at 37°C at 120 rpm shaking. For selection of cells containing the plasmid, 50 and 100 µL as well as the remaining pellet of the cell suspension were each plated on antibiotic containing agar plates and incubated at 37°C for 1 to 2 days.

2.3.10 General procedures for incorporation of genes into plasmid pPICZαA

All transgenic genes were incorporated behind the native *S. cerevisiae* α-factor secretion signal using restriction site EcoR1. The second restriction site was chosen from the multiple cloning site of the vector pPICZαA. Before a restriction site was selected it was ensured that the same restriction site is not contained in the corresponding gene insert to avoid destruction of gene upon digestion. Further, EcoR1 was excluded as second restriction site to avoid false incorporation of the gene into the plasmid. For incorporation into the vector genes and pPICZαA were digested with the corresponding restriction enzymes at 37°C for 30 minutes. Digested plasmids and generated fragments were separated by agarose gel electrophoresis and plasmid isolated from gel using peqGOLD gel extraction kit (peqlab, Erlangen, Germany). Digested genes were purified using E.Z.N.A. Cycle Pur Kit (Omega bio-tek, GA, USA). Ligation was performed using T4 DNA ligase with T4 DNA ligase buffer. For each ligation a new, frozen cap of T4 ligase buffer was used, since contained ATP was not stable and the number of positive ligation events decreased with the number of thawing/freezing cycles Therefore, ligase buffer was aliquoted upon purchasing and only frozen and thawed once to prevent degradation of ATP. Reaction was carried out using a ratio of 1:5 between concentrations of plasmid and insert. Ligation was performed by incubation of the reaction mixture in a water bath at 22°C, which was cooled down to 4°C overnight.

2.3.11 Cloning of *nsltp1* gene from *H. vulgare*

For recovery of barley DNA, kernels of *H. vulgare* cv. Marnie were put into humid potting soil and grown for five days at room temperature. Sprouts were harvested and frozen at -20°C until further processing. DNA was isolated from sprouts using the PrestoSpin D DNA isolation kit (Molzym, Bremen, Germany). Design of primers was performed according to the GenBank entry for nsLTP1, accession no. X59253.1 on NCBI nucleotide database, whereas the sequence for the signal peptide for secretion was excluded. Prediction of the secretion factor was performed using SignalP 3.0 Server. (http://www.cbs.dtu.dk /services/SignalP/). The nsLTP1 gene was amplified by PCR using primers (EcoR1-LTP1-f, LTP1-Age1-r, for sequences see Table 1). Restriction sites EcoR1 and Age1 were attached to the 5'- and 3'-end by using this pair of primers. The native gene comprised two exons and one intron, whereas the second exon consisted of only 10 nucleotides. An intron free gene of nsLTP1 was obtained by addition of the exon 2 nucleotide sequence to the 3'-end of exon 1 using an extended reverse primer (LTP1-Age1-r) containing the exon 2 nucleotide sequence.

2.3.12 Cloning of the *afpA* gene from *F. graminearum*

Gene coding for *afpA* was used as found in *F. graminearum* (TMW 4.0157). Primer EcoR1-AfpA-f and AfpA-Xba1-r were derived from studies of Zapf (2006) on AfpA from *F. culmorum*. The sequence of the signal peptide was excluded, which was predicted using SignalP 3.0 Server. Intron free nucleotide sequence was obtained by isolation of total RNA from a 3 days culture, which was cultivated at 28°C at 160 rpm on a horizontal shaker. Isolation of RNA, transcription into cDNA and sequence analysis was performed as described above. The gene was incorporated into pPICZαA using restriction sites EcoR1 at the 5'-end and Xba1 at the 3'-end. Both restriction sites were attached via extended primers (see Table 1).

2.3.13 Cloning of the *hfb2* gene from *T. reesei*

The intron free nucleotide sequence of *hfb2* from *T. reesei* was used as found in the EMBL data base under accession no. Y11894. For an optimal expression of the corresponding protein in *P. pastoris* the native nucleotide sequence was adapted to the codon usage of *S. cerevisiae*. Together with this step the two introns were removed from the gene sequence. The gene was *in-vitro* synthesized and incorporated into plasmid 1002685_HFB2_pMA by Geneart AG (Regensburg). For backup and multiplication the plasmid was transferred into chemically competent *E. coli* TOP 10 cells by heat shock transformation as described above. Selection of plasmid carrying cells was performed on LB agar plates containing ampicillin. Plasmid isolation was performed with an overnight culture using peqGOLD plasmid miniprep kit (peqlab, Erlangen, Germany). Gene for *hfb2* was amplified from the plasmid with primer pair EcoR1-HFB2-f and HFB2-Xba1-r (see Table 1) while restriction sites EcoR1 and Xba1 were attached to the 3'- and 5'-end, respectively. Primers were designed omitting the secretion signal as predicted by the online tool SignalP 3.0 Server.

2.3.14 Cloning of *fchyd3* from *F. culmorum*

Intron free nucleotide sequence of *fchyd3* from *F. culmorum* was taken from plasmid 052549pPCR-Script as described by Zapf *et al.* (2006b). This plasmid contained the *in-vitro* synthesized and intro free nucleotide sequence of *fchyd3* adapted to the codon usage of *S. cerevisiae* and was delivered by Geneart AG (Regensburg, Germany). For backup and multiplication the plasmid was transferred into chemically competent *E. coli* TOP 10 cells by heat shock transformation as described above. Selection of plasmid carrying cells was performed on LB agar plates containing ampicillin. The gene coding for *fchyd3* was

amplified with primer pair EcoR1-FcHyd3-f and FcHyd3-Age1-r (see Table 1) while restriction sites EcoR1 and Age1 were attached to the 3'- and 5'-end, respectively. Primers were designed omitting the secretion signal as predicted by the online tool SignalP 3.0 Server.

2.3.15 Transformation of genes in *P. pastoris* X33

Transformation of genes into *P. pastoris* X33 was performed using plasmid pPICZαA (EasySelect^{TM} *Pichia* Expression Kit, Invitrogen, Paisley, UK) containing the native *S. cerevisiae* α-factor secretion signal. Introduction of the plasmid into *P. pastoris* X33 was performed in electroporation cuvettes by electroporation using the GenePulser II Electroporation System (Bio-Rad, Hercules, USA) at 25 µF, 200 Ω, 2 kV. For an efficient integration of the genes into the yeast genome vector pPICZαA was linearized using restriction enzyme *Sac*1 prior to transformation. Immediately after transformation cells were diluted in 1 mL ice cold sorbitol (1 M), transferred into a 15 mL Sarstedt-tube and incubated at 30°C for 1.5 hours without shaking. To screen the culture for cells, in which the plasmid was integrated into the genome, cell suspension was plated on YPDS agar plates containing Zeocin, wrapped in aluminum foil for photo protection and incubated at 30°C for 3-7 days.

2.3.16 Class II hydrophobin FcHyd5p from *F. culmorum*

P. pastoris X33 [pPICZαA-FcHyd5] (TMW 3.213) carrying the *fchyd5* gene from *F. culmorum* was established by Stübner *et al.* (2010). Expression of the recombinant protein was proved by the authors via RNA isolation and generation of cDNA. Detection of *fchyd5* was performed using PCR, excluding contamination with chromosomal DNA by DNAse treatment of isolated RNA. This strain was used for production of the class II hydrophobin FcHyd5p and represented the basis of this study.

2.4 Proteomics

2.4.1 One-dimensional gel electrophoresis

Sample testing for determination of purity and molecular sizes of contained proteins was performed by SDS-PAGE according to Schägger and Jagow (1987). This system was chosen due to the improved separation of small proteins below 20 kDa as compared to other SDS-PAGE protocols. SDS-PAGE was carried out in a Mini Protean III Cell (Bio-Rad Laboratories, Hercules, CA, USA), using buffers and solutions as mentioned in section 2.1.7. As separating and stacking gels were casted successively no glycerol was added to the separating gel substituting the volume by water. For gel casting short plate and 0.75 mm spacer plate were placed into the casting frame, sealed with parafilm (Pechiney Plastic Packaging, Menasha, Wi, USA) at the bottom and mounted on the casting stand. TEMED and freshly prepared APS-solution were added immediately prior to casting. For a clear border between both gel sections separating gel was covered by isopropanol until it was polymerized. After 30 minutes isopropanol was removed and stacking gel was casted. To prevent the formation of bubbles between stacking gel and comb, which was used to form sample slots, glass plates were sealed with melted agarose. After 30 minutes gels were used for electrophoresis or wrapped in a humid tissue and aluminum foil and stored at 4°C until used. Prior to separation fluid samples were mixed 1:1 (v/v) with incubation buffer and incubated for 10 minutes at 75°C. If freeze dried or dry samples were applied, they were dissolved in deionized water before mixing with incubation buffer. Prior to sample application the gel was conditioned at 90 V, 9 mA for 10 minutes in the ready assemble Mini Protean III Cell (Bio-Rad Laboratories, Hercules, CA, USA). After loading of the samples into the gel pockets proteins were led into the stacking gel at 60 V, 9 mA for 15 minutes. Further separation was performed at 120 V and 18 mA. Electrophoresis was stopped 15 min after the bromophenol blue front had left the gel.

2.4.2 Staining of polyacrylamide gels

2.4.2.1 Colloidal Coomassie Blue

Colloidal Coomassie Blue staining was performed using diluted Roti®Blue solution as described above. Fixation and staining were performed together by addition of 1 % (v/v) of phosphoric acid (85 %) to the staining solution. Staining of gels was carried out over night on a horizontal shaker at 65 rpm. Destaining was performed using destaining solution. Destaining was stopped by washing the gel with deionized water as soon as background was destained.

2.4.2.2 Hot Coomassie Blue

For a rapid detection of protein bands, staining with hot coomassie blue was applied. An advantage of this staining method was that loss of alcohol soluble proteins could be reduced, since no methanol or ethanol was involved. Staining was achieved by incubation of gels in a water bath at 60°C in the solution as mentioned above for 30 minutes. Destaining was performed using destaining solution. Destaining was stopped by washing the gel with deionized water, as soon as background was removed.

2.4.2.3 Silver staining according to Blum *et al.* (1987)

As a very sensitive staining method silver staining according to Blum *et al.* (1987) was employed to detect lowest amounts of protein. Staining was carried out following the steps below. During all procedures of the staining hands were protected by gloves to avoid contact with formaldehyde, staining of the skin with silver as well as contamination of the gels. Developing step was stopped, when bands on the gel became visible. If no bands could be seen on the gel, developing was stopped as soon as protein size marker bands were fully stained. In both cases staining was continued only until background staining was getting too dark.

Step	Reagent	Duration
Fixation	Fixative	>3 h or over night
Washing	Wash solution	2x 20 min
Washing	Deionized water	20 min
Sensitizer	Thiosulfate reagent	1 min
Washing	Deionized water	3x 20 sec
Silver	Silver nitrate reagent	20 min
Washing	Deionized water	3x 20 sec
Development	Developer	3-5 min
Washing	Deionized water	3x 20 sec
Stop	Stop reagent	5 min
Wash	Deionized water	3x 10 min

2.4.2.4 Negative staining with imidazole zinc according to Görg *et al.* (2004)

For negative staining gels were transferred into sodium carbonate solution after electrophoresis and incubated for 5 minutes. This solution was replaced by imidazole solution and the gel incubated for a further 15 minutes. After a rinse with deionized water for 10 seconds the gel was agitated in the zinc acetate solution for 60 seconds. In this step the gel became white and opaque. Finally the gel was rinsed with deionized water for at least 5 x 2 minutes and 3 x 15 minutes. All steps were performed at room temperature and gels were handled with powder free nitrile gloves.

2.4.2.5 Lumitein staining

Lumitein staining is a luminescent staining for proteins after gel electrophoresis with a detection limit of 1 ng per spot. Before application to staining 40 mL Lumitein 5X (Biotium, Hayward, CA, USA) solution were diluted by addition of 110 mL deionized water and 50 mL methanol. For fixation and staining gels were transferred into the diluted Lumitein solution and incubated for 90 minutes on a shaker at 70 rpm. Destaining was achieved by incubating the gels in destaining solution for 5 minutes on a shaker at 70 rpm. Afterwards gels were washed and agitated in deionized water for further 5 minutes. Gels were made visible in an UV-chamber at a wavelength of 320 nm and documented by video camera using Intas® GDS equipment and software.

2.4.3 Verification of protein expression

For *P. pastoris* X33 [pPICZαA-FcHyd3] protein expression was verified by RNA detection after induction, since the protein could not be visualized on SDS-PAGE gels using coomassie blue. RNA isolation and cDNA generation was performed as described above. PCR was performed using primer EcoR1-FcHyd3-f and FcHyd3-Age1-r (see Table 1). For control and detection of residual DNA molecules in RNA samples, PCR was also performed with RNA, DNAse treated RNA (RQ1 RNAse-free DNAse, Promega, Madison, WI, USA) and plasmid pPICZαA containing *fchyd3*.

For *P. pastoris* X33 [pPICZαA-AfpA], *P. pastoris* X33 [pPICZαA-LTP1] and *P. pastoris* X33 [pPICZαA-HFB2] protein expression was verified by peptide mass finger print (Protein analysis unit, Ludwigs-Maximilians-Universität München, Germany). Prior to measurement SH-groups of the proteins were alcylated with iodoacetamide and digested with trypsin. Determination of the peptide masses was performed using LC-MS/MS. Processing of data

was performed using mascot algorithms. *In silico* translation of nucleotide sequences into corresponding amino acid sequences was performed using the Translate online tool from http://www.expasy.org/tools/dna.html. *In silico* digestion and prediction of peptides resulting from trypsin treatment as well as calculation of isoelectric points and masses for proteins and peptides was performed using the PeptideMass online tool from http://www.expasy.org/tools/peptide-mass.html.

2.4.4 Expression and preparation of recombinant proteins

For expression of the recombinant proteins, transformed yeasts were cultivated in 50 mL BMGY medium overnight at 30°C and 180 rpm to result in an optical density of OD_{600} = 2.0 to 6.0. After removal of the protein containing supernatant, cells were transferred into a Fernbach flask containing 200 mL protein free BMM medium or a protein containing complex medium (BMMY) and cultivated for 4 days at 30°C (180 rpm). Expression of the recombinant proteins was induced by adding 0.05 % (v/v) methanol as sole carbon source every 24 hours. Sufficient aeration was achieved by using Fernbach flasks, which provide a high ratio between surface and volume due to the large diameter of their glass bottom. Flasks were agitated by horizontal shaking at 180 rpm and air supply was ensured by sealing the flask with a gauze bandage. Following incubation cells were removed by centrifugation at 6000 x g for 30 minutes at 10°C. For culture media of cells, which were grown in protein free minimal medium, cell free supernatants were further processed by dialysis for 4 days at 4°C against daily changed deionized water using tubes with a molecular weight cut off of 3500 Da (Serva Electrophoresis GmbH, Heidelberg, Germany). Subsequently, supernatants were freeze dried in a lyophilisator (WKF, Modautal, Germany) after centrifugation at 6000 x g for 15 minutes at ambient temperature. Wild type strain *P. pastoris* X33 was cultivated and the supernatant processed in the same way as described above. This lyophilisate was used as negative control.

2.4.5 Semi-Dry Blotting and Western Blotting

For immunochemical detection proteins were blotted from acrylamide gels onto PVDF or nitrocellulose membranes according to Matsudaira (1987). For blotting experiments prestained protein ladders were used instead of unstained molecular weight markers. After separation of proteins by SDS PAGE gels were incubated in ice cold blotting buffer for 20 minutes. If a PVDF membrane was applied, it was made wettable by incubation in methanol for 2 minutes before equilibrating in ice cold blotting buffer for 5 minutes. Nitrocellulose membrane was only equilibrated in ice cold blotting buffer for 5 minutes. In

the meantime 4 pieces of blotting paper were trimmed to fit on the gel and soaked in the blotting buffer. After equilibration of the blotting membrane, papers and gel were assembled as shown in Figure 5 on the cathode of the blotting chamber.

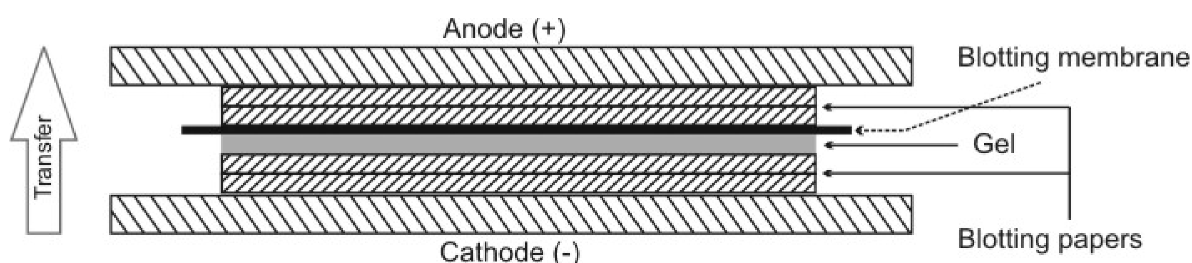

Figure 5: Assembly of the semi-dry blotting system;

Before starting the protein transfer, blotting sandwich was over-rolled carefully with a 50 mL Sarstedt tube, to remove enclosed air cushions. Anodic electrode plate was placed on the sandwich and fixed. Screw nuts were fixed finger-tight. Blotting was performed at 0.8 mA/cm^2 and 20 V for 1.5 hours.

For detection of the transferred proteins the blotting membrane was removed from the assembly and further processed according to the steps described as follows.

Step	Treatment
1	Wash membrane in TBS buffer for 1 minute.
2	Block membrane by shaking in blocking buffer containing 1 % BSA at 37°C for 1 hour.
3	Incubate membrane by shaking in 25 mL TBS buffer containing primary antibodies (dilution of 1:2000) for 1.5 hours.
4	Wash membrane by shaking in TBS-T buffer at 37°C for 5 min; repeat step 3 times.
5	Incubate membrane by shaking in 25 mL TBS buffer containing secondary antibodies (dilution of 1:2000) for 1.5 hours.
6	Wash membrane by shaking in TBS-T buffer at 37°C for 5 min; repeat step 3 times.
7	Incubate membrane in staining solution until bands are visible (30 – 120 s).
8	Stop staining by addition of EDTA solution for 2 minutes.
9	Wash membrane in deionized water and air dry it in the dark at room temperature.

Immunoglobulins from rabbit with affinity to the corresponding protein were used as primary antibodies. Secondary antibodies were goat-anti-rabbit immunoglobulins. Staining was

achieved by alkaline phosphatase, which was attached to secondary antibodies. If proteins were detected, blue bands became visible on the membrane.

2.4.6 Concentration of protein solutions

Concentration of proteins solutions was performed by centrifugation using Amicon centrifugal filter units with a molecular weight cut off of 5 kDa. Permeate was discarded and retentate was used for further tests. For removal of high molecular weight proteins filter units with a cut off of 30 kDa were used. In this case samples were applied on the filter unit and centrifuged according to the manufacturers manual. In a second step permeate was recovered and retentate was diluted with deionized water and washed by another centrifugation step. All permeate was collected and concentrated using a 5 kDa filter unit.

2.4.7 Hydrophobic interaction chromatography

For purification of FcHyd5p from cultures of the transformed yeast strain *P. pastoris* X33 [pPICZαA-FcHyd5] cell free supernatant was dialyzed overnight against start buffer using tubes with a molecular weight cut off of 3500 Da (Serva Electrophoresis GmbH, Heidelberg, Germany). Prior to separation samples were centrifuged at 13000 x g and room temperature for 10 minutes to remove any precipitated proteins and particles present. Injection was performed using 5 mL sample. Cell free supernatant of a culture of the unmodified strain *P. pastoris* X33 was also subjected to HIC as negative control. Chromatography was performed on a Biologic HR FPLC system (Bio-Rad Laboratories, Hercules, CA, USA) using a HiLoad 16/10 Phenyl Sepharose High Performance column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). The protocol for HIC followed the steps as shown below. As start buffer 1 M ammonium sulfate, 100 mM Tris, pH 7.0 was used. Elution buffer was identical but ammonium sulfate was omitted. Both buffers as well as the deionized water as used in step 6 were filtered at a pore size of 0.2 µm and degassed by application of vacuum prior to application.

Step	mL	Action	
1	0	Set zero base line	UV Detector
2	0	UV Lamp	Turn on
3	0	Load/inject sample	1.6 mL/min; 5 mL

4	5	Isocratic flow	3.2 mL/min; 30 mL;	Start buffer 100 % Elution buffer 0 %%
5	35	Linear gradient	3.2 mL/min; 200 mL;	Start buffer 100 % →0 % Elution buffer 0 % →100 %
6	235	Isocratic flow	3.2 mL/min; 60 mL	H_2O
7	295	End of protocol		

Detection of eluting proteins was performed using a UV detector at 280 nm. Fractions were collected with a fraction collector (Model 2128, Bio-Rad Laboratories, Hercules, CA, USA) from 195 mL up to 295 mL. Fraction volume was 2 mL each. Fractions containing eminent peaks in the chromatogram were stored at 4°C and subsequently subjected to SDS-PAGE to screen the molecular weight of the contained proteins. Additionally, from all fractions of the run protein concentrations were measured using the Bio-Rad Protein Assay (Bradford-test, Bio-Rad Laboratories, Hercules, CA, USA).

2.4.8 Isolation of nsLTP1 from barley

Fractionated precipitation of nsLTP1 from barley using ammonium sulfate was performed according to Leisegang and Stahl (2005). 250 g of barley (*H. vulgare* cv. Marnie) were ground in a DLFU disk mill (Bühler, Braunschweig, Germany) at a gap width of 0.2 mm. The raw flour was suspended in 1250 mL deionized water and stirred overnight at 4°C for a sufficient extraction of water soluble proteins. Subsequently, particles were removed by centrifugation at 6000 x g for 30 minutes. For fractionated precipitation ammonium sulfate was added to the supernatant until a saturation of 40 % was reached. Solution was stirred for 4 hours at 4°C and precipitated proteins were removed by centrifugation. The relevant protein fraction was obtained by increasing ammonium sulfate saturation to 75°% followed by a precipitation step overnight at 4°C on a stirrer. After centrifugation at 6000 x g for 30 minutes supernatant was discarded and the pellet resuspended in 40 mL deionized water. Amounts of ammonium sulfate necessary for precipitation were calculated according to temperature and volume increase. Further purification of nsLTP1 was performed by HIC on a Biologic HR FPLC system (Bio-Rad Laboratories, Hercules, CA, USA) using a HiLoad 16/10 Phenyl Sepharose High Performance column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). After fractionated precipitation by ammonium sulfate as described above, the pellet was resuspended in start buffer (HIC) (100 mM disodium hydrogen phosphate; 1.5 M ammonium sulfate; pH 7.0) instead of deionized water and centrifuged at 13000 x g for removal of particles and precipitated proteins. For injection 5 mL of resuspended nsLTP1 were used. Elution was performed with elution buffer (HIC), which

was identical to start buffer (HIC) but without ammonium sulfate. Separation was performed according to the HIC protocol as shown above. Fractions at eminent peaks were examined by SDS-PAGE. A second approach for further purification of nsLTP1 was performed by anion exchange chromatography (IEX) using a HiLoad 16/10 Q Sepharose High Performance column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). In order to remove residual ammonium sulfate the protein extract obtained from ammonium sulfate fractionation was dialyzed against deionized water overnight. Subsequently it was dialyzed overnight against start buffer (IEX) at pH 11.0 in correspondence to the *I*p of nsLTP1 (*I*p = 8.2) in order to provide a negative net charge of the protein. Column was handled according to the manufacturer´s instructions. Protein solution and buffers were filtered (0.2 µm) to remove particles and degassed by application of vacuum. Prior to injection pH of protein solution was checked and reset to pH 11.0 if necessary. After equilibration of the column, 5 mL of the protein solution were loaded into the sample loop. As elution buffer (IEX) start buffer (IEX) with addition of 1 M sodium chloride was used. FPLC run was performed comprising the steps as shown below.

<table>
<tr><th>Step</th><th>mL</th><th>Action</th><th></th><th></th></tr>
<tr><td>1</td><td>0</td><td>Set zero base line</td><td>UV Detector</td><td></td></tr>
<tr><td>2</td><td>0</td><td>UV Lamp</td><td>Turn on</td><td></td></tr>
<tr><td>3</td><td>0</td><td>Load/inject sample</td><td>1.6 mL/min; 5 mL</td><td></td></tr>
<tr><td rowspan="2">4</td><td rowspan="2">5</td><td rowspan="2">Isocratic flow</td><td rowspan="2">3.2 mL/min; 30 mL;</td><td>Start buffer 100 %</td></tr>
<tr><td>Elution buffer 0 %%</td></tr>
<tr><td rowspan="2">5</td><td rowspan="2">35</td><td rowspan="2">Linear gradient</td><td rowspan="2">3.2 mL/min; 250 mL;</td><td>Start buffer 100 % →0 %</td></tr>
<tr><td>Elution buffer 0 % →100 %</td></tr>
<tr><td>6</td><td>285</td><td>End of protocol</td><td></td><td></td></tr>
</table>

Detection of eluting proteins was performed using a UV detector at 280 nm. Fractions were collected with a fraction collector (Model 2128, Bio-Rad Laboratories, Hercules, CA, USA) from 0 mL up to 180 mL. Fraction volume was 2 mL each. Fractions at eminent peaks in the chromatogram were stored at 4°C and subsequently subjected to SDS-PAGE to screen the molecular weight of the contained proteins.

2.5 Freeze drying of dialyzed culture supernatant

Culture supernatants containing the secreted proteins were freeze dried to concentrate proteins and to enhance storability and handling. After dialysis supernatants were filled in 50 mL Sarstedt tubes and centrifuged at 5000 x g for 15 min and 21°C. Prior to freeze drying samples were transferred into round bottom flasks and frozen in an ethanol bath at permanent rotation to minimize the thickness of the ice layer of frozen protein solution in the flask. Temperature of the ethanol bath was -17°C. Thoroughly frozen samples immediately were fixed to the outside of the drying unit and connected to the vacuum. Thawing of samples was prevented by precooling of the lyophilisator to -40°C. Freeze drying was stopped, when samples were apparently dry and no water condensation was visible at the outside of the flasks. Lyophilisate was collected and stored in sealed flasks at room temperature until further processing.

2.6 Gushing experiments with carbonated beverages

For examination of their behavior in carbonated beverages recombinant proteins were added to several different lots of bottled German lager beer. During set up of the experiments, lager beer from different German breweries were used for comparison. Beer from one brewery was chosen and used throughout the experiments. Also carbonated water (Bonaqa®) as used for the modified Carlsberg test (Radau *et al.*, 1995) and several carbonated fruit juices from retail markets were used for the gushing experiments. Since gushing instability was found in some beer lots, the gushing tendency of each lot was tested before purchasing greater quantities. This was performed by rotating bottles for 16 h at ambient temperature in a modified cement mixer (Atika, Ahlen, Germany) at 28 rpm and opening after 1 h of rest. Only beer lots that showed no over-foaming after the treatment were used for further experiments. Prior to addition of the recombinant proteins bottles were chilled to 0°C in an ice-salt bath to minimize CO_2 loss upon opening. After opening, transgenic proteins were added and bottles were resealed with sterile crown caps. If lyophilisate was added, it was aliquoted in reaction tubes containing the desired amount each and poured into the corresponding bottle. If a solution of hydrophobins was added, bottles were inoculated from a stock solution, which was stirred thorough the experiment to guarantee a homogeneous distribution of hydrophobin molecules in the solution. Dissolved proteins were added to the bottles using pipettes. Prior to addition 4-5 mm were cut away from the pipette tips to enlarge the outlet hole. Thereby, the formation of bubbles due to the rapid injection of hydrophobin solution could be reduced. In order to provide and to enhance the formation of gas bubbles in treated and resealed beverages the gas volume of

the head space was led through the beverage again and again. This was performed by rotation over head for 16 hours at ambient temperature (28 rpm). After one hour of rest bottles were weighed and opened to determine the gushing potential. Loss of volume was measured by reweighing bottles after opening.

2.7 Addition of hop products to hydrophobin treated beverages

To investigate the influence of hop on hydrophobin treated beer, hop products were added to beer alone or in combination with transgenic hydrophobins. Since it was impossible to obtain hop free beer only bottom fermented German lager beer containing hop was used for the tests. The following hop products were tested: Linalool 97 % (Sigma–Aldrich, Steinheim, Germany), Hop Oil Type Dry (complete range of hop essential oils, main components are myrcene, humulene and caryophyllene; HOTD); Rho 35 % (potassium salts of reduced iso-α-acids); Tetra 10 % (potassium salts of tetrahydro-iso-α-acids); Iso 30 % (isomerized α-acids) (all products above obtained from Hopsteiner, Mainburg, Germany); Effects of hop compounds on gushing were calculated as percentaged change in lost beer volume. For comparison, the gushing volume of reference bottles containing only the respective hydrophobin was set 100 % as reference. The amounts of hop components added were based on the recommendations of the manufacturer, for HOTD amounts were higher than the recommended concentrations. For comparison with hop oil type dry amounts of linalool were also higher than recommended. Apart from beer hop compounds were also tested in carbonated water containing hydrophobin. This was done to investigate the ability of each hop compound to influence gushing in carbonated water as a model system.

2.8 Determination of foam stability

The ability to stabilize bubbles is an important feature for potential gushing-inducing proteins. As an indirect way to test proteins for this property the foam-building and -stabilizing capacity was evaluated. This was performed for all recombinant proteins, which were used in this study by direct testing the cell free spent culture media of the yeast clones studied here. Since the transformed yeasts were cultivated in a protein free medium and *P. pastoris* is known to secrete only very small amounts of native proteins, the majority of protein in the medium can be assumed to be mainly represented by the protein of the transformed gene (Barr *et al.*, 1992). After removal of cells by centrifugation at 6000 x g for 30 minutes, culture media were divided in 50 mL aliquots in flasks. Prior to the test one aliquot of each culture medium was heat treated by incubation in boiling water for 1 hour.

Homogenization of heat treated and untreated culture supernatant was performed using a rotor-stator system (Miccra D-8, ART Prozess- & Labortechnik, Mühlheim, Germany) for 1 minute at 10500 rpm. Subsequent foam degradation and clearing up of the culture supernatant in each flask was examined.

2.9 Ultrasonication treatment of FcHyd5p solution

Due to the tendency of hydrophobins to self-assemble, ultrasonication was applied as a disintegrative condition to mechanically separate hydrophobin polymers. Treated samples were used for gushing tests to examine the influence of ultrasonication on the gushing potential of class II hydrophobin FcHyd5p. Freeze dried FcHyd5p was dissolved in deionized water to a concentration of 2 mg per mL and subjected to ultrasonication treatment. This was performed using an ultrasonic probe (Sonoplus, Bandelin, Berlin, Germany) or an ultrasonic bath (Sonorex, Bandelin, Berlin, Germany). For the ultrasonic probe, two repetitions of 30 s each were applied at 70 % power and 7 x 10 % cycle, while samples were kept on ice. For the ultrasonic bath, samples were placed on a floater in the bath and treated for 3 min at room temperature with permanent operation. The FcHyd5p solutions were treated immediately prior to addition to beer or other carbonated beverages to minimize the risk of re-aggregation.

2.10 Heat treatment of surface-active proteins

To test the heat stability of the transgenic proteins and to imitate conditions as prevailing under wort boiling, FcHyd5p, FcHyd3p, AfpA, Hfb2 and nsLTP1 were subjected to thermal treatment. Lyophilisate was dissolved in deionized water to a concentration of 2 mg per mL. Samples were filled in glass flasks, tightly closed and incubated in boiling water for 1 hour. Since it was impossible to remove all proteins from conventional wort, lyophilisate was also boiled as a solution in a protein free synthetic wort. This wort contained acid and sugar components in their characteristic concentrations and was mixed according to Zapf (2006a). Prior to addition to carbonated beverages, treated samples were cooled down to room temperature and flasks were carefully inverted for several times to provide a homogeneous solution of hydrophobins.

3 Results

3.1 Isolation and purification of FcHyd5p

An isolation procedure for the class II hydrophobin FcHyd5p was designed with two objectives. One aim was, to purify the protein. The second aim was to obtain sufficient amounts of this protein to test its potential to induce gushing in carbonated beverages and to examine its potential as a gushing inducer in beer. *P. pastoris* X33 [pPICZαA-FcHyd5] was cultivated in a buffered complex medium containing methanol as inducer of gene expression (BMGY). For examination of the culture medium for the excreted protein samples were taken each day of cultivation and subjected to SDS-PAGE. After separation of the cell free supernatant combined with colloidal coomassie blue staining (Roti-Blue) no bands could be seen on the gel at the expected molecular weight of 8.4 kDa or at 10 kDa representing FcHyd5p with signal peptide for secretion. Several alternative staining techniques were applied for visualization of the recombinant protein comprising hot coomassie staining, silver staining, lumitein staining and negative staining. However, none of the staining protocols used gave rise to a detectable protein band of the expected size. In order to break down possible aggregates of FcHyd5p and to improve its accessibility to staining protocols, samples were mixed 1:1 with 100 % trifluoroacetic acid (TFA). After removal of TFA with nitrogen the remaining dry matter was resuspended in deionized water and separated by electrophoresis. Again, no corresponding band could be detected on the gel indicating that either no proteins had been secreted by the yeast clone or the concentration of recombinant protein in the culture medium was too low to be detected. In a second approach, cultivation was performed in a protein free culture medium. Cells were removed after cultivation and the supernatant dialyzed and concentrated by freeze drying. The white lyophilisate obtained proved to be soluble in water. The protein powder was dissolved in deionized water and again subjected to SDS-PAGE. At concentrations higher than 10 mg per mL a band was detected between 6.5 and 12.5 kDa when the gel was stained with colloidal coomassie blue. For comparison the *P. pastoris* X33 wild type strain containing no hydrophobin gene also was cultivated in protein free medium and the cell free supernatant processed in the same way and subjected to SDS-PAGE. No bands were detected in the low molecular weight area after separation of this lyophilisate. From the results obtained it was assumed that the band observed in the lyophilisate of the transformant *P. pastoris* X33 [pPICZαA-FcHyd5] represented hydrophobin FcHyd5p. Comparison of the yield of lyophilisate from both strains revealed significantly higher amounts in the transformed yeast. An examination of several production cycles of FcHyd5p lyophilisate from *P. pastoris* X33 [pPICZαA-FcHyd5] and lyophilisate from the supernatant

of *P. pastoris* X33 wild type resulted in an average yield of 200-400 % lyophilisate from the transgenic yeast as compared to the amount of lyophilisate obtained from *P. pastoris* X33 (= 50-75 % FcHyd5p in lyophilisate of *P. pastoris* X33 [pPICZαA-FcHyd5]). Due to the high amounts needed to obtain a visible protein band of FcHyd5p upon colloidal coomassie blue staining, FcHyd5p lyophilisate was again subjected to SDS-PAGE and stained with silver. The gels obtained showed no bands at the expected molecular weight, indicating that FcHyd5p was not accessible to the silver staining protocol as applied. Figure 6 shows a colloidal coomassie stained gel after separation of lyophilisate from both yeast strains. The sample from the transformed yeast strain (FcHyd5p) contains a band between 6.5 and 12.5 kDa. This corresponds to the size of the monomeric molecule of the class II hydrophobin FcHyd5p.

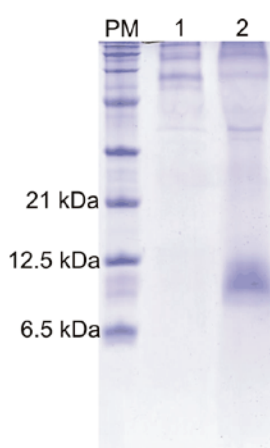

Figure 6: SDS-PAGE of lyophilisate obtained from *P. pastoris* X33 (1) and *P. pastoris* X33 [pPICZαA-FcHyd5] (2) after colloidal coomassie blue staining; PM = protein marker (Serva, Heidelberg, Germany);

This singular band in the low molecular weight area was observed in several subsequent SDS-PAGEs made with FcHyd5p lyophilisate upon protein production. However, in some cases a shift to higher protein masses was detected.

Culture supernatants of the FcHy5p producing *P. pastoris* strain and the unmodified strain were subjected to hydrophobic interaction chromatography (HIC) in combination with UV-detection at 280 nm for further purification. In the HIC diagrams obtained from both supernatants no differences between transformant and the wild type could be detected. Therefore, no further purification was achieved by this method. A test on the absorption of FcHyd5p lyophilisate dissolved in deionized water revealed that the absorbance of the solution was very low at 280 nm. At 220 nm a much higher absorption was found as shown in Figure 7. However, this wavelength could not be applied during HIC. The relation between absorption and increasing amounts of FcHyd5p lyophilisate in deionized water for

both wavelengths 220 nm and 280 nm confirmed this finding as shown in Figure 8. A test of the fractions obtained from HIC using the Bradford test revealed no fractions with eminent protein contents.

An *in silico* determination of the absorption of FcHyd5p was performed using the online tool ProtParam (http://www.expasy.org/tools/protparam.html) for a 1 mg/mL solution at 280 nm. It resulted in an absorbtion of 0.394 when all cysteine residues were assumed to be paired in disulfide bonds and in 0.337 under the assumption that all cysteine residues were unpaired.

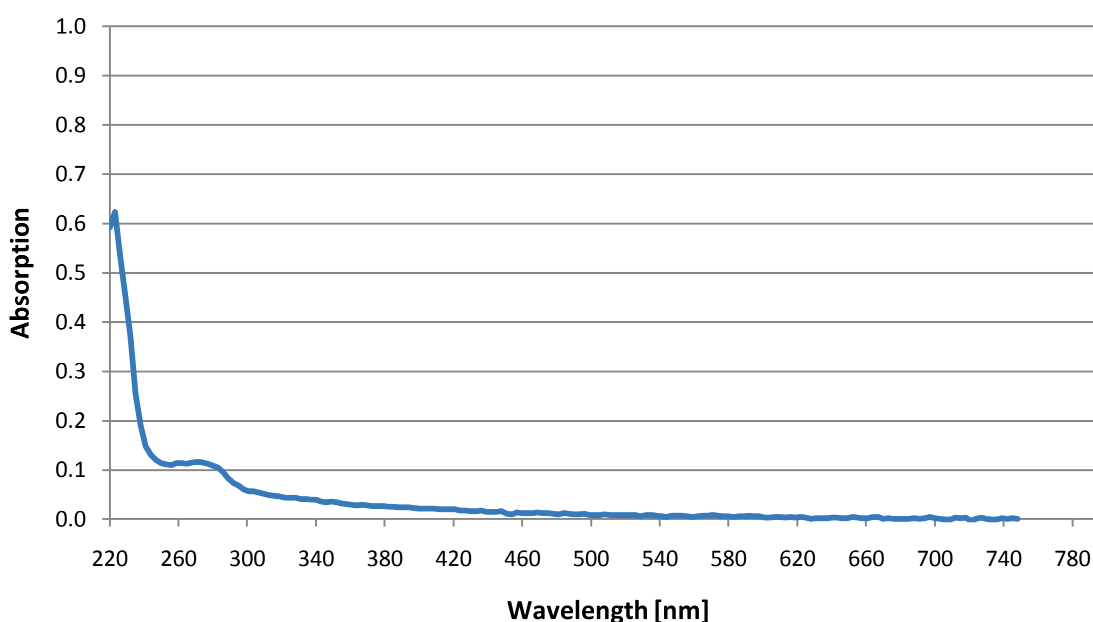

Figure 7: Wavelength scan of a 5 mg per mL solution of FcHyd5p lyophilisate in deionized water;

This calculation was performed assuming a complete cleavage of the signal peptide. Under the assumption that four amino acids of the signal peptide (EAEA) were left uncleaved at the N-terminus the absorption values changed slightly to 0.377 and 0.323, respectively. The actual absorption found by measuring a 1 mg/mL solution at 280 nm was 0.018. This finding indicated that the content of FcHyd5p in the solution was 5 % (w/w).

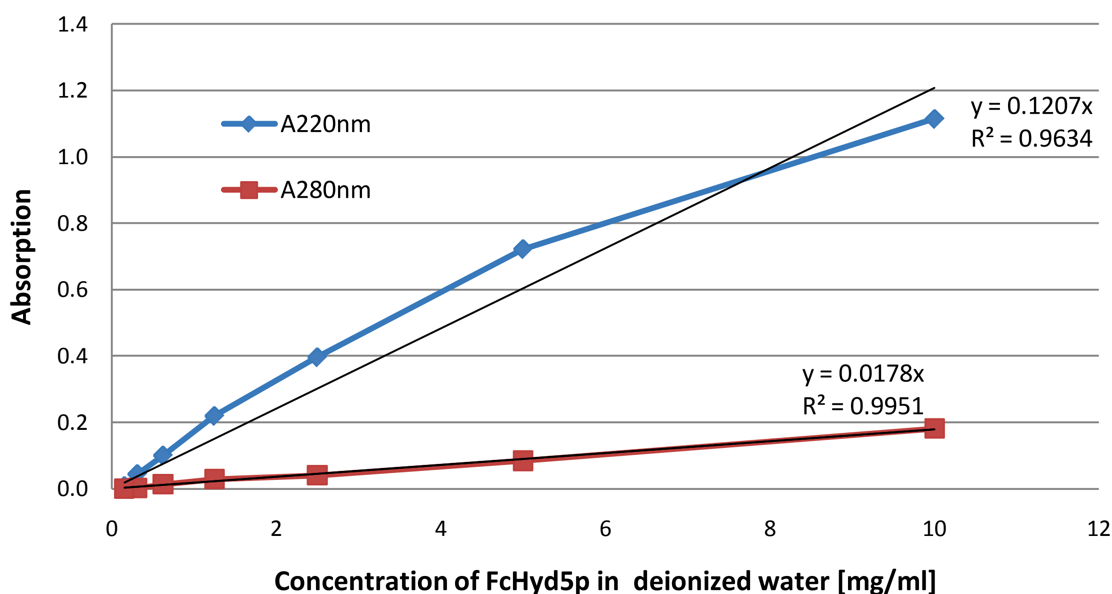

Figure 8: Absorption of increasing concentrations of FcHyd5p lyophilisate at two different wavelengths (220 and 280 nm);

3.2 Isolation and purification of nsLTP1 from barley

The beer foam protein nsLTP1 is central to the current discussion on inducers of beer gushing. In order to test its influence on over-foaming in carbonated beverages the protein was isolated from barley grains. This was accomplished by aqueous extraction and fractionized precipitation of the protein using ammonium sulfate. The proteins, which were recovered between 40 and 75 % saturation were resuspended in water and separated by gel electrophoresis. After colloidal coomassie blue staining, a band between 6.5 and 10 kDa was found on the gel. However, several other protein bands were still present. Also, it was observed that the band at the size of nsLTP1 became weaker and disappeared, if the protein was stored for a few days at 4°C. To increase the purity and shelf life of nsLTP1 isolations, the protein fraction was subjected to a further purification step using HIC on a FPLC system. It was loaded on a phenyl sepharose column in the presence of 1.5 M ammonium sulfate at pH 7.0 and eluted with the a buffer lacking ammonium sulfate. As shown in Figure 9 two major protein peaks were obtained. When the fractions obtained from peak 1 were subjected to SDS-PAGE, it was found that still a lot of other proteins were contained in the sample as shown in Figure 10 (gel A). For further purification, proteins with molecular masses >30 kDa were removed using Amicon centrifugal filter units. Gel B in Figure 10 shows the result after concentrating the resulting protein solution. When the protein content of this sample was determined using the Bio-Rad Protein Assay,

an absorption equal to a concentration of 0.1 mg/mL BSA was found. Since the amount of purified nsLTP1 was too low to apply it to tests in carbonated beverages, IEX using a Q sepharose column was applied as another method for purification of nsLTP1. For this, the protein fraction was loaded on the column at pH 11 and eluted with an increasing gradient of elution buffer. After optimization of the chromatography protocol it was found that nsLTP1 eluted at 25 % elution It was revealed that around 25 % elution buffer actually two substances eluted from the column, whose peaks covered each other in the previous settings. A diagram of an IEX run obtained with the optimized purification protocol for nsLTP1 is shown in Figure 11. The two peaks mentioned above were assigned as peak 3 and peak 4. Analysis of their corresponding fractions by SDS-PAGE showed that both peaks contained proteins of a similar molecular weight. A western blot with antibodies directed against LTP1500 from wheat but showing cross-reactivity with barley nsLTP1, indicated that peak 4 could be assigned to nsLTP1. SDS-PAGEs of the protein fraction from ammonium sulfate precipitation and from peak 3 and 4 from IEX as well as a western blot from peak 3 and 4 are shown in Figure 12.

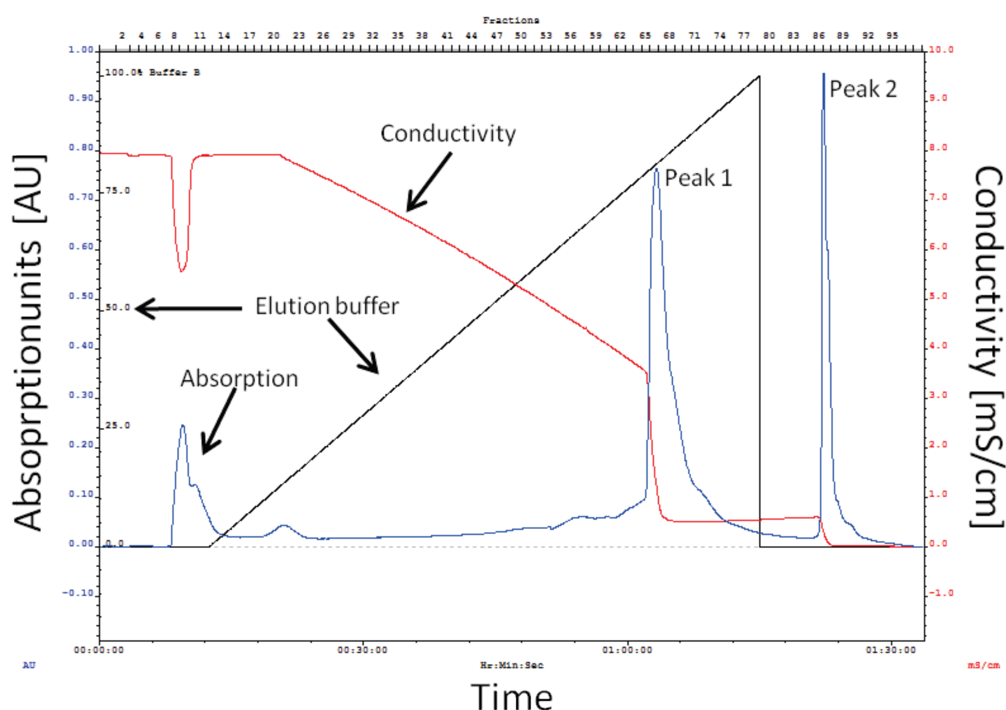

Figure 9: Diagram of hydrophobic interaction chromatography of barley-water extract fractionized by ammonium sulfate precipitation; Peak 1 containing nsLTP1;

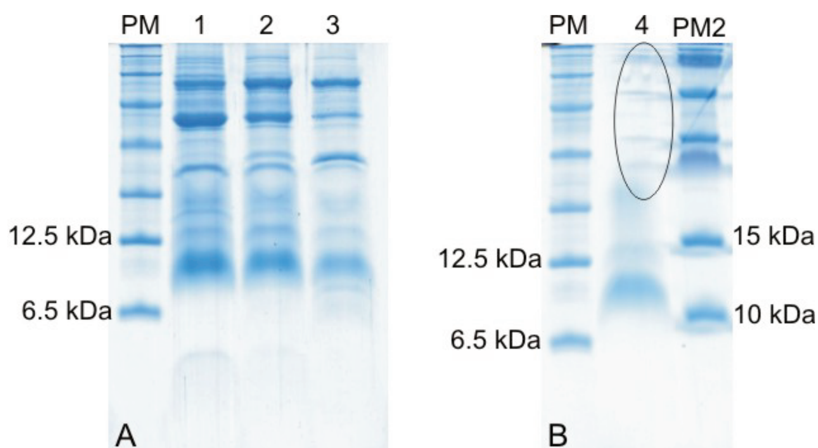

Figure 10: Gel A = SDS-PAGE of fractions collected from peak 1 of hydrophobic interaction chromatography of barley-water extract fractionized by ammonium sulfate precipitation at 40-75 % saturation (1, 2, 3). Lowest band in each lane represents nsLTP1. Remaining bands represent barley proteins, which eluted at the same percentage of elution buffer. PM = protein marker (Serva, Heidelberg, Germany); Gel B = SDS-PAGE after removal of proteins great than 30 kDa and concentration of protein solution using Amicon centrifugal filter units; Weak bands in lane 4 (marked with an ellipse) represent contaminations from neighboring lane; PM2 = protein marker (Fermentas, St. Leon-Rot, Germany);

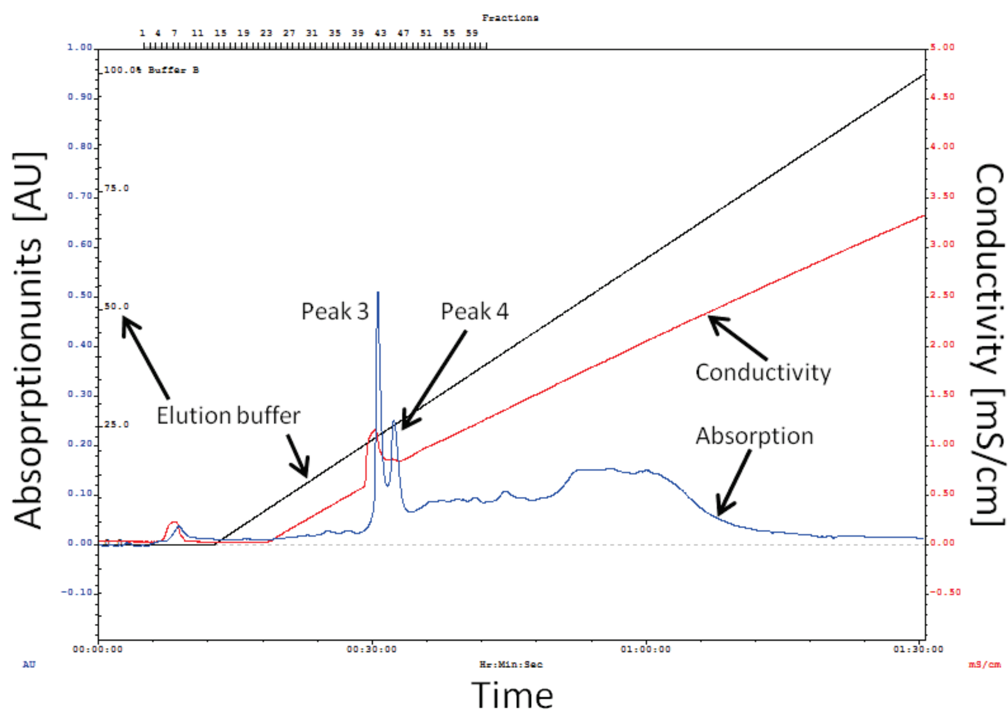

Figure 11: Diagram of anion exchange chromatography of barley-water extract fractionized by ammonium sulfate precipitation; Peak 4, which elutes at 25 % of buffer B, represents nsLTP1;

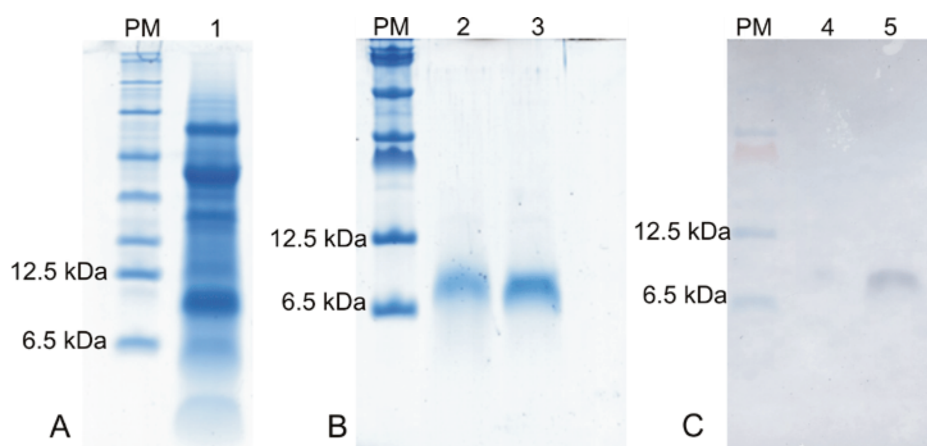

Figure 12: SDS-PAGE of nsLTP1 obtained from fractionated ammonium precipitation (A) and after further purification by anion exchange chromatography (IEX) (B); gels were stained by colloidal coomassie blue; PM = protein marker (Serva, Heidelberg, Germany); 1 = fraction of aqueous extract of barley between 40 and 75 % ammonium sulfate saturation; 2 = peak 3 from (IEX) as shown in the diagram above; 3 = peak 4 from (IEX) as shown in the diagram above, confirmed as nsLTP1 by peptide mass finger print and western blot using antibodies against nsLTP1500 from wheat (C); 4 = peak 3 from (IEX); 5 = peak 4 from (IEX);

In order to proof the identity of peak 4 from IEX with nsLTP1, a peptide mass finger print of the corresponding protein band was made after tryptic digestion. The protein band as shown in Figure 12 gel B lane 3 was excised from the acrylamide gel and subjected to the analysis. Out of eight theoretical peptides, five were found by mass spectroscopy resulting in a sequence coverage of 68 % relative to the amino acid sequence of nsLTP1. The amino acid sequence of nsLTP1 from barley is shown below. Underlined letters indicate those peptides, which were identified in the analyzed sample.

L N C G Q V D S K M K P C L T Y V Q G G P G P S G E C C N G V R D L H N Q A Q S S G D R Q T V C N C L K G I A R G I H N L N L N N A A S I P S K C N V N V P Y T I S P D I D C S R I Y

Purified nsLTP1 showed better shelf life than the protein fraction obtained by ammonium sulfate precipitation. However, the yield obtained by extraction from barley grain was not sufficient for an application of the purified protein to test its influence on carbonated beverages. Even further concentrating of the IEX-purified nsLTP1 by using Amicon centrifugal filter units resulted in an absorption equal to only 0.25 mg/mL BSA when measured with the Bio-Rad Protein Assay.

3.3 Cloning of surface-active proteins in *P. pastoris*

In order to produce high quantities and to evaluate their gushing-inducing potential, the class I hydrophobin FcHyd3p from *F. culmorum*, the class II hydrophobin Hfb2 from *T. reesei*, the alkaline foam protein A (AfpA) from *F. graminearum* and nsLTP1 from *H. vulgare* cv. Marnie (barley) were heterologously expressed in *P. pastoris*. In the sections transformation experiments and the resulting transgenic proteins are described.

3.3.1 Class I hydrophobin FcHyd3p from *F. culmorum*

For transformation of the *fchyd3* gene from *F. culmorum* in *P. pastoris* a modified nucleotide sequence was used, which was adapted to the codon-usage for *S. cerevisiae* for optimal expression in yeast. The intron free nucleotide sequences of the original and the modified gene are shown in Figure 13. The upper line represents the original gene, whereas the modified version is aligned below for comparison. Positions with identical nucleotides in both sequences are marked with an asterisk.

```
fchyd3_orig   ATGCAGTTCTCTACTCTCACCACTGTCTTCGCCCTCGTCGCCGCTGCCGTCGCTGCTCCC 60
fchyd3_mod    ATGCAATTTTCTACTTTGACTACAGTTTTTGCTTTGGTTGCTGCTGCTGTTGCTGCTCCA 60
              ***** ** ****** * ** ** ** ** **  * ** ** ***** ** ********

fchyd3_orig   CACGGCAGCAGCGGAGGCAACAACCCCGTCTGCTCTGCTCAGAACAACCAGGTCTGCTGT 120
fchyd3_mod    CATGGTTCTTCAGGTGGTAACAATCCAGTTTGTTCTGCTCAAAACAATCAAGTTTGCTGT 120
              ** **       ** ** ***** ** ** ** ******** ***** ** ** ******

fchyd3_orig   AACGGACTCCTCAGCTGTGCCGTCCAGGTTCTCGGCAGCAACTGCAATGGCAACGCTTAC 180
fchyd3_mod    AATGGTTTATTGTCTTGTGCTGTTCAAGTTTTGGGTTCTAATTGTAATGGTAATGCTTAT 180
              ** **  *  *    ***** ** ** *** * **    ** ** ***** ** *****

fchyd3_orig   TGCTGCAACACTGAGGCCCCTACTGGCACTCTCATCAACGTTGCTCTCCTCAACTGTGTC 240
fchyd3_mod    TGTTGTAATACTGAAGCTCCAACTGGTACTTTGATTAATGTTGCTTTGTTGAATTGTGTT 240
              ** ** ** ***** ** ** ***** *** * ** ** ****** *  * ** *****

fchyd3_orig   AAGCTCCTCTAG 252
fchyd3_mod    AAATTGTTGTAA 252
              **  *  * **
```

Figure 13: Comparison of original and modified nucleotide sequence of *fchyd3* from *F. culmorum*. Modification was applied to achieve an optimal expression of the *fchyd3* gene in *P. pastoris* by adapting it to the codon-usage of *S. cerevisiae*. *fchyd3_orig* = original nucleotide sequence, *fchyd3_mod* = modified nucleotide sequence;

After *in silico* translation of the nucleotide sequence into the corresponding amino acid sequence, the signal peptide of FcHyd3p was predicted to have a length of 18 amino acids with a corresponding cleavage site between AVA – APH. The total length of the amino acid

of FcHyd3p without signal peptide was found to be 65 amino acids resulting in a molecular weight of 6.5 kDa and a theoretical isoelectric point of 6.7. In the amino acid sequence shown below the sequence of the signal peptide is written in bold letters.

M Q F S T L T T V F A L V A A A V A A P H G S S G G N N P V C S A Q N N Q V C C N G L L S C A V Q V L G S N C N G N A Y C C N T E A P T G T L I N V A L L N C V K L L

Since the gene was integrated into the plasmid pPICZαA behind the *S. cerevisiae* secretion factor, which is known to be recognized by *P. pastoris*, the nucleotide sequence of *fchyd3* was used starting at nucleotide 55 (GCTCCA...) omitting the original signal peptide. After cloning of pPICZαA containing *fchyd3* into *E. coli,* clones were picked from LB-agar plates with Zeocin added for selection of positive transformation events. Clones, which were taken for plasmid back up and multiplication were streaked on LB-Zeocin agar plates in 3 generations to ensure that the cells carry the plasmid pPICZαA with the *Sh ble* gene conferring resistance against Zeocin. After plasmid isolation from these clones, proper incorporation of the *fchyd3* gene into the plasmid and sequence identity with the optimized gene could be confirmed by sequence analysis using the primer pair AOX1-primer_f and AOX1-primer_r (see Table 1 for sequences). After successful transformation of *P. pastoris* X33 with the linearized plasmid and selection of Zeocin resistant yeast clones, sequence identity was again confirmed by sequence analysis of the 605 bp amplicon obtained by using yeast clone genomic DNA and primer pair AOX1-primer_f and AOX1-primer_r for PCR amplification. Confirmed FcHyd3p yeast clones were used for protein production experiments. The lyophilisate obtained after cultivation, dialysis of cell free supernatant and freeze drying was subjected to SDS-PAGE. No bands of the expected size could be detected after separation and coomassie blue and silver staining. Since no distinct protein band was obtained after electrophoresis, expression and secretion of FcHyd3p by the tested transformant cultures could not be verified by peptide mass finger printing. However, production of FcHyd3p was confirmed indirectly by analyzing transcription of the *fchyd3* gene. RNA isolation combined with cDNA generation showed that *fchyd3* was expressed following induction of the alcohol oxygenase promoter AOX1 by addition of methanol into the growth medium. In Figure 14 amplicons obtained from PCR with DNA and RNA samples using primer pair EcoR1-FcHyd3-f and FcHyd3-Age1-r are shown. Lane 4, in which products from PCR using untreated RNA as template were applied, contained a band of 218 bp, indicating that DNA contaminations were still present in the isolated RNA sample. In contrast to this, no band of the size of *fchyd3* was observed in lane 1, in which PCR products of DNAse treated RNA had been applied. Positive results are shown in lanes

2 and 3, which contain products from PCR using cDNA made from DNAse treated RNA (lane 2) and plasmid pPICZαA containing *fchyd3* as positive control (lane 3).

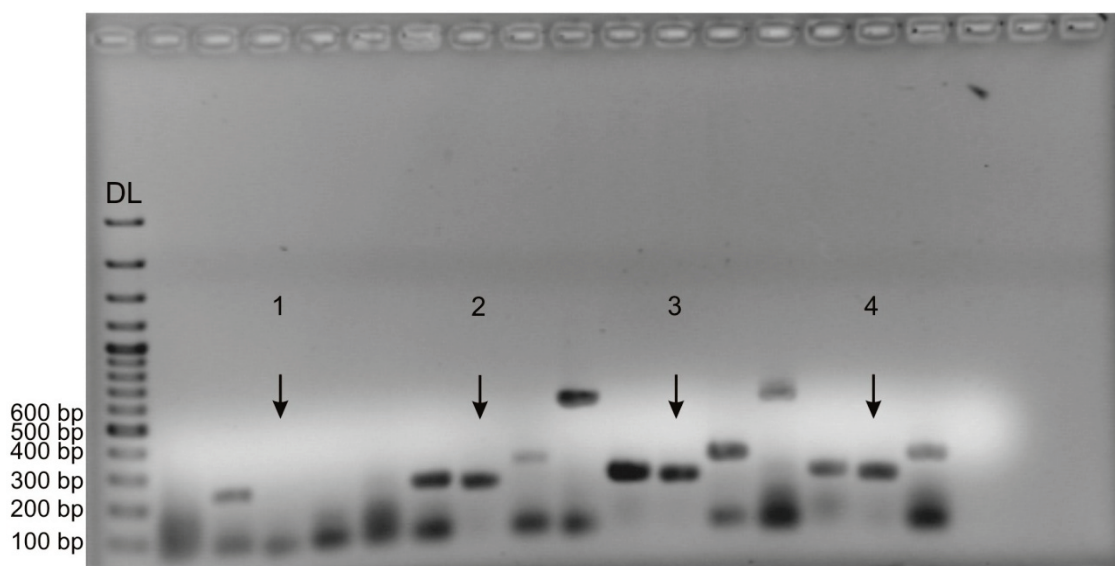

Figure 14: Verification of gene expression for *fchyd3* using reverse transcription PCR; Agarose gel electrophoresis of following PCR amplicons; DL = DNA ladder; 1 = PCR with RNA after DNAse treatment; 2 = PCR with cDNA (transcribed from DNAse treated RNA); 3 = PCR with plasmid DNA containing *fchyd3* (positive control); 4 = PCR with RNA (without DNAse treatment);

3.3.2 Class II hydrophobin Hfb2 from *T. reesei*

For transformation of *hfb2* from *T. reesei* in *P. pastoris* a modified nucleotide sequence with adaption to the codon-usage of *S. cerevisiae* for optimal expression in yeast was used. The intron free sequences of the original and the modified gene are shown in Figure 15. The upper line represents the original gene, whereas the modified version is aligned below for comparison. Positions with identical nucleotides in both sequences are marked with an asterisk.

```
hfb2_orig    ATGCAGTTCTTCGCCGTCGCCCTCTTCGCCACCAGCGCCCTGGCTGCTGTCTGCCCTACC 60
hfb2_mod     ATGCAGTTCTTCGCTGTTGCTTTGTTCGCTACTTCCGCTTTGGCTGCTGTTTGTCCAACT 60
             ************** ** **  * ***** **   ***  ********** ** ** **

hfb2_orig    GGCCTCTTCTCCAACCCTCTGTGCTGTGCCACCAACGTCCTCGACCTCATTGGCGTTGAC 120
hfb2_mod     GGTTTGTTCTCCAACCCATTGTGTTGTGCTACAAACGTTTTGGACTTGATCGGTGTTGAC 120
             **  * ***********  **** ***** ** *****  * *** * ** ** ******

hfb2_orig    TGCAAGACCCCTACCATCGCCGTCGACACTGGCGCCATCTTCCAGGCTCACTGTGCCAGC 180
hfb2_mod     TGTAAGACTCCAACTATCGCTGTTGACACTGGTGCTATTTTCCAGGCTCACTGTGCTTCT 180
             ** ***** ** ** ***** ** ******** ** ** *****************
```

```
hfb2_orig    AAGGGCTCCAAGCCTCTTTGCTGCGTTGCTCCCGTGGCCGACCAGGCTCTCCTGTGCCAG 240
hfb2_mod     AAGGGTTCCAAGCCTTTGTGCTGTGTTGCTCCAGTTGCTGACCAGGCTTTGTTGTGTCAG 240
             ***** ********* * ***** ******** ** ** ********* *  **** ***

hfb2_orig    AAGGCCATCGGCACCTTCTAA 261
hfb2_mod     AAGGCTATCGGTACTTTCTAA 261
             ***** ***** ** ******
```

Figure 15: Comparison of original and modified nucleotide sequence of *hfb2* from *T. reesei*. Modification was performed for an optimal expression in *S. cerevisiae* by adapting the codon usage. *hfb2_orig* = original nucleotide sequence, *hfb2_mod* = modified nucleotide sequence; Positions with identical nucleotides in both sequences are marked with an asterisk.

After *in silico* translation of the nucleotide sequence into the corresponding amino acid sequence, the signal peptide of Hfb2 was predicted to have a length of 15 amino acids with a cleavage site between ALA – AVC. The total length of Hfb2 without signal peptide was found to be 71 amino acids resulting in a molecular weight of 7.2 kDa and a theoretical isoelectric point of 6.7. In the amino acid sequence shown below, the sequence of the signal peptide is written in bold letters.

M Q F F A V A L F A T S A L A A V C P T G L F S N P L C C A T N V L D L I G V D C K T P T I A V D T G A I F Q A H C A S K G S K P L C C V A P V A D Q A L L C Q K A I G T F

Since the gene was integrated into plasmid pPICZαA having the secretion factor of *S. cerevisiae*, which is known to be recognized by *P. pastoris*, gene *hfb2* was transformed starting at nucleotide 46 (GCTGTT...) omitting the original signal peptide. Following cloning of pPICZαA containing *hfb2* into *E. coli*, clones were picked from LB-agar plates with Zeocin added for selection of positive transformation events. Clones, which were used for plasmid back up and multiplication, were streaked on LB-Zeocin agar plates in three generations to ensure that the cells carry the plasmid pPICZαA with the *Sh ble* gene conferring resistance against Zeocin. After plasmid isolation from these clones, proper incorporation of the *hfb2* gene into the plasmid and sequence identity with the optimized gene were confirmed by sequence analysis using the primer pair AOX1-primer_f and AOX1-primer_r. After successful transformation of *P. pastoris* X33 with the linearized plasmid and selection of Zeocin resistant yeast clones, sequence identity was confirmed as well by sequence analysis of the 748 bp amplicon obtained by using yeast clone genomic DNA and primer AOX1-primer_f and AOX1-primer_r for PCR. Confirmed Hfb2 yeast clones were used for protein production experiments. Lyophilisate, which was obtained after

cultivation, dialysis of cell free supernatant and freeze drying, was subjected to SDS-PAGE. Hfb2 was well stainable by colloidal coomassie blue. The recombinant protein also proofed to be accessible to silver staining. Verification of the identity of the protein secreted by *P. pastoris* clones with Hfb2 was accomplished by peptide mass finger printing of the corresponding band cut from a SDS-PAGE gel after coomassie staining. After alkylation and tryptic digestion of proteins contained in the excised band, fragments generated by trypsin treatment were measured by mass spectrometry. Peptides detected are underlined in the amino acid sequence of Hfb2 given above. Omitting the natural signal peptide, 53.5 % of the amino acid sequence of Hfb2 as shown above could be recovered by the fragments found with peptide mass finger printing.

3.3.3 Non specific lipid transfer protein 1 (nsLTP1) from *H. vulgare* cv. Marnie

In contrast to cloning of *fchyd3* and *hfb2*, the *nsltp1* gene sequence used for cloning was not adapted to the codon-usage of *S. cerevisiae*. In order to recover the gene, DNA was isoltated from from barley sprouts. The intronic sequence present at the end of the sequence was removed from the nucleotide sequence of *nsltp1* using PCR primers designed to bridge the intron and connect both exons of the gene. The intron free gene was multiplied by PCR using the primer pair EcoR1-LTP1-f and LTP1-Age1-r. Figure 16 shows the complete native nucleotide sequence of the *nsltp1* gene. Nucleotide sequence of the intron is underlined.

```
1      ATGGCCCGCG CTCAGGTACT GCTCATGGCC GCCGCCTTGG TGCTGATGCT CACGGCGGCC
61     CCGCGCGCTG CCGTGGCCCT CAACTGCGGC CAGGTTGACA GCAAGATGAA ACCTTGCCTG
121    ACCTACGTTC AGGGCGGCCC CGGCCCGTCC GGCGAATGCT GCAACGGCGT CAGGGATCTC
181    CATAACCAGG CGCAATCCTC GGGCGACCGC CAAACCGTTT GCAACTGCCT GAAGGGGATC
241    GCTCGCGGCA TCCACAATCT CAACCTCAAC AACGCCGCCA GCATCCCCTC CAAGTGCAAT
301    GTCAACGTCC CATACACCAT CAGCCCCGAC ATCGACTGCT CCAGGTGATT AAATTTACAC
361    TCATCCAGAG TGAAATCTTT AAAAAGAACT ATATTTACGA ACGGAGTGAG TATATAGGAA
421    CATTCATCCA CGTAAAATTT GTTGATATTA ACATTAACAC GCATGATTGA CCTGCAGGAT
481    TTACTGA
```

Figure 16: Nucleotide sequence of the native *nsltp1* gene from *H. vulgare* (barley). The contained intron is underlined;

According to *in silico* translation of the nucleotide sequence into the corresponding amino acid sequence, the signal peptide of nsLTP1 was predicted to have a length of 26 amino acids with a cleavage site between AVA – LNC. The total length of nsLTP1 without signal

peptide was found to be 91 amino acids resulting in a molecular weight of 9.7 kDa and a theoretical isoelectric point of 8.2. The sequence of the signal peptide is written in bold in the amino acid sequence shown below.

M A R A Q V L L M A A A L V L M L T A A P R A A V A L N C G Q V D S K M K P C L T Y V Q G G P G P S G E C C N G V R D L H N Q A Q S S G D R Q T V C N C L K G I A R G I H N L N L N N A A S I P S K C N V N V P Y T I S P D I D C S R I Y

Since the gene was integrated into plasmid pPICZαA having the secretion factor of *S. cerevisiae*, which is known to be recognized by *P. pastoris*, the *nsltp1* gene was transformed starting at nucleotide 79 (CTCAAC...) omitting the original signal peptide. After cloning of pPICZαA containing *nsltp1* into *E. coli*, clones were picked from LB-agar plates with Zeocin added for selection of positive transformation events. Clones, which were taken for plasmid back up and multiplication, were streaked on LB-Zeocin agar plates in 3 generations to ensure that the cells carry the plasmid pPICZαA with the *Sh ble* gene conferring resistance against Zeocin. Following plasmid isolation from these clones, proper incorporation of the *nsltp1* gene into the plasmid and sequence identity with the spliced gene was confirmed by sequence analysis using the primer pair AOX1-primer_f and AOX1-primer_r. After successful transformation of *P. pastoris* X33 with the linearized plasmid and selection of Zeocin resistant yeast clones, sequence identity was confirmed again by sequence analysis of the 683 bp amplicon obtained from amplifying yeast clone genomic DNA with primers AOX1-primer_f and AOX1-primer_r in a PCR. Confirmed nsLTP1 yeast clones were used in protein production experiments. Lyophilisate, which was obtained after cultivation, dialysis of cell free supernatant and freeze drying, was subjected to SDS-PAGE. As described under 3.2, nsLTP1 was moderately stainable with colloidal coomassie blue. The recombinant protein also proofed to be accessible to silver staining. Verification of protein expression and secretion was performed by peptide mass finger printing of an excised SDS-PAGE band of appropriate size after coomassie staining. Following alkylation and tryptic digestion of proteins contained in the excised band, fragments generated by trypsin treatment were measured by mass spectrometry. Recovered peptides are underlined in the amino acid sequence given above. Omitting the natural signal peptide resulted in recovery of 73.6 % of the amino acid sequence of nsLTP1.

3.3.4 Alkaline foam protein A from *F. graminearum*

Similar to barley nsLTP1, the *afpA* gene for the alkaline foam protein A (AfpA) from *F. graminearum* was cloned in *P. pastoris* without previous adaption of the nucleotide sequence to the codon usage of *S. cerevisiae*. Primers were designed on the basis of AfpA from *F. culmorum* omitting the native signal peptide. In order to obtain an intron free nucleotide sequence of the *F. graminearum afpA* gene, RNA was isolated from a *F. graminearum* culture and reversely transcribed into cDNA. After amplification of cDNA by PCR using primer EcoR1-AfpA-f and AfpA-Xba1_r, a band was observed at the expected size of 540 bp. The forward primer used was designed to omit the native signal peptide of the AfpA protein.

```
afpA_gram      GCCCCCGAGCCCAAGACCTTTGGTCTCGTCGCTCTTCGCTCAGGCAGCCCTTTCCACCTC 60
afpA_culm      GCCCCCGAGCCCAAGACCTTTGGTCTCGTCGCTCTCCGCTCAGGCAGTCCTTTCCACCTC 60
               *********************************** *********** ************

afpA_gram      TCCAGCGTCAGCGCCTCTGAGAGCGGCTTCTCGCTTCTCCTCCCCAAGGGCAAGCAGGGT 120
afpA_culm      TCCAGCGTCAGTGCCTCTGAGAGCGGCTTCTCGCTTCTCCTCCCCAAGGGCAAGCAGGGT 120
               *********** ************************************************

afpA_gram      GCCAAGTGCGCCGACAACAAGAAGGAGGACTTTGCTACTTTCCGCATCAGCAAGGACAAG 180
afpA_culm      GCCAAGTGCGTCGACAACAAGAAGGAGGACTTTGCTACTTTCCGCATCAGCAAGGACGGC 180
               ********** **********************************************

afpA_gram      AAGCTTGTCCTCTACCACAAGGGCAAGGAGCAGCAGATTGCCTACACTGACCGATCCGGC 240
afpA_culm      AAGCTTGTCCTCTACCACAAGGGCAAGGAGCAGCAGGTTGCCTACACTGACCGATCCGGC 240
               ************************************ ***********************

afpA_gram      ATGGGCCAAGGTGTTCTCCAGTACACTGGCCAGAAGAACTACCCTCGCAACGCCGAGACT 300
afpA_culm      ATGGGCCAAGGTGTTCTCCAGTACACTGGCCAGAAGAACTACCCTCGCAACGCCGAGACT 300
               ************************************************************

afpA_gram      GAGGGCTGGAAGGTCGACAAGGACGGCAACCTCGTCTTTGGCAGCAACAACGCTGGCTTC 360
afpA_culm      GAGGGCTGGAAGGTCGACAAGGACGGCAACCTCGTCTTTGGCAGCAACAACGCTGGCTTC 360
               ************************************************************

afpA_gram      ATGGCCTGCCCTGGTCTCAAGTCCACTGACCCCTGGAGCATCTGGGTCGCCACCGGTACC 420
afpA_culm      ATGGCCTGCCCTGGTCTCAAGTCCACTGACCCCTGGAGCATCTGGGTCGCCACCGGTACC 420
               ************************************************************

afpA_gram      GACCACCCCGGTAACAGCGAGAAGGAGTGCTACAGCTTCAGCGCCCGCGTCGCTGAGACC 480
afpA_culm      GACCACCCCGGTAACAGCGAGAAGGAGTGCTACAGCTTCAGCGCCCGCGTCTCTGAGACC 480
               *************************************************** ********

afpA_gram      AAGAAGCCTGTTAGCTGCATCTACTCCCAGTACAGCAACTGA 522
afpA_culm      AAGAAGCCTGTTAGCTGCATCTACTCCCAGTACAGCAACTGA 522
               ******************************************
```

Figure 17: Comparison of nucleotide sequences of the *afpA* gene from *F. graminearum* and *F. culmorum; afpA_gram* = nucleotide sequence *afpA* from *F. graminearum*; *afpA_culm* = nucleotide sequence *afpA* from *F. culmorum;* Positions with identical nucleotides in both sequences are marked with an asterisk.

Figure 17 compares the nucleotide sequences of the *afpA* gene from *F. graminearum* and *F. culmorum*. Both sequences differ from each other in 9 nucleotide positions corresponding to a sequence homology of 98.3 %. The translated amino acid sequence of the *F. graminearum* strain used here was found to differ in 3 positions from *F. culmorum* and was found to be identical to the amino acid sequence of FG08122.1, a hypothetical protein from *G. zeae* (strain PH-1, anamorph: *F. graminearum*, Gene ID 2790083). Figure 18 shows an alignment of the three amino acid sequences.

```
AfpA_gram       APEPKTFGLVALRSGSPFHLSSVSASESGFSLLLPKGKQGAKCADNKKEDFATFRISKDK 60
FG08122.1       APEPKTFGLVALRSGSPFHLSSVSASESGFSLLLPKGKQGAKCADNKKEDFATFRISKDK 60
AfpA_culm       APEPKTFGLVALRSGSPFHLSSVSASESGFSLLLPKGKQGAKCVDNKKEDFATFRISKDG 60
                *******************************************.***************

AfpA_gram       KLVLYHKGKEQQIAYTDRSGMGQGVLQYTGQKNYPRNAETEGWKVDKDGNLVFGSNNAGF 120
FG08122.1       KLVLYHKGKEQQIAYTDRSGMGQGVLQYTGQKNYPRNAETEGWKVDKDGNLVFGSNNAGF 120
AfpA_culm       KLVLYHKGKEQQVAYTDRSGMGQGVLQYTGQKNYPRNAETEGWKVDKDGNLVFGSNNAGF 120
                ************:***********************************************

AfpA_gram       MACPGLKSTDPWSIWVATGTDHPGNSEKECYSFSARVAETKKPVSCIYSQYSN 173
FG08122.1       MACPGLKSTDPWSIWVATGTDHPGNSEKECYSFSARVAETKKPVSCIYSQYSN 173
AfpA_culm       MACPGLKSTDPWSIWVATGTDHPGNSEKECYSFSARVSETKKPVSCIYSQYSN 173
                *************************************:***************
```

Figure 18: Comparison of amino acid sequence of AfpA from different soureces. AfpA_gram = *F. graminearum*, FG08122.1 = *G. zea* (strain PH-1, anamorph: *F. graminearum*, Gene ID 2790083) and AfpA_culm = *F. culmorum;* Positions with identical nucleotides in both sequences are marked with an asterisk.

Calculation of molecular weight resulted in a size of 18.9 kDa and an isoelectric point of 9.1. After cloning of pPICZαA containing *afpA* into *E. coli*, clones were picked from LB-agar plates with Zeocin added for selection of positive transformation events. Clones , were taken for plasmid back up and multiplication, were streaked on LB-Zeocin agar plates in three generations to ensure that cells carry the plasmid pPICZαA with the *Sh ble* gene conferring resistance against Zeocin. After plasmid isolation from these clones, proper incorporation of the *afpA* gene into the plasmid and sequence identity with the cDNA was confirmed by sequence analysis using the primer pair AOX1-primer_f and AOX1-primer_r. After successful transformation of *P. pastoris* X33 with the linearized plasmid and selection of Zeocin resistant yeast clones, sequence identity was confirmed again by sequence analysis of the 923 bp amplicon obtained by using yeast clone genomic DNA and primer AOX1-primer_f and AOX1-primer_r for PCR. Confirmed *afpA* containing yeast clones were used in protein production experiments. Lyophilisate, which was obtained after cultivation, dialysis of cell free supernatant and freeze drying was subjected to SDS-PAGE. AfpA was well stainable by colloidal coomassie blue. However, it proofed to be not accessible to

silver staining. Verification of protein expression and secretion was accomplished by peptide mass finger printing subsequent to SDS-PAGE and coomassie staining. After alkylation and tryptic digestion of proteins contained in the excised band, fragments generated by trypsin treatment were measured by mass spectrometry. Recovered fragments of the protein are underlined in the amino acid sequence below. 85.0 % of the amino acid sequence of AfpA were covered by the fragments measured.

E A E A E F A P E P K T F G L V A L R S G S P F H L S S V S A S E S G F S L L L P K G K Q G A K C A D N K K E D F A T F R I S K D K K L V L Y H K G K E Q Q I A Y T D R S G M G Q G V L Q Y T G Q K N Y P R N A E T E G W K V D K D G N L V F G S N N A G F M A C P G L K S T D P W S I W V A T G T D H P G N S E K E C Y S F S A R V A E T K K P V S C I Y S Q Y S N

Analysis of the peptide mass fingerprints of the recombinant AfpA showed that one of the recovered peptides contained four amino acids of the signal peptide (EFEF) and an additional two amino acids representing the restriction site EcoR1 (EF), which was used for integration of the *afpA* gene (highlighted with a grey background). This indicates that the α-factor for secretion is not removed completely from the secreted protein in this case.

3.4 Accessibility of the transgenic proteins to silver and coomassie staining

As described above, the proteins produced by the yeast transformants differed considerably in their accessibility to staining. Figure 19 shows all heterologously expressed proteins from this work as visualized by colloidal coomassie blue and silver staining, respectively. Proteins were applied to SDS-PAGE in concentrations >10 mg lyophilisate per mL. Gel A shows colloidal coomassie blue staining of nsLTP1 (1), AfpA (2), Hfb2 (3), FcHyd5p (4) and FcHyd3p (5). The first lane represents a protein size standard (PM2). Gel B contains the same samples and was subjected to silver staining. In the coomassie blue stained gel (A) several bands in the high molecular region above 30 kDa are present. As they can be found in each sample, these proteins can be assumed to be native to the wild type *P. pastoris* X33. Considering that more than 10 mg per mL lyophilisate were applied in each sample, the quantities of these proteins in the lyophilisate must be very low. It can be seen that in each lane the bands of the recombinant proteins differ from the expected size, when compared with the protein size marker. However, all bands obtained from the

lyophilisate of the transformed yeast could be related to the corresponding proteins by peptide mass finger printing, except for FcHyd3p, since no band could be found upon staining with coomassie blue. For AfpA it was observed that it is not accessible to silver staining, whereas colloidal coomassie blue staining gave a good response (lane 2).

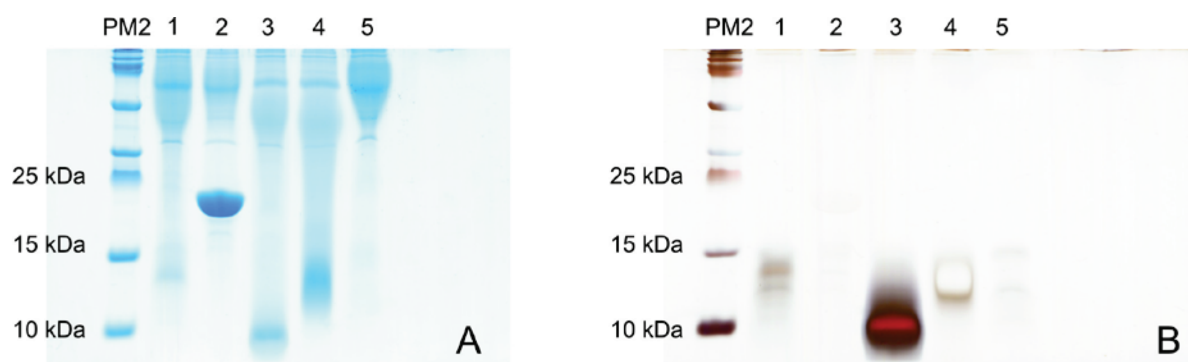

Figure 19: Comparison of colloidal coomassie blue staining (A) and silver staining (B) after separation of transgenic proteins nsLTP1 (1), AfpA (2), Hfb2 (3), FcHyd5p (4) and FcHyd3p (5); PM2 = protein marker (Fermentas, St. Leon-Rot, Germany);

The behavior found for the class II hydrophobin Hfb2 was, however, in contrast to the other proteins. It was found that this protein was highly accessible to silver staining, whereas staining with colloidal coomassie blue gave only moderate response (lane 3). nsLTP1 (lane 1) revealed to be similarly accessible to both staining methods. The lyophilisate containing FcHyd5p gave a very diffuse band upon staining with coomassie blue. Silver staining of FcHyd5p in most cases led to gels with no band at the expected molecular weight. In some cases undefined structures were observed on the gel as shown in gel B lane 4. Class I hydrophobin FcHyd3 showed no bands after coomassie blue staining, whereas after silver staining two very weak bands resulted between 10 and 17 kDa.

3.5 Gushing potential of class II hydrophobin FcHyd5p

In order to investigate the gushing-inducing potential of the class II hydrophobin FcHyd5p, lyophilisate obtained from *P. pastoris* X33 [pPICZαA-FcHyd5] was applied to gushing experiments in several carbonated beverages comprising black currant juice, apple juice, carbonated water, elder juice, apple/grape juice and beer. Bottles were opened after chilling to 0°C and lyophilisate was added. Resealed bottles were rotated overhead for 16 hours and gushing volume was measured after 1 hour of rest. In all beverages tested gushing could be induced upon addition of lyophilisate. All control bottles containing no

lyophilisate showed no gushing. Figure 20 shows the resulting gushing volumes in percent of the bottle volume. Since no data on the content of carbon dioxide in the beverages were available, no conclusions on differences in the susceptibility to FcHyd5p induced gushing of individual beverages can be drawn.

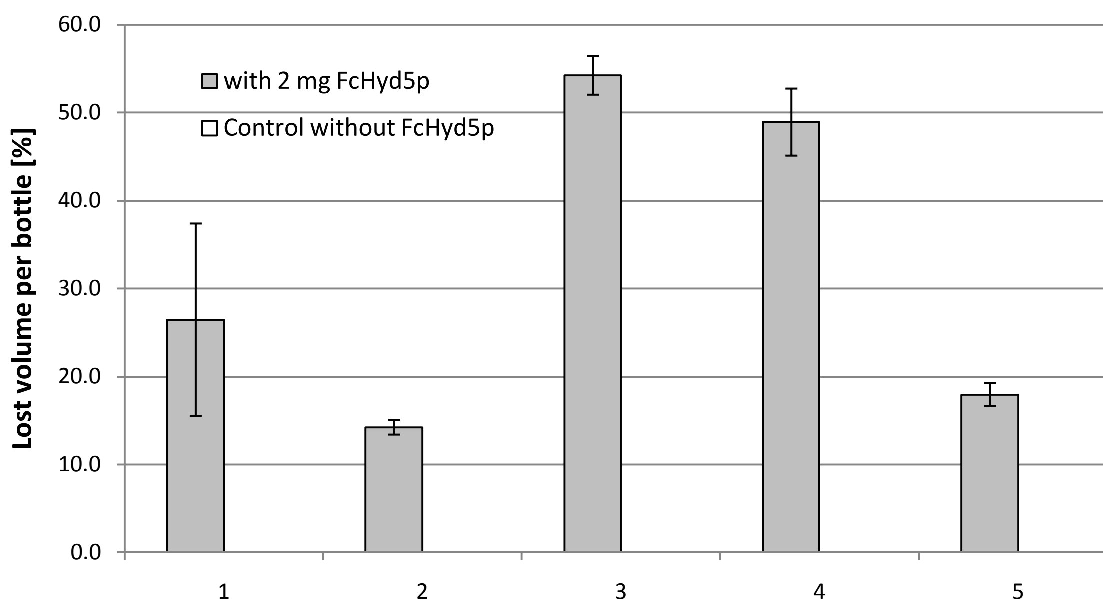

Figure 20: Gushing volumes of carbonated beverages given in percent of the bottle volume upon addition of 2 mg FcHy5p lyophilisate per bottle; 1 = black currant juice; 2 = apple juice; 3 = carbonated water; 4 = elder juice; 5 = apple/grape juice;

Since hydrophobins are suspected to induce gushing in beer, all gushing experiments were carried out using bottom fermented German lager beer as a model system. Carbonated water served as a reference system, since no surface-active substances are supposed to be present in sparkling water. A test, in which increasing quantities of FcHyd5p lyophilisate were added to beer, showed that there is a relation between the amount of FcHyd5p and the obtained gushing volume. It was found that addition of amounts up to 2 mg per beer bottle resulted in an increase of the over-foaming volume. Raising the amount to 5 mg per bottle did not further increase the gushing volume. Figure 21 shows the relation found between amounts of FcHyd5p lyophilisate added and the resulting over-foaming volume in beer bottles. As a result of these findings, 2 mg of lyophilisate per bottle of beer (0.5 l) were applied for further experiments. In general, dosing of the hydrophobin was performed by dissolving the freeze dried protein in deionized water at a concentration of 2 mg per mL prior to addition. Experiments showed that there was no difference in gushing volumes when adding the lyophilisate as a powder or as an aqueous solution of appropriate concentration. Addition of a solution provided easier handling and also minimized weighing

error. Despite this optimization, a high standard deviation of gushing volumes was still observed in beer. It was found that the gushing volumes differed between different brands of German lager beer, but it was also seen that different lots of the same brand and even bottles from the same lot showed different over-foaming volumes after addition of the standard amount of 2 mg per bottle.

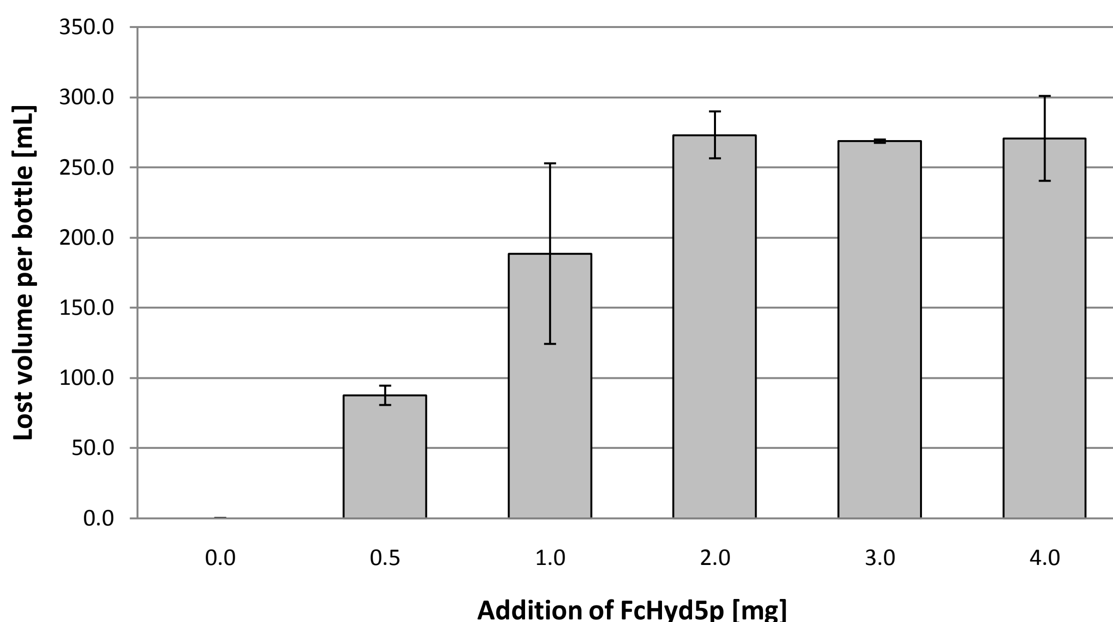

Figure 21: Relation between amount of added, transgenic hydrophobin FcHyd5p (lyophilisate) and gushing volume in bottom fermented beer (0.5 l per bottle);

German lager beer produced by another brewery was tested and appeared to be more resistant to hydrophobins and showed no gushing upon addition of 2 mg FcHyd5p. However, addition of higher amounts revealed a principally similar relationship between added quantities and gushing volume as compared to the results shown in Figure 21. The average gushing volume calculated from all gushing experiments with addition of 2 mg FcHyd5p to German lager beer was found to be 211 mL with a standard deviation of 57 mL. Control tests, which were performed with the same amount of lyophilisate obtained from the non hydrophobin producing strain *P. pastoris* X33 in beer or carbonated water, resulted in bottles without any gushing tendency.

When the relation between the amount of hydrophobin and gushing volume in carbonated water was tested, it was found that carbonated water was much more sensitive than beer to the induction of gushing by the class II hydrophobin FcHyd5p. Addition of increasing amounts of FcHyd5p lyophilisate to carbonated water revealed that a quantity of 0.1 mg

was able to cause gushing with an over-foaming volume of 161 mL. Further increase of the added amount to 2 mg resulted in a lost volume of 179 mL. At opening of the conditioned bottles of carbonated water an abrupt burst out of water, causing a fountain of at least 3 m in height, was observed, indicating the high sensitivity of carbonated water to FcHyd5p. Further gushing experiments with carbonated water were performed with a standard dosage of 1 mg FcHyd5p lyophilisate per bottle, which was added from a 2 mg per mL aqueous solution. The average gushing volume of carbonated water after addition of 1 mg was found to be 177 mL with a standard deviation of 8 mL. Figure 22 shows the dependence of the over-foaming volume of carbonated water from the amount of FcHyd5p lyophilisate added.

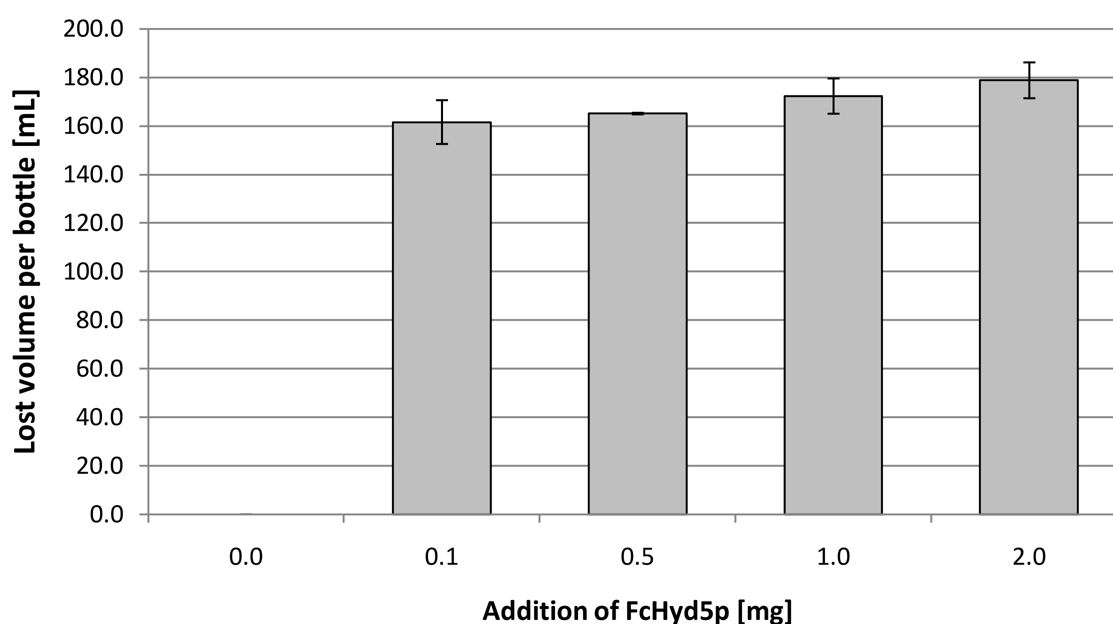

Figure 22: Relation between amount of added, transgenic hydrophobin FcHyd5p (lyophilisate) and gushing volume in carbonated water (0.33 l per bottle);

To demonstrate the influence of the introduction of gas bubbles into a carbonated beverage for the induction of gushing, tests were made without rotation of the pre-conditioned bottles. Bottles of beer and carbonated water were treated with FcHyd5p in a solid state as lyophilisate or as a solution dissolved in deionized water. When FcHyd5p was added as lyophilisate and bottles were incubated at ambient temperature overnight in an upright position, gushing was observed both in beer and carbonated water. However, the resulting over-foaming volumes were very low as compared to bottles, which were rotated prior to opening. Addition of the same amount of FcHyd5p as an aqueous solution without

subsequent rotation led only to over-foaming in carbonated water. Addition of dissolved FcHyd5p lyophilisate to beer without subsequent rotation resulted in no gushing.

3.6 Influence of heat treatment on the gushing potential of FcHyd5p

It must be assumed that potentially gushing-inducing agents must either remain in their active form after processing at the conditions prevailing at wort boiling or be even activated under such conditions. To test its heat stability, FcHyd5p lyophilisate was dissolved in deionized water, filled in a flask, sealed and incubated in boiling water for 1 hour. No protein precipitation was observed in the flask after the heat treatment, indicating that the protein was resistant to denaturation during boiling. After cooling down to room temperature the solution was added to beer (2 mg per 500 mL bottle) and the gushing volume was measured after rotation of the bottles overnight. The results demonstrated that the gushing potential of the heat treated hydrophobin was unchanged as compared to beer treated with an unboiled control solution. The same situation was observed in carbonated water. In order to imitate a real wort boiling process without interfering proteins, FcHyd5p lyophilisate was dissolved in a protein free synthetic wort with the characteristic sugar and acid composition of conventional wort. After incubation for 1 hour in boiling water, again no protein precipitation was observed. However, a reduction in the induced gushing volume by 40 % was found after addition to beer resulting in a lost volume of 123 mL with a standard deviation of 63 mL.

3.7 Influence of ultrasonication of hydrophobin solutions on gushing

Since it was observed in the experiments described above that FcHyd5p was able to induce gushing without rotation of the bottles after hydrophobin addition and as it is known that hydrophobins tend to self-assemble, ultrasonication was applied to FcHyd5p solutions in order to disintegrate assembled hydrophobin molecules, which may possibly act as condensation nuclei for carbon dioxide. In order to exclude the influence of iron derived from ultrasonication equipment as a possible gushing-inducing factor, water was subjected to ultrasonication prior to addition to non-gushing beer as a control. No gushing was observed in these samples. Subsequent gushing experiments showed that ultrasonication treatment of FcHyd5p solutions did not result in a significant change in beer volume loss. As an effect of ultrasonication treatment, it was observed that standard deviations were decreased in samples treated with an ultrasonic probe as compared to samples with addition of untreated hydrophobin. Treatment of hydrophobins in an ultrasonic bath however, showed less reproducible results.

3.8 Influence of hop compounds on gushing induced by FcHyd5p

The ability of hop products to influence the severity of gushing in beer has been described in several studies. This issue was addressed by application of hop compounds to beer pre-conditioned with FcHyd5p lyophilisate. Figure 23 shows the influence of iso-α acid products on the gushing volume. The diagram shows gushing volumes relative to reference bottles, to which only FcHyd5p lyophilisate was added with no addition of hop products. These control bottles (FcHyd5p) were set 100 %. It was observed that only the modified iso-α-acids were able to reduce gushing induced by the class II Hydrophobin FcHyd5p. Reduced iso-α-acid (Rho 35 %) and tetrahydro iso-α-acid (Tetra 10 %) showed an inhibitory effect on gushing volume, whereas no such property could be detected with the unmodified iso-α-acid (Iso 30 %). The average reduction of lost volume for both modified iso-α-acids was more than 50 %. However, these results were subject to considerable variation. Addition of Rho 35 % showed the highest reduction, when 20 µL per bottle were applied, which led to a reduction to 20 % as compared to the untreated positive control. Addition of 10 µL or 30 µL of the same product resulted in lower reduction values. However, due to the high standard deviations of the results, no optimum value could be determined upon addition of Rho 35%. The gushing volume was also reduced by addition of the Tetra 10 % product. As shown in Figure 23, addition of 60 µL revealed the highest reduction of gushing volumes. However, due to the high standard deviations of the results, there was again no significant trend. In contrast to the products containing modified iso-α-acids, addition of unmodified iso-α-acid led to an increase in the gushing volume (data not shown). Negative controls containing only iso-α-acids and no hydrophobin resulted in beer without gushing for Rho 35 % and Tetra 10 %. However, gushing was observed when the Iso 30 % product was added as a negative control to beer.

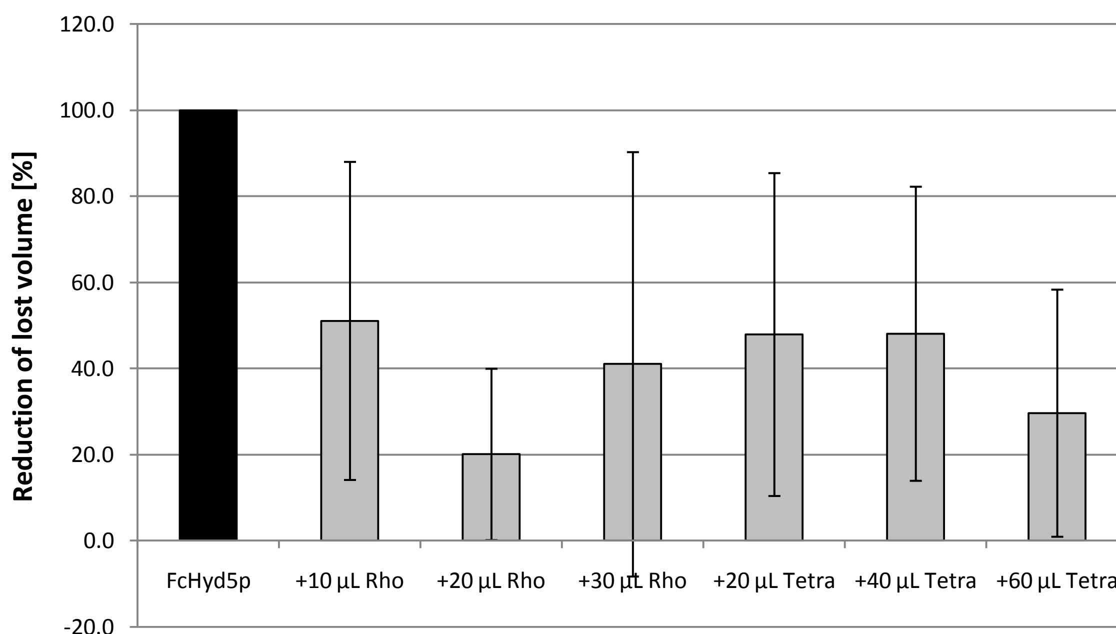

Figure 23: Relative inhibition of gushing after addition of transgenic hydrophobin FcHyd5p (lyophilisate) and iso-α-acid products to German lager beer (0.5 l per bottle); FcHyd5p = Gushing induced by 2 mg of transgenic FcHyd5p without inhibition (=100 %); Rho = inhibition of gushing by addition of iso-α-acid product Rho 35 %; Tetra = inhibition of gushing by addition of iso-α-acid product Tetra 10 %;

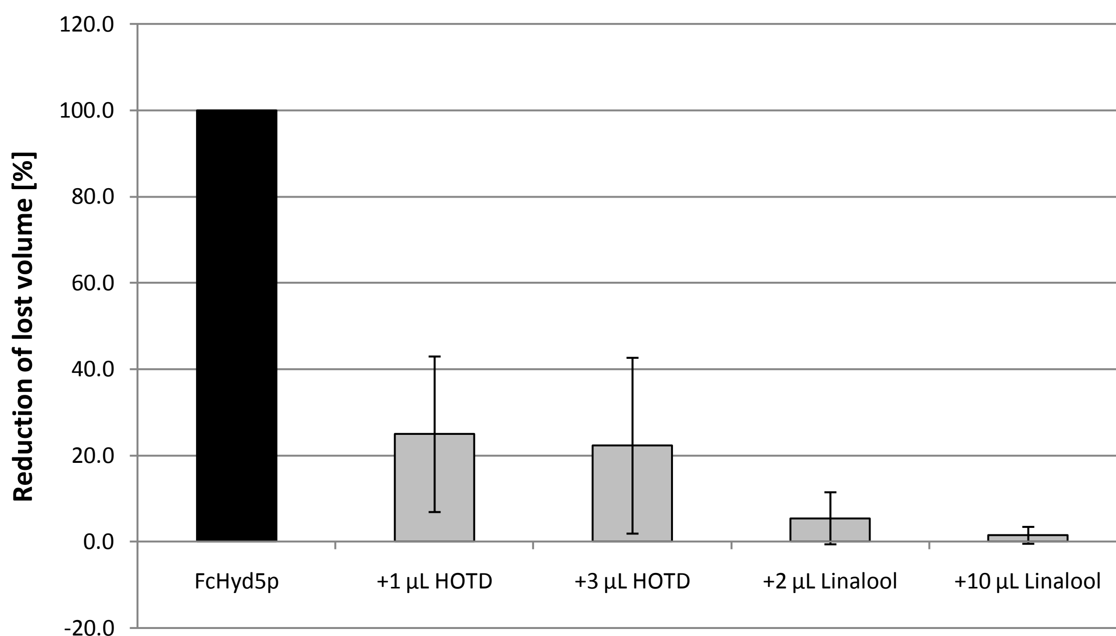

Figure 24: Relative inhibition of gushing after addition of transgenic hydrophobin FcHyd5p (lyophilisate) and hop oils to German lager beer (0.5 l per bottle); FcHyd5p = Gushing induced by 2 mg transgenic FcHyd5p without inhibition (=100 %); HOTD = inhibition of gushing by addition hop oil product hop oil type dry; Linalool = inhibition of gushing by addition of linalool;

Next to iso-α acid products, also two hop oil products were tested as shown in Figure 24. In the diagram the reduction of the gushing volume is given in relation to bottles, to which only FcHyd5p lyophilisate was added and no hop products. These control bottles (FcHyd5p) were set 100 %. The remaining values denote the relative gushing volumes as compared to the control bottles. Addition of linalool resulted in a strong reduction of the over-foaming of beer. 2 µL of linalool reduced the lost beer volume by more than 90 % as compared to the untreated control containing only the hydrophobin. When 10 µL of linalool were added, an almost complete inhibition of gushing was observed. Samples with linalool levels >10 µL showed no gushing. Addition of the hop oil type dry (HOTD) product to beer also had an inhibitive effect on hydrophobin induced gushing. Addition of 1 µL of HOTD reduced the lost volume to 25 % as compared to samples without HOTD. The addition of 3 µL of the product resulted in further reduction of the gushing volume to 22 %. Negative controls with only HOTD or linalool added showed no gushing.

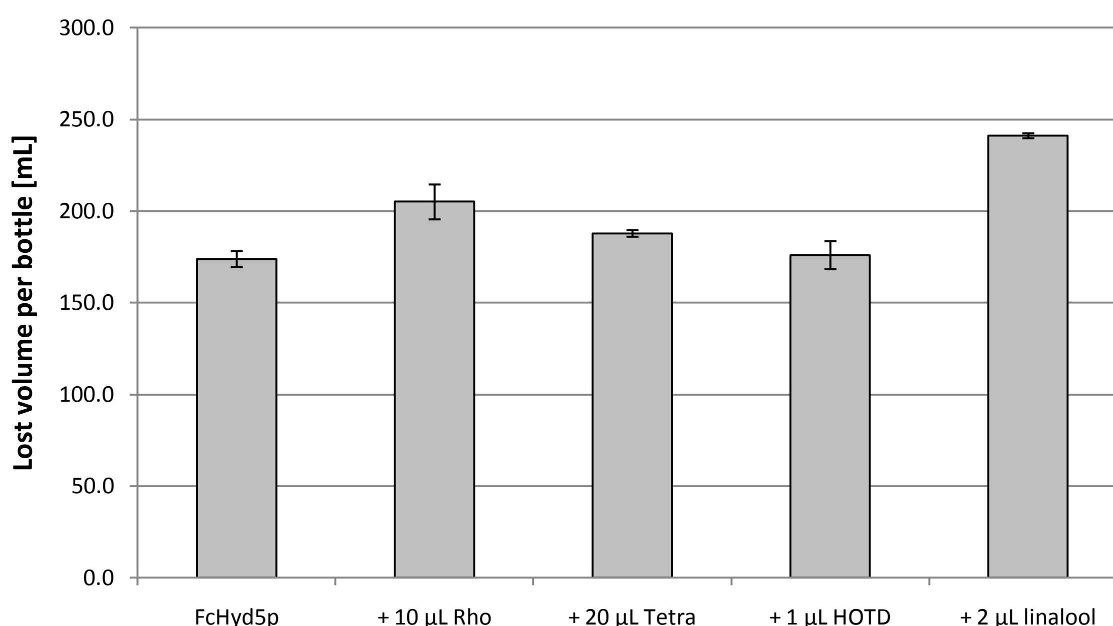

Figure 25: Effect of hop products on transgenic hydrophobin FcHyd5p (1 mg per bottle, lyophilisate) induced gushing in carbonated water; results shown as absolute gushing volumes for each hop product after addition of FcHyd5p and hop product; Rho = iso-α-acid product Rho 35 %, Tetra = iso-α-acid product Tetra 10 %, Iso = iso-α-acid product Iso 30 %, HOTD= hop oil product hop oil type dry, linalool = hop oil component linalool;

The influence of hop compounds on hydrophobin-induced gushing was also tested in carbonated water (see Figure 25). This was conducted to investigate if the hop compounds used would be able to inhibit hydrophobin gushing by themselves or if the presence of other substances from beer was necessary for this effect. The amounts of hop products

added were identical to the lowest amounts that were added to beer. Addition of hop products to hydrophobin-treated carbonated water resulted in an increase in the gushing volume for each hop product, as compared to samples solely inoculated with the hydrophobin. Addition of linalool to hydrophobin-treated carbonated water resulted in the highest increase in water emission. An increase in volume loss of 40 % was observed after addition of 1 µL of linalool. Amounts of up to 100 µL of linalool per bottle resulted in the same gushing volume. Addition of 1 µL of HOTD showed no significant increase in the gushing volume. If 3 µL were applied, the increase became significant (data not shown). Addition of the other hop products resulted in gushing volumes >200 mL, whereas an increase of added hop compound resulted in a slight increase of gushing volume. Negative controls, containing one hop product each, resulted in gushing for the iso-α-products. Addition of the hop oil components did not result in gushing.

3.9 Gushing potential of class II hydrophobin Hfb2

As a second representative of the group of class II hydrophobins, Hfb2 from *T. reesei* was heterologously expressed by *P. pastoris.* It was applied to gushing experiments and its gushing potential was examined. It was found that addition of Hfb2 lyophilisate was able to induce gushing in the carbonated beverages tested. For these tests carbonated water and beer were used. Figure 26 shows the relation, which was found between the addition of increasing amounts of Hfb2 lyophilisate and the obtained gushing volume. It was revealed that gushing could be induced by addition of 1 mg Hfb2 and the over-foaming volume could be increased by addition of quantities up to 4 mg per 0.5 l bottle. Higher amounts of Hfb2 lyophilisate did not further raise the over-foaming volume. Based on these findings, subsequent gushing experiments in beer were done with addition of 4 mg per bottle as standard dosage, exceptions indicated. Prior to addition, lyophilisate was dissolved in deionized water at a concentration of 2 mg per mL.

When Hfb2 was added to carbonated water a relation between the amounts added and the gushing volume was found as well. Results from this experiment are shown in Figure 27. Addition of 0.1, 0.5 and 1.0 mg Hfb2 lyophilisate resulted in a constant increase of gushing volumes. However, addition of higher amounts only led to a very slight increase of the effect.

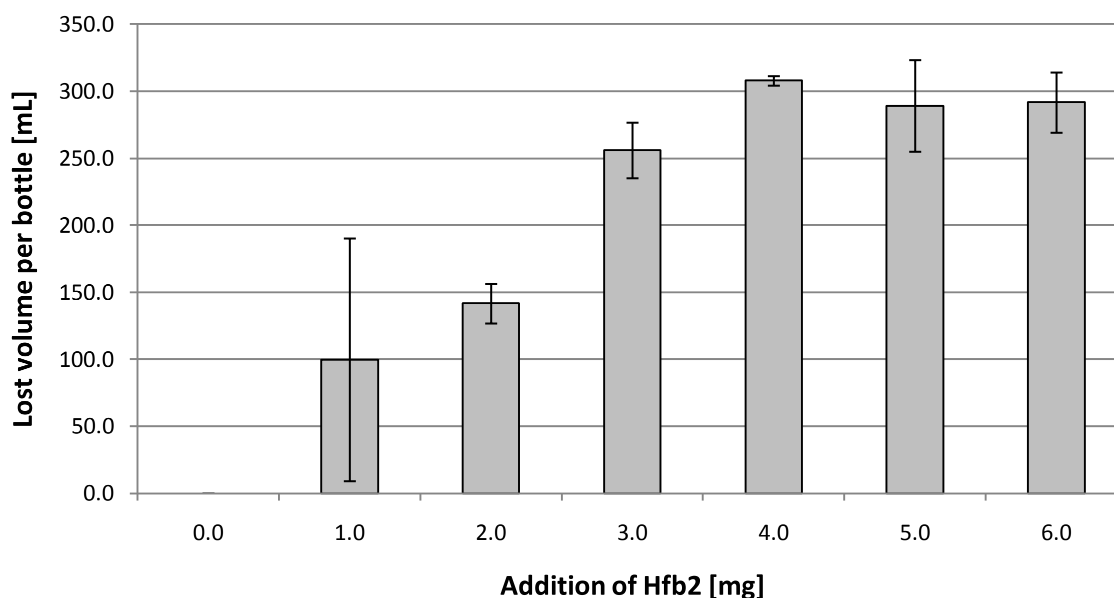

Figure 26: Relation between amount of added, transgenic hydrophobin Hfb2 (lyophilisate) and gushing volume in German lager beer (0.5 l per bottle);

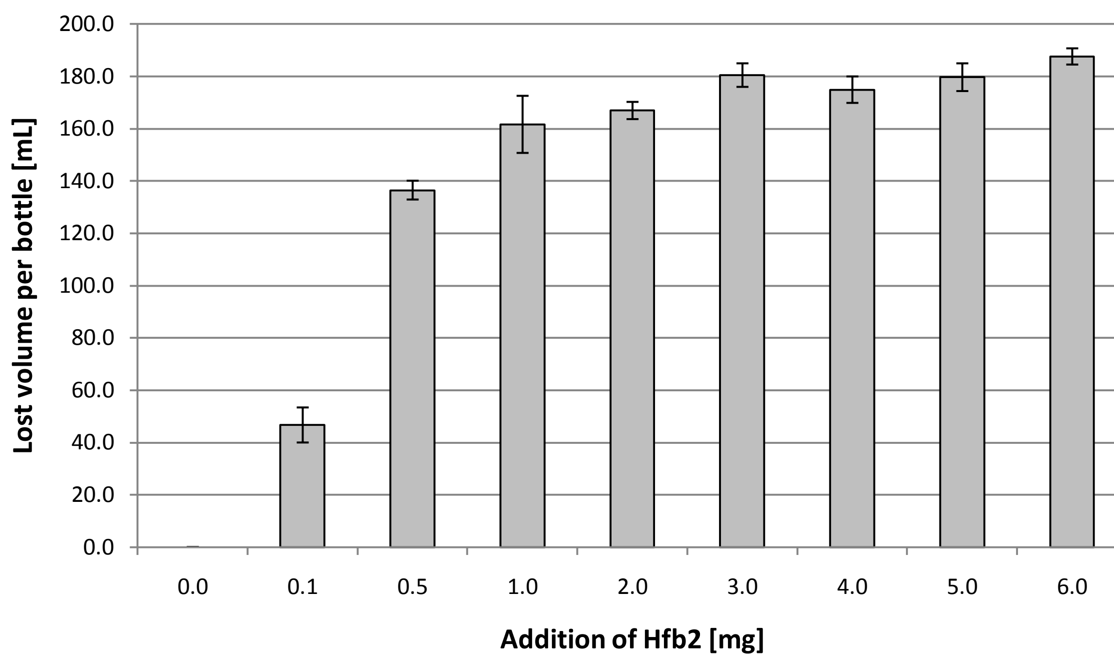

Figure 27: Relation between amount of added, transgenic hydrophobin Hfb2 (lyophilisate) and gushing volume in carbonated water (0.33 l per bottle);

When Hfb2 lyophilisate was dissolved in synthetic wort and incubated in a boiling water bath for 1 hour, the gushing activity of the protein in beer was reduced by 27 % as compared to non-boiled lyophilisate. However, heat treatment of Hfb2 did not affect its

gushing activity in carbonated water. In order to test the influence of hop compounds on gushing induced by Hfb2, hop oil or iso-α acid products were added to beer in combination with Hfb2 lyophilisate. Figure 28 shows the values obtained for reduction of gushing upon application of hop products. In the diagram reduction of the gushing volume is given as percentage relative to bottles, to which Hfb2 lyophilisate was added with no addition of hop products. It was observed that addition of iso-α acid product Rho resulted in a reduction of the over-foaming volume to 32 % and 36 %, for 20 and 30 µL of Rho as compared to the control, respectively. When 2 µL of linalool, a hop oil product, were added to beer, a reduction of the gushing volume by 12 % relative to the control was observed. Addition of 10 µL of linalool resulted in a reduction by only 6 % as compared to the control. Addition of hop oil type dry resulted in a decrease of the over-foaming volume by 65 % relative to the control after addition of 3 µL of the product per bottle. Application of 10 µL of the product revealed complete elimination of gushing. For addition of Rho 35% and linalool, no clear trends could be observed due to very high variation between the results observed for different amounts added to the beer.

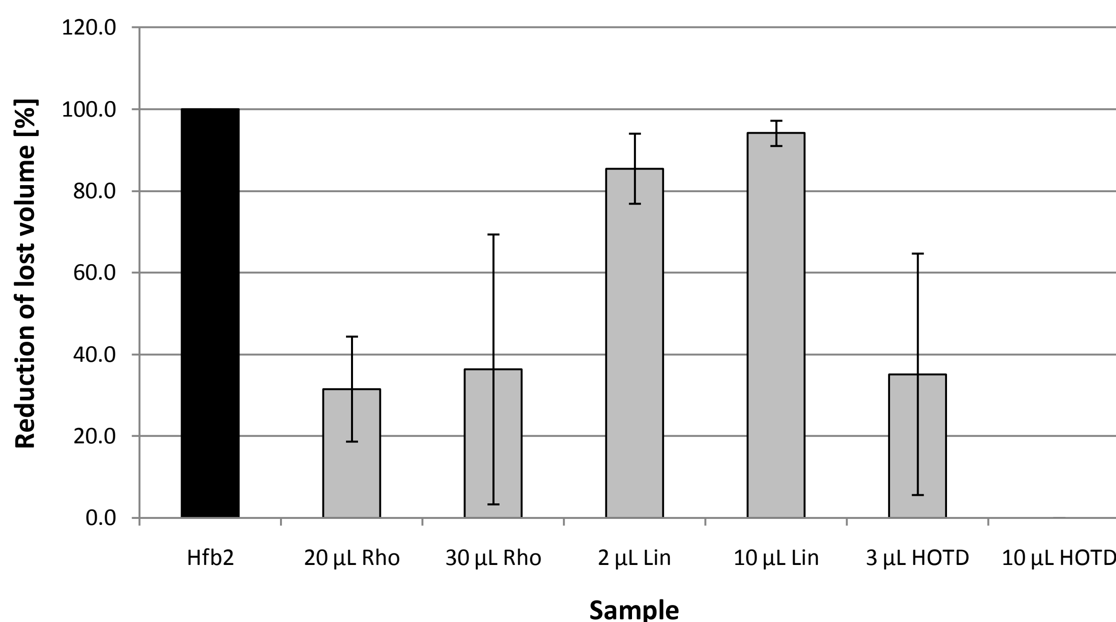

Figure 28: Relative inhibition of gushing after addition of transgenic hydrophobin Hfb2 (lyophilisate) and hop products to German lager beer (0.5 l per bottle); Hfb2 = Gushing induced by 4 mg of transgenic Hfb2 without inhibition (=100 %); Rho = inhibition of gushing by addition of iso-α-acid product Rho 35 %; Lin = inhibition of gushing by addition of hop oil product linalool; HOTD = inhibition of gushing by addition of hop oil product hop oil type dry;

3.10 Gushing potential of class I hydrophobin FcHyd3p

Next to class II hydrophobins FcHyd5p and Hfb2 the class I hydrophobin FcHyd3p from *F. culmorum* was applied in gushing experiments in order to examine its potential to induce gushing in beer and carbonated water. Addition to beer showed no gushing, when it was applied as lyophilisate. This test was done using amounts of up to 5 mg per bottle. An addition as aqueous solution at a concentration of 2 mg per mL also showed no gushing, when quantities up to 6 mg per 500 mL bottle were added. After addition of FcHyd3p to carbonated water as aqueous solution of 2 mg per mL, gushing was observed as shown in Figure 29. Starting from a gushing volume of 20 mL upon addition of 1 mg of FcHyd3p a slight increase of over-foaming volumes was observed upon addition of higher FcHyd3p amounts. Addition of 6 mg hydrophobin resulted in an over-foaming volume of 36 mL. When 2 mg of FcHyd3p were added to carbonated water in a solid state as lyophilisate, an increase of the gushing volume to 60 mL was observed. When 2 mg of FcHyd3p were added as lyophilisate to carbonated water an increase of the gushing volume to 60 mL was observed.

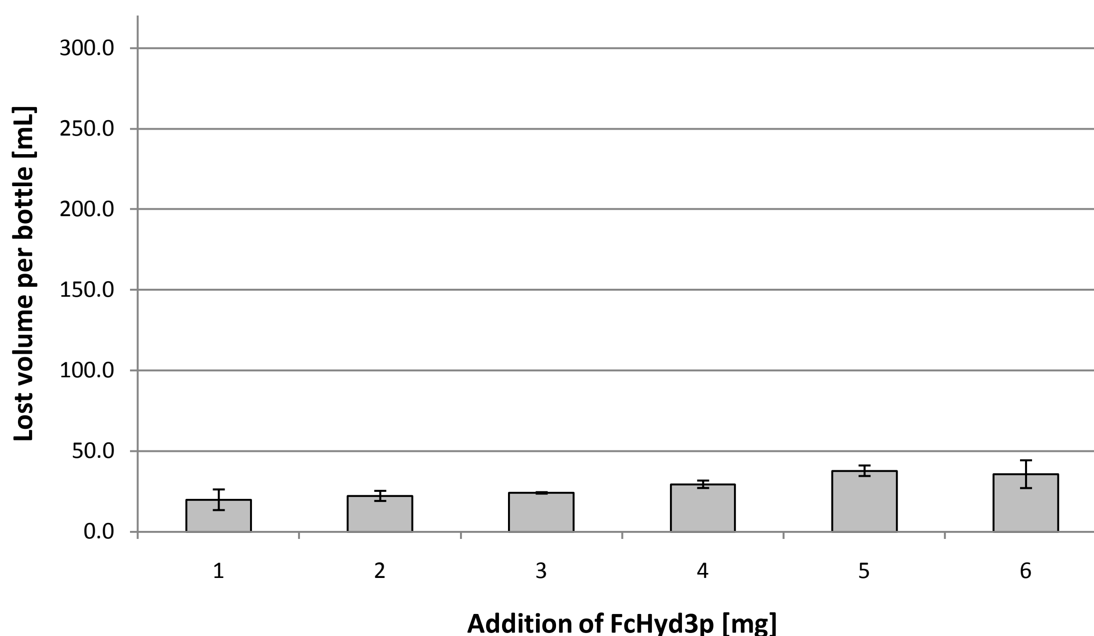

Figure 29: Relation between amount of added, transgenic hydrophobin FcHyd3p (lyophilisate) and gushing volume in carbonated water (0.33 l per bottle);

Addition of FcHyd3p, which was dissolved in synthetic wort and incubated for 1 hour in a boiling water bath showed that heat treatment of FcHyd3p in the presence of wort sugars leads to a reduction of its gushing potential. As depicted in Figure 30, it was observed that

gushing started at a concentration of 3 mg FcHyd3p lyophilisate per bottle, which led to a gushing volume of 6 mL. The over-foaming volume, which resulted from addition of 5 mg of heat treated lyophilisate, was nearly identical with the gushing volume resulting from addition of 1 mg of untreated lyophilisate.

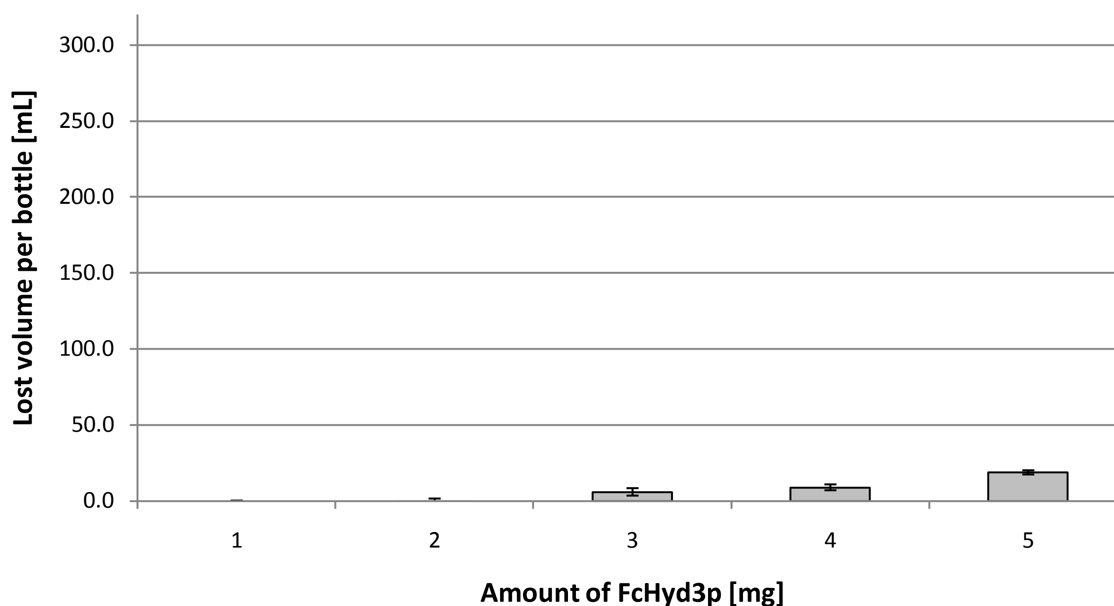

Figure 30: Influence of heat treatment on the gushing potential of FcHyd3p. Lyophilisate was dissolved in synthetic wort, incubated in boiling water for 1 hour and added in increasing amounts to carbonated water (0.33 l per bottle);

3.11 Gushing potential of AfpA and nsLTP1

To test their behavior in carbonated beverages, each of the non-hydrophobin surface-active proteins nsLTP1 from barley and AfpA from *F. graminearum* was applied to gushing experiments in carbonated water and beer. When AfpA lyophilisate was added to beer from a 2 mg per mL aqueous solution, no gushing was observed for amounts up to 6 mg per 500 mL bottle. The same was observed when it was applied in carbonated water in quantities up to 4 mg per bottle. A 2 mg per mL solution of AfpA lyophilisate in synthetic wort, which was incubated for 1 hour in a boiling water bath, also did not induce gushing when added to beer in amounts of up to 6 mg per bottle. In order to test the gushing potential of nsLTP1 lyophilisate, 16 mg were added to beer from a 2 mg per mL solution in synthetic wort, which was incubated in a boiling water bath for 1 hour prior to addition. No gushing was observed in these bottles. Addition of 6 mg nsLTP1 lyophilisate, which was also dissolved in synthetic wort and incubated in a boiling water bath for 1 hour, did not lead to gushing in

carbonated water. In all experiments done with addition of AfpA and nsLTP1, no induction of gushing was observed.

3.12 Influence of nsLTP1 on gushing induced by FcHyd5p

Since nsLTP1 lyophilisate did not show gushing-inducing activity according to the results shown under 3.11, the ability of the protein to influence gushing in beer, which had previously been conditioned with FcHyd5p was investigated.

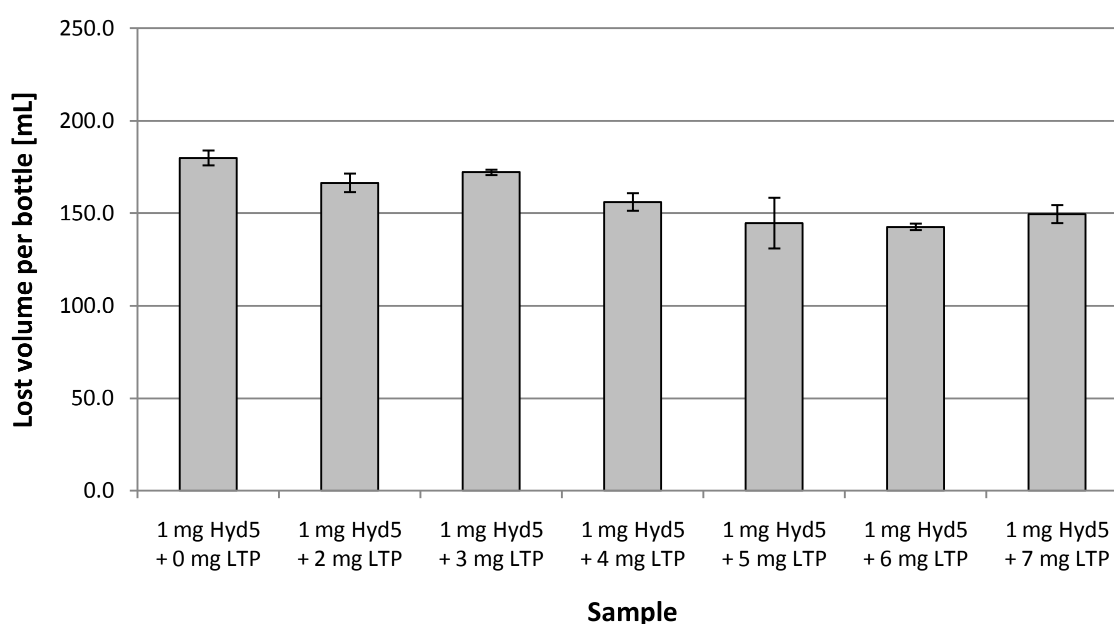

Figure 31: Effect of nsLTP1 on the gushing volume of FcHyd5p treated, carbonated water. Increasing amounts of nsLTP1 lyophilisate (LTP) were added to carbonated water, which was constantly conditioned with 1 mg of FcHyd5p lyophilisate (Hyd5).

Figure 31 shows the over-foaming volumes obtained when increasing amounts of nsLTP1 lyophilisate were added to bottles of carbonated water, which were pre-conditioned with 1 mg of FcHyd5p lyophilisate. It was observed that the gushing volumes decreased with increasing levels of nsLTP1 lyophilisate. A reduction of the over-foaming volume of 30 mL relative to the untreated control was obtained by addition of 7 mg of nsLTP1 lyophilisate, which was previously dissolved in synthetic wort and incubated for 1 hour in a boiling water bath. Since beer naturally contains nsLTP1 amounts exceeding 50 mg per liter (Leisegang and Stahl, 2005), higher levels of nsLTP1 were necessary for testing with beer. When a solution of boiled nsLTP1 was added to beer, which was pre-treated with FcHyd5p

lyophilisate, different reactions were observed. One out of three bottles tested showed a slight increase of the gushing volume in the pre-conditioned beer. A second bottle displayed a reduction by 33 % of the original gushing volume, whereas in the third bottle only a slight reduction was observed after addition of 16 mg of nsLTP1. In average a reduction to 77 % with a standard deviation of 37 % was measured. These results were supported by observations made with naturally gushing beer, in which addition of heat treated nsLTP1 led to a complete elimination of a moderate form of over-foaming.

3.13 Influence of AfpA on gushing induced by class II hydrophobins

AfpA lyophilisate, which showed no gushing-inducing potential in previous tests, was applied in combination with FcHyd5p and Hfb2 lyophilisate in beer and carbonated water to test its potential to influence gushing induced by class II hydrophobins. Figure 32 shows the effect of different amounts of AfpA lyophilisate on the gushing volume of carbonated water, which was pre-treated with 1 mg of FcHyd5p lyophilisate. A clear decrease of gushing volume was observed upon addition of 2 mg of AfpA lyophilisate. This trend continued with increasing amounts of AfpA added to pre-treated carbonated water. Upon addition of 6 mg of lyophilisate a reduction of the lost volume of 74 mL, which corresponds to a reduction by 39 % as compared to the control, was measured. When the combination of both proteins was tested in beer, that trend became even more obvious as is shown in Figure 33. It shows gushing volumes, which were obtained when beer pre-conditioned with 2 mg of FcHyd5p lyophilisate was treated with AfpA lyophilisate. The gushing volume decreased steadily, starting at an addition of 1 mg AfpA lyophilisate. An almost complete reduction of gushing from 207 mL to 18 mL was accomplished by addition of 3 mg AfpA lyophilisate. Further tests revealed, that AfpA also showed gushing inhibiting activity when it was applied in combination with Hfb2 lyophilisate (see Figure 34). In these experiments beer was pre-conditioned with 2 mg of Hfb2 lyophilisate and increasing amounts of AfpA were added.

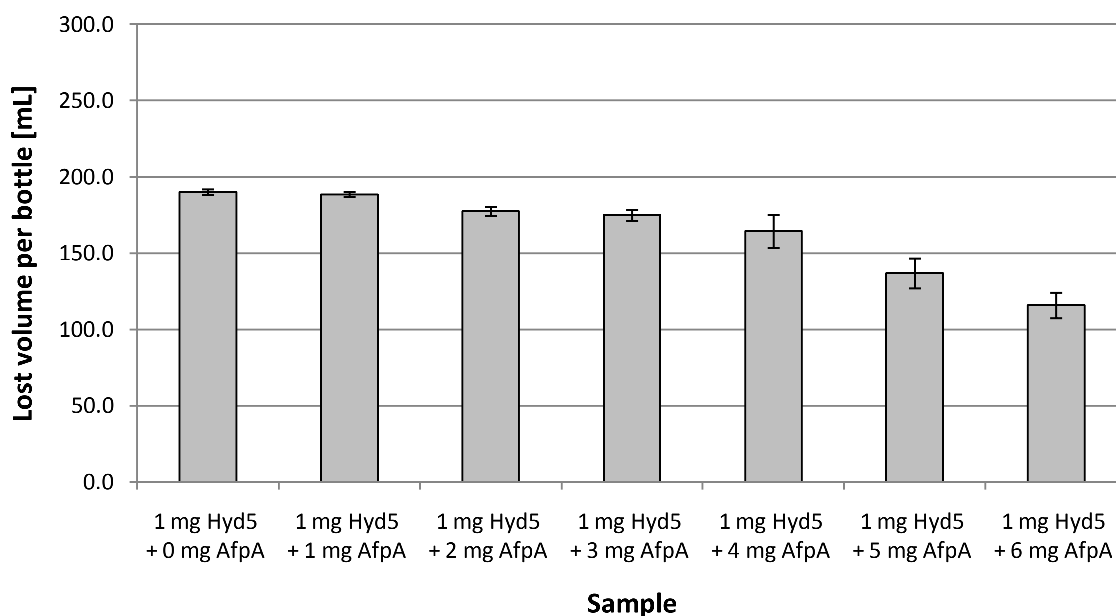

Figure 32: Effect of AfpA on the gushing volume of FcHyd5p treated, carbonated water. Increasing amounts of AfpA lyophilisate were added to carbonated water, which was constantly conditioned with 1 mg of FcHyd5p lyophilisate.

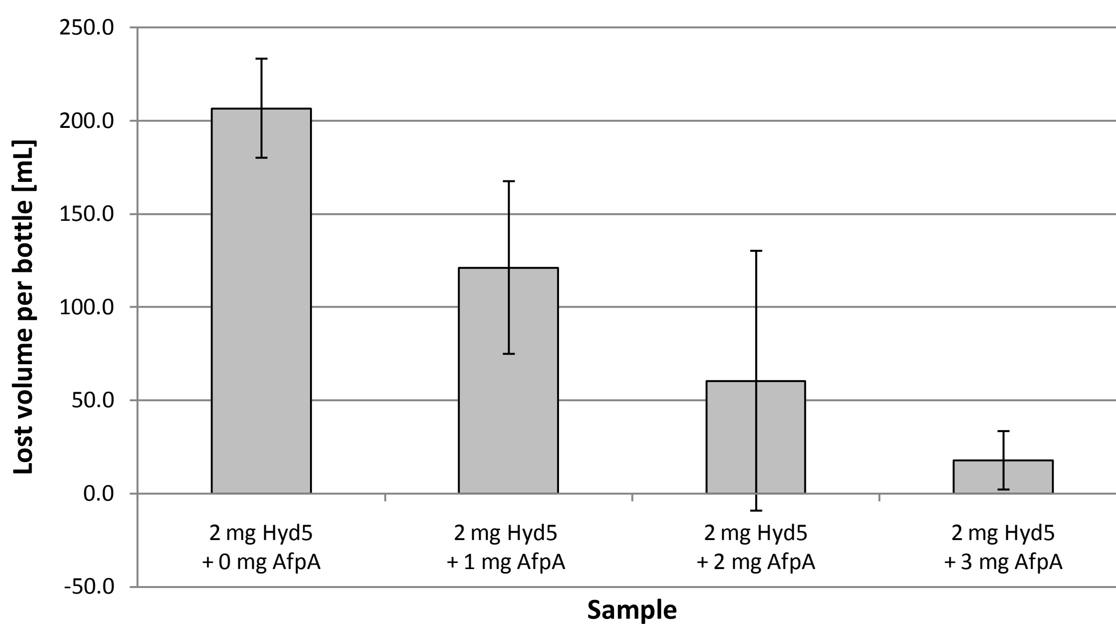

Figure 33: Effect of AfpA on the gushing volume of FcHyd5p treated beer. Increasing amounts of AfpA lyophilisate were added to beer, which was constantly conditioned with 2 mg of FcHyd5p lyophilisate (Hyd5).

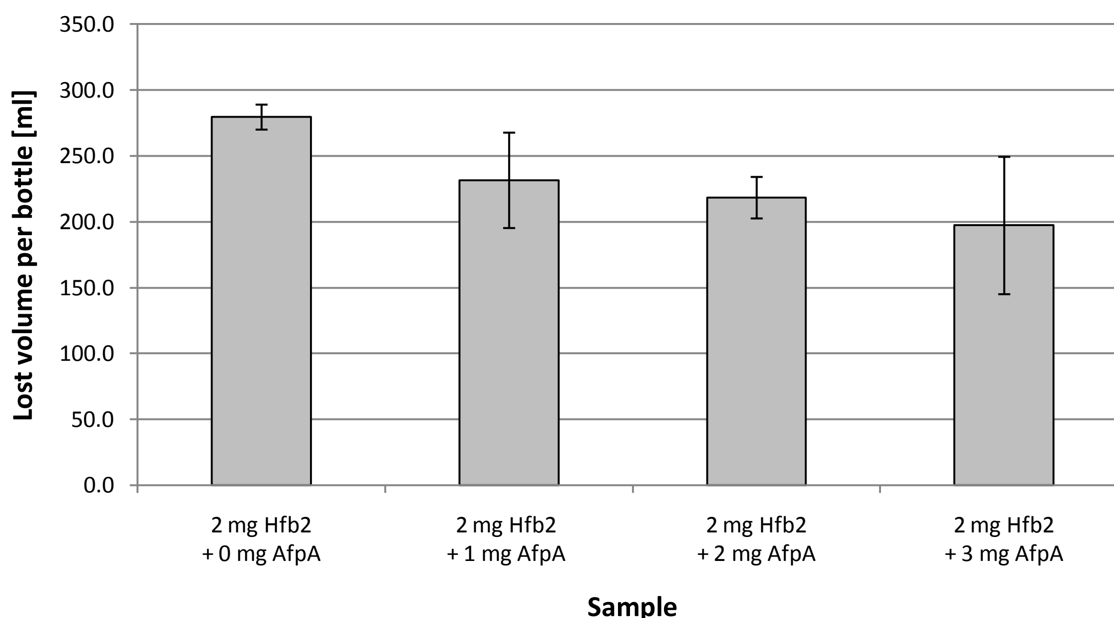

Figure 34: Effect of AfpA on the gushing volume of Hfb2 treated beer. Increasing amounts of AfpA lyophilisate were added to beer, which was constantly conditioned with 2 mg of Hfb2 lyophilisate.

3.14 Comparison of foam-stabilizing potential of recombinant proteins

All transgenic proteins, including the class II hydrophobin FcHyd5p, were tested for their ability to stabilize foam and to form a stable turbidity in solutions. Cell free culture supernatants of the minimal medium used for production of the proteins were subjected to a homogenization treatment using a rotor-stator system. Supernatants from cultures of each recombinant yeast strain were divided in two aliquots. One was heat treated for one hour in a boiling water bath, whereas the other one was left untreated. None of the heat treated supernatants showed protein precipitation as a result of the treatment. Subsequent homogenization of the supernatants resulted in the formation of a foam covered hazy liquid. Degradation of the foam started immediately following homogenization in all samples analyzed. However, after 1 hour there were clear differences visible when the supernatants of hydrophobins and non-hydrophobin proteins were compared. The foam covering heat treated and non heat treated supernatants of AfpA and nsLTP1 had completely disintegrated and the initially turbid liquid turned clear. In contrast, samples of the class II hydrophobins FcHyd5p and Hfb2 showed a very much slower clearing of the liquid and the foam covering remained stable until the experiment was terminated after 1 week. Samples of the class I hydrophobin FcHyd3p cleared faster than those of the class II hydrophobins. Only the non heat treated sample retained a ring of foam bubbles where the liquid touched

the glass-wall of the vessel. The heat treated sample completely lost its foam and turbidity after 24 hours. Figure 35 shows the homogenized samples after 24 hours.

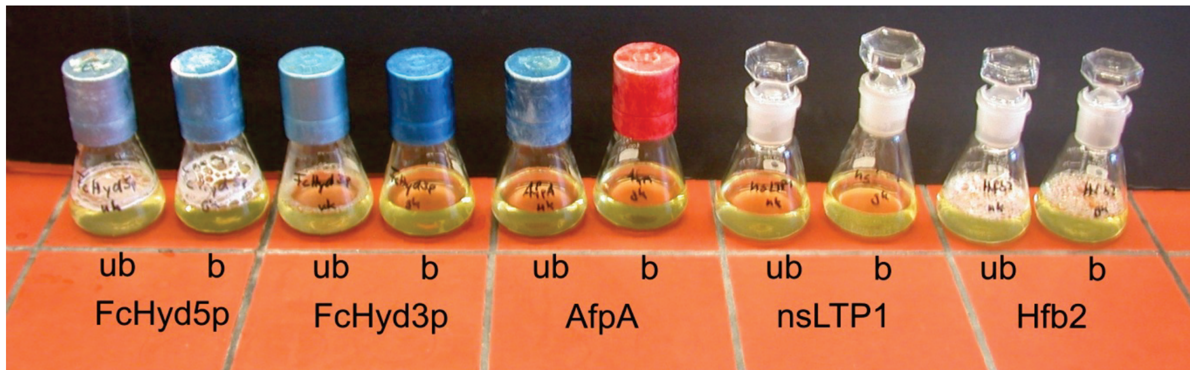

Figure 35: Samples of cell free minimal medium after growth of transformed *P. pastoris* strains 24 hours after homogenization; ub = unboiled, b = boiled;

4 Discussion

In this study a system was set up, in which the gushing-inducing potentials of recombinantly produced surface-active proteins from *F. culmorum*, *F. graminearum*, *T. reesei* and barley were determined. It was found that the gushing-inducing potential was characteristic only for the class II hydrophobins tested, whereas a class I hydrophobin and two moderately surface-active non-hydrophobin proteins were excluded as gushing inducers in beer. Next to the influence of a single protein on the formation of gushing, influences on the gushing-inducing activity of potential gushing inducers could be determined with this system. It was found that the class II hydrophobins retained their gushing potential during the wort boiling process, while the presence of wort sugars and acid caused a reduction of the over-foaming volumes. Also hop oils and modified iso-α acids were found to be able to reduce the gushing potential of the class II hydrophobins. Combinations of AfpA or nsLTP1 with class II hydrophobins revealed that these moderately surface-active proteins – instead of enhancing the gushing problem – are able to reduce the gushing potential. In spite of the extremely low stainability and difficulties in the quantification of some proteins the system established proved to be stable and enables further characterization of gushing influencing compounds.

4.1 Isolation of nsLTP1 from barley

Preparation of nsLTP1 from barley was achieved in two different ways. The first method using HIC led to a removal of most of the undesired proteins from the sample and the almost pure protein was obtained by a combination of HIC with Amicon centrifugal filter units. The second method using IEX directly delivered the pure protein after elution from the column, giving a higher purity as compared to HIC. Moreover, the latter method was considerably time saving as compared to HIC. As a further advantage of this method it was found that an uncharacterized protein of nearly the same molecular size was separated from nsLTP1. It remained unclear during the studies whether the uncharacterized protein is removed during HIC or is still present in the purified sample. When the protein concentrations of both HIC and IEX isolations were analyzed using BSA as reference protein, very low values were found. However, according to the amino acid composition of nsLTP1 and BSA, these values may underestimate the concentrations found for the LTP1 protein, because much more basic and aromatic amino acid residue are present in BSA as compared to nsLTP1. This fact may lead to a comparably higher accessibility of BSA to coomassie blue. The contents of these amino acid residues are shown In Table 2 for both

proteins. Analysis reveals that nsLTP1 contains 13 and BSA 157 of these residues. Deduction of a correction factor is difficult, since the amino acids do not all contribute to same degree to the dyability of a protein. In consideration of the tests, for which nsLTP1 should be prepared, the amounts obtained from fractionated ammonium sulfate precipitation and purification by HIC or IEX were not sufficient, even if the nsLTP1 concentrations of the isolations are assumed to be much higher than the actual values found. The expenditure of time, work, material and equipment resulting in 4 mL of low concentrated protein solution per purification run did not enable to perform tests in carbonated water and beer, which naturally contains high amounts of this protein. In order to still be able to test the influence of nsLTP1 on gushing in carbonated beverages, the gene coding for nsLTP1 was transformed into *P. pastoris* and expressed.

4.2 Heterologous expression of gushing-related proteins in *P. pastoris*

P. pastoris X33 was chosen as host for the production of recombinant surface-active proteins during the current study, since the non transformed cells display only low rates of natural protein secretion into the medium during cultivation (Barr *et al.*, 1992). This fact was confirmed by SDS-PAGE of a freeze dried culture supernatant of the wild type yeast strain. The amount of total protein produced under these conditions was very low as compared to the transformed *P. pastoris* strains. Further confirmation was brought by comparison of the absolute yield of lyophilisate from both types, transformed and non-transformed strains, respectively. Whereas cultivation of *P. pastoris* X33 led to very low amounts of lyophilisate, significantly higher levels were obtained from all recombinant strains produced during the current study. The low rates of naturally secreted proteins of the unmodified *P. pastoris* X33 strain in combination with cultivation using a protein free culture medium for expression served as a first step of purification. Negative controls using lyophilisate of the unmodified yeast also showed that gushing is not induced by the proteins naturally secreted from the yeast. Another important point was that the use of recombinant yeast strains for production of the proteins guaranteed that no other surface-active fungal or plant proteins were present in the protein preparations used for the gushing tests.

Proper insertion of the gene constructs into the genome of the transformed yeast strains was confirmed by analysis of the nucleotide sequence of inserts. Expression and excretion of the transgenic proteins were demonstrated and confirmed by SDS-PAGE and peptide mass finger print for the transformant strains *P. pastoris* X33 [pPICZαA-AfpA], *P. pastoris* X33 [pPICZαA-nsLTP1] and *P. pastoris* X33 [pPICZαA-Hfb2], which produced proteins of the anticipated size. Since no FcHyd3p protein band could be visualized on gels using coomassie blue staining, heterologous expression of the transformed gene was indirectly

confirmed by reverse transcription PCR in cells of the transformant strain *P. pastoris* X33 [pPICZαA-FcHyd3].

It was observed that the recombinant proteins showed different behavior when they were subjected to staining procedures. AfpA proofed to be highly accessible to colloidal coomassie staining, whereas class I hydrophobin FcHyd3p could not at all be stained by this method. Class II hydrophobins FcHyd5p and Hfb2 as well as plant protein nsLTP1 showed moderate dyability. This is in accordance with the finding that coomassie brilliant blue mainly binds on basic amino acid residues arginine, lysine and histidine as well as aromatic residues tryptophan, tyrosine and phenylalanine as reported previously (Compton and Jones, 1985). Table 2 gives the content of these amino acid residues in the corresponding proteins and BSA.

Table 2: Content of amino acid residues, which are accessible to coomassie brilliant blue, in the heterologously expressed proteins and bovine serum albumin (BSA, according to GenBank entry CAA76847.1);

Protein	Count	Residue	Protein	Count	Residue	Protein	Count	Residue
AfpA	5	arginine	nsLTP1	4	arginine	Hfb2	0	arginine
	18	lysine		4	lysine		4	lysine
	3	histidine		2	histidine		1	histidine
	3	tryptophan		0	tryptophan		0	tryptophan
	7	tyrosine		3	tyrosine		0	tyrosine
	8	phenylalanine		0	phenylalanine		3	phenylalanine
FcHyd5p	3	arginine	FcHyd3p	0	arginine	BSA	26	arginine
	1	lysine		1	lysine		60	lysine
	0	histidine		1	histidine		17	histidine
	0	tryptophan		0	tryptophan		3	tryptophan
	2	tyrosine		1	tyrosine		21	tyrosine
	1	phenylalanine		0	phenylalanine		30	phenylalanine

It proved that out of the recombinant proteins, AfpA (44 residues) possesses the highest number of coomassie blue accessible amino acids, whereas FcHyd3p has only 3 of them. nsLTP1, Hfb2, and FcHyd5p have 13, 8 and 7 of such residues, respectively. These facts explain why no eminent protein concentrations could be measured in the fractions obtained from HIC purification of FcHyd5p using the Bradford test, since it is also based on protein staining with the coomassie blue dye.

Deviations and trends towards higher molecular masses of the respective protein bands on were observed on gels during the protein analyses using SDS-PAGE. This may have two reasons. First, the sample preparation comprised the reduction of disulfide bridges through addition of DTT and TCEP. During separation of the proteins on the gel, re-formation of

such bridges may occur. During such re-formation, the SH-groups may not necessarily build the same pairs as can be found in the native molecule resulting in a protein with a different 3D-structure. Such changes may contribute to apparent variations in the molecular size of a protein. During the study two different molecular size markers were used. In Figures 6, 10 and 12 proteins were detected at the expected size using protein marker PM. However, the bands observed after SDS-PAGE as shown in Figure 19 differ from the expected size by 3-4 kDa towards higher masses. Such a shift was also found, when both protein markers were compared as shown in Figure 10 B. Strongly differing patterns of the molecular sizes from one protein marker in relation to the gel composition are described in the manual of PM2. Those differences were found in relation to varying gel concentrations and differing gel types, as for example Tris-Glycine, Tris-Acetate or Bis-Tris gels. In the current study Tris-Tricine gels were used, meaning that the deviations found above are most probably related to the gel type. Therefore, the protein bands found to display a shift to higher masses were nonetheless identified as the respective recombinant proteins.

4.3 Isolation and purification of FcHyd5p

Heterologous expression of the class II hydrophobin FcHyd5p from *F. culmorum* in *P. pastoris* followed two aims. The first objective was to purify the protein from the cell free culture medium of the recombinant yeast strain *P. pastoris* X33 [pPICZαA-FcHyd5] to homogeneity. The second objective was to develop an isolation protocol, which provides access to sufficient amounts of FcHyd5p for application in gushing tests. However, as shown under results detection of FcHyd5p by SDS-PAGE followed by several staining techniques was not successful until amounts >10 mg FcHyd5p lyophilisate per mL were applied to electrophoresis. Furthermore, the protein proved to be not accessible to the Bradford test in solutions with low concentrations of FcHyd5p for the same reason as it was not stainable by coomassie blue. Also photometrical protein detection at 280 nm as used during HIC could not detect the protein. This situation led to difficulties in establishing a proper purification protocol, since protein preparations could not be analyzed for the presence of FcHyd5p. It was only after carrying out protein production in a protein free growth medium in combination with freeze-drying, a lyophilisate was obtained, which contained enough FcHyd5p to detect it after SDS-PAGE and colloidal coomassie blue staining. However, even after the manipulations described it still was not possible to properly identify the protein band as FcHyd5p by mass spectroscopy. Nevertheless, several reasons pleaded for the protein band obtained from freeze dried culture supernatant of the yeast transformant *P. pastoris* X33 [pPICZαA-FcHyd5] to be identical with FcHyd5p: 1. The correctness of the gene insert in *P. pastoris* X33 [pPICZαA-FcHyd5]

as well as expression of the *fchyd5* gene in the transformant were confirmed by Stübner *et al.* (2010). 2. This protein band was only found for *P. pastoris* X33 [pPICZαA-FcHyd5], the unmodified strain showed no band of corresponding size. 3. Amounts of lyophilisate obtained from *P. pastoris* X33 [pPICZαA-FcHyd5] were much higher than those from the unmodified strain. 4. Addition of lyophilisates from both strains to carbonated beverages resulted only in gushing for the lyophilisate obtained from cultures of *P. pastoris* X33 [pPICZαA-FcHyd5].

When comparing the amounts of lyophilisates obtained from both strains the content of FcHyd5p was estimated to be 50-75 % (w/w) of total lyophilisate mass. In contrast, comparison of the measured absorption of a 1 mg/mL solution of FcHyd5p lyophilisate in water at 280 nm with the absorption, which was theoretically calculated based on the amino acid composition of FcHyd5p resulted in a content of FcHyd5p of only 5 % (w/w). It is well known that the absorption as calculated by ProtParam may often differ from the measured values if no tryptophan is present in the protein (Pace *et al.* 1995), which is the case for FcHyd5p. This difference is caused by the fact that tryptophan contributes much more to the over-all extinction coefficient than tyrosine or cystine do (cystine is the amino acid resulting, when two cysteines form a disulfide bridge). However, such great deviations were not found by the authors during establishing their calculation algorithm. Even if both values found for the content of FcHyd5p in the lyophilisate are subjected to high errors in measurement, there will be still a gap, which has to be filled with substances to obtain such high amounts of lyophilisate. Those substances hardly will be other proteins, since they would have increased the overall absorption of the solution of FcHyd5p lyophilisate. The real content of FcHyd5p and the identity of the accompanying substances have to be determined in future studies. Nevertheless, purification of FcHyd5p from the supernatant of *P. pastoris* X33 [pPICZαA-FcHyd5] was successful, even if it could not be purified to homogeneity. Quantities sufficient for testing its gushing-inducing potential could be provided by the isolation protocol established during the current study and an influence of accompanying substances could be excluded.

4.4 Gushing experiments

The influence of hydrophobins and surface-active proteins on the gas release in carbonated beverages was examined by measuring the over-foaming volume, which occurred after conditioning of a carbonated drink with the respective protein. High standard deviations were observed when beer was used, whereas deviations were low in tests with carbonated water. As a consequence of the way of measuring, there are several influencing factors on the resulting gushing volume, which cannot be determined exactly.

Those factors are mainly related to the composition of the beer used, in respect to its content of surface-active substances as well as gushing-favoring or gushing-inhibiting compounds. Also the qualities of the inner wall of the bottle and the content of carbon dioxide have an influence on the volume of lost beer. Nevertheless, due to the lack of other ways to determine the influence of the proteins tested on the gas release in carbonated beverages, the gushing volume was used for examination.

For tests with beer or carbonated water, reusable glass bottles were used in both cases. Since it was found that standard deviations in the experiments with carbonated water were very low as compared to beer, the influence of the quality of the inner wall of the bottle should be low. Concerning the content of carbon dioxide, the probability of variations of this parameter should also be low, since only bottled German lager beer produced by a defined brewery were used in all experiments. These findings indicate that the raw material, the transfer of gushing-enhancing and gushing-inhibiting substances from it into the beer and the process of beer production have the highest influence on the standard deviations of the gushing volumes. They may be the main levers for manipulating and even inhibiting gushing in beer.

4.5 Gushing potential of hydrophobins

4.5.1 Class II hydrophobin FcHyd5p

Gushing experiments clearly showed that hydrophobin FcHyd5p is capable of inducing gushing when added to beer. Since in practice, a beer with a volume loss of 20 mL would already be termed a gushing beer, concentrations of FcHyd5p sufficient to induce gushing to an extent unwanted by breweries would be even lower than what was observed during the current experiments. The tendency, according to which the gushing volume is dependent on the amount of FcHyd5p added, is supported by previous studies (Christian *et al.*, 2009a, 2009c). The authors stated that gushing could only be observed from a minimum concentration of gushing-inducing substances. In the experiments of the current study gushing could be induced by the mere addition of freeze-dried hydrophobin FcHyd5p, without rotating the bottles, and hence without bringing bubbles mechanically into the beer. This indicates that microbubbles were brought into the beer by the lyophilisate. These microbubbles could be the result of air, which is trapped in the porosity of the lyophilisate and stabilized by hydrophobin molecules upon dissolving in the beverage. This effect disappeared and no gushing occurred when the hydrophobin was dissolved in water prior to addition, and bottles were not rotated. For carbonated water, both cases led to reduced gushing. It was assumed that this effect was caused by the higher sensitivity of that model

system as compared to beer. In beer, new bubble interfaces are immediately covered by the surface-active substances present as reported by Liger-Belair (2005), whereas in carbonated water no such substances are available. Therefore, bubbles in beer, which are formed and introduced during opening and addition of FcHyd5p solution, are covered by hydrophobin molecules and surfaces active molecules from beer. In carbonated water, those bubbles can be stabilized exclusively by the hydrophobin molecules leading to gushing, even after addition of dissolved FcHyd5p. Christian *et al.* (2009b) were able to induce gushing in carbonated water by the addition of a surface-active substance called CTAC. They further observed that gushing also occurred without shaking of the bottles. The authors assumed that this is caused by diffusion of CO_2 into the evacuated micelles of CTAC.

It was demonstrated that the FcHyd5p protein is heat stable and maintains its gushing potential when boiled in water. However, a slight reduction of the gushing potential in beer was found after boiling the hydrophobin in synthetic wort, as well. This reduction could possibly be caused by glycosylation of the protein during heat treatment in the presence of wort sugars as was observed for nsLTP1 (Jégou *et al.*, 2001). In this case such glycosylation has to take place in the hydrophobic part of the protein, which would reduce its surface activity and explain the lower gushing potential. This matter, however, has to be elucidated in more detail in future studies.

Ultrasonication treatment, which was applied as disintegrative conditioning to break down assembled hydrophobin molecules, showed no reducing influence on the gushing volume. This is in accordance with the results of the boiling tests with water-dissolved hydrophobin and indicates that monomerized molecules are able to induce gushing by migration to water-air interfaces where a concentration of these molecules takes place and stabilized bubbles are formed.

4.5.2 Class II hydrophobin Hfb2

Trichoderma spp. are known as soil fungi, which are prevalent in the litter of humid, mixed hardwood forests (Domsch *et al.*, 1980; Eveleigh, 1985). Therefore, they may be found on kernels of barley or grain, but deep infections of those grains by *Trichoderma* spp. are not common. As a consequence, Hfb2 from *T. reesei* will not play a role in the actual induction of gushing in beer. However, this protein is one of the best characterized class II hydrophobin proteins (Askolin *et al.*, 2005; Kisko *et al.*, 2008, 2009; Nakari-Setälä *et al.*, 1997; Sarlin *et al.*, 2005; Torkkeli *et al.*, 2005) and could help a lot in finding characteristics, which are crucial for gushing induction. Hence, this protein was integrated in this study. It

was demonstrated in the gushing experiments performed that the class II hydrophobin Hfb2 from *T. reesei* is a strong gushing inducer in beer. This is in accordance with reports from Sarlin *et al.* (2005), who made the same observation upon addition of native Hfb2 to beer. However, the relations found between the amounts of hydrophobin added and the gushing volume obtained greatly differs between previous reports and the current study. These differences may be due to the fact that in the previous studies Hfb2 was isolated from cultures of *T. reesei,* and not from a transgenic source. Also the concentrations of the hydrophobin in the solutions used are not readily comparable. Comparison of the relation between hydrophobin quantity and resulting gushing volume for Hfb2 with the results found for hydrophobin FcHyd5p showed a lower gushing potential of Hfb2 in beer as compared to FcHyd5p. A similar tendency was observed when Hfb2 was added to carbonated water. Both in water and in beer, higher dosages of Hfb2 were necessary to induce similar gushing volumes. The finding that heat treatment reduced the gushing potential of Hfb2 indicates that the class II hydrophobin is moderately heat sensitive, since the protein partially retained its gushing-inducing potential under the conditions prevailing at the wort boiling step during beer production. Similar to FcHyd5p the gushing potential of Hfb2 in carbonated water is not influenced by heat treatment when dissolved in deionized water or synthetic wort prior to addition to the beverage. However, the gushing potential of FcHyd5p in beer was less influenced by heat treatment.

4.5.3 Class I hydrophobin FcHyd3p

When the transgenic class I hydrophobin FcHyd3p was added to beer, no gushing was observed, whereas in carbonated water a slight gushing-inducing potential was detected. However, the volumes measured were much lower as compared to those found with class II hydrophobins Hfb2 or FcHyd5p. Heat treatment of FcHyd3p further reduced its gushing potential. These findings suggest that FcHyd3p is not a relevant gushing-inducing protein in beer. The findings are in agreement with results reported by Zapf *et al.* (2006b) who examined the gushing potential of FcHyd3p in a wort fermentation using a transgenic *S. cerevisiae* strain, which produced FcHyd3p during fermentation. No gushing tendencies were found in the resulting beer.

4.6 Gushing potential of AfpA and nsLTP1

AfpA is a moderately surface-active alkaline protein produced by *F. graminearum*. Testing the influence of transgenic AfpA in carbonated beverages revealed that this protein did not induce gushing under the conditions applied during the current study. The observation that

AfpA has gushing-enhancing properties as published previously (Zapf *et al.*, 2007) could not be confirmed here. Addition of the protein to carbonated beverages pre-conditioned with either of the two gushing active class II hydrophobins revealed a gushing reducing character of the protein. The same observations were made when nsLTP1 was added to beer and to carbonated water pre-conditioned with class II hydrophobins. Also this protein did not induce gushing when added to beer or carbonated water alone. In the case of nsLTP1, reduction of the gushing volume could only be determined in carbonated water. As shown above, results also point to the ability of nsLTP1 to reduce gushing in beer. However, this finding could not be definitely confirmed and should be further elucidated in future studies.

4.7 Influence of surface-active compounds on hydrophobin treated, carbonated water

A feature common to both class II hydrophobins tested here was that combined addition together with either surface-active AfpA or nsLTP1 both reduced the gushing volumes in beer and in carbonated water. This observation may help to explain the differences of gushing volumes found between beer and carbonated water when hydrophobins were added at the same level. An increase of the content of these surface-active proteins (AfpA or nsLTP1) in the respective beverage provided a higher resistance against gushing induced by class II hydrophobins. Since carbonated water is free of surface-active proteins, no such gushing reducing agents are present in this beverage. The absence of those substances could be the reason for the high gushing sensitivity of carbonated water to treatment with hydrophobins. The fact that Hippeli and Hecht (2008) detected reduced amounts of nsLTP1 in bottles of gushing beer as compared to non gushing beer of the same brand would explain the increased gushing instability and support the assumptions made above. Based on the current results it is suggested, that beer with low levels of nsLTP1 is more susceptible to gushing as compared to beer with normal levels of this surface-active protein. Therefore, the reason for the coincidence of gushing and low levels of nsLTP1 in beer is not necessarily a result of nsLTP1 degradation products as postulated by Hippeli and Hecht (2008). It may possibly be caused by a shift in the relative concentrations of gushing-inducing hydrophobins and nsLTP1 in favor of the hydrophobins. Such a shift could result in a changed composition of the bubble skin. It is suggested that an addition of FcHyd5p or Hfb2 to carbonated water leads to single-domain bubble skins, which are exclusively covered with hydrophobin molecules and possess a high gushing potential. A combined addition with proteins such as AfpA or nsLTP1 results in formation of multi-domain bubble skins with a reduced gushing potential. The higher the percentage of

surface-active proteins such as nsLTP1 and AfpA in a carbonate beverage is, the lower the susceptibility to gushing will be. This would explain, why gushing in beer could be much better reduced by addition of AfpA than in water. In beer already a great amount of surface-active substances are present giving a basic resistance to gushing, whereas in carbonated water the lack of such a basic resistance must be compensated by addition of higher amounts of surface-active proteins.

It was observed during the tests that such a resistance completely prevented gushing upon addition of FcHyd5p in some bottles of beer from different breweries. Apparently, there is a strongly varying intrinsic gushing inhibition in beer from different breweries. Obviously, some combinations of brewing process parameters, hopping and the nature of the raw materials used are able to increase the resistance in beer against gushing. It is an important point of future investigations, to determine, which substances present in beer are able to provide resistance against gushing and how their concentrations can be increased by the choice of raw material or by modification of process parameters during beer production.

4.8 Foam stability of recombinant proteins

Class II hydrophobins FcHyd5p and Hfb2 were demonstrated to be highly foam active proteins. Foams produced by homogenization of cell free supernatants of transformant cultures excreting either hydrophobin proved to be highly stable over days. In contrast to that finding, the class I hydrophobin FcHyd3p possessed only a low foam stabilizing ability. Other fungal (AfpA) and plant surface-active proteins (nsLTP1) possessed no such stabilizing properties. These findings are in correspondence with the gushing-inducing potentials found for the proteins during the current study. According to the theory published by Fischer (2001), the induction of gushing by surface-active substances should be related to their ability to stabilize bubbles at diameters, which enable them to grow after pressure release of a saturated CO_2 solution. If the ability to stabilize foams at this extent is conferrable to the ability to stabilize bubbles in carbonated beverages at radii greater than the critical diameter, the foam stabilizing test may be applied as a further tool to analyze the gushing-inducing potential of substances added to beer.

4.9 Influence of hop components on hydrophobin induced gushing

During the current study further parameters for inhibition of gushing were investigated. One such parameter was the addition of hops, which might be a modulating factor of gushing

activity in beer. It should be mentioned that the beer utilized here already contained hops. However, responses to the addition of FcHyd5p sometimes differed greatly from lot to lot. This suggests that the gushing inhibiting behavior of such beer cannot only be ascribed to the presence of hop compounds in these lots. In order to address this question, commercial products containing enriched hop components were added to beer after addition of class II hydrophobins FcHyd5p or Hfb2. In the case of hop oils (linalool and HOTD) as well as iso-α acid products (Rho 35 %, potassium salts of reduced iso-α-acids, and Tetra 10 %, potassium salts of tetrahydro-iso-α-acids), addition led to a reduction of gushing volumes with the highest reduction achieved by the addition of hop oils for FcHyd5p. This is in accordance with results published previously (Gardener *et al.*, 1973; Krause, 1936). The findings are also supported by the very recent work of Hanke *et al.*, (2009). The effects observed could be caused by the foam negative properties of the oil. However, a reduction of gushing volume was also achieved by the addition of the Tetra 10 % product, which possesses foam-promoting properties, leaving unsolved the mechanisms behind the reduction of the gushing potential of class II hydrophobins. The gushing-enhancing effect of Iso 30 % could have been caused by the mode of addition. It is recommended by the manufacturer that hop products should be added before filtration to ensure any contained particles are removed from the bottled beer. However, filtration could not be performed in the tests. The addition of hop products to class II hydrophobin FcHyd5p treated carbonated water in the same amounts as used in beer resulted for each product in an increase of the gushing volume. This increase could be explained by a surface-active effect of the products leading to a controlled gushing, in which the throttling property of the bottleneck is reduced. These findings also indicate that none of the hop products are capable of inhibiting gushing on their own. Further substances from beer may be needed to provide gushing inhibiting properties. For Hfb2 reduction of the gushing volumes was observed for iso-α acid product Rho 35 % and hop oil products HOTD and linalool. However, addition of linalool, which had the strongest reducing effect when added to beer in combination with FcHyd5p, showed only a slight reduction when applied with Hfb2. Further, this effect could not be intensified upon addition of higher quantities of linalool as was observed with FcHyd5p. This finding represents an essential difference between both class II hydrophobins.

4.10 Differences between class II hydrophobins FcHyd5p and Hfb2

In the gushing tests it was found that both class II hydrophobins FcHyd5p and Hfb2 are strong gushing-inducers. However, differences were found concerning their resistance against heat treatment, the extent of their gushing potential and as a main point the different influence of linalool on the resulting gushing volume in beer. Such distinctions may

be caused by structural differences of both proteins. Comparison of the structure in combination with comparison of their amino acid composition may help to find general characteristics crucial for a potentially gushing-inducing protein in beer. A relation between the ability to induce gushing and the structure of potentially gushing-inducing agents was previously reported by Christian *et al.* (2009b) for a different class of macromolecules. The authors examined the induction of gushing in carbonated water by addition of pure aliphatic surfactants. They found that gushing occurred exclusively if a surface-active substance called CTAC was added. The gushing activity was correlated to the chemical structure of the compound.

4.11 Differences between class I and class II hydrophobins

In the experiments gushing could be induced in beer and carbonated water upon addition of class II hydrophobins Hfb2 and FcHyd5p. However, this strong gushing-inducing potential was not observed for the class I hydrophobin FcHyd3p, which showed only a low gushing-inducing ability in carbonated water. Concerning the classes, in which hydrophobins are categorized, it would be interesting to know, if the strong gushing-inducing potential is related to class II hydrophobins or exclusively to specific hydrophobins of both classes. More information about gushing-inducing potentials of other class I hydrophobins would help to confine the search of gushing relevant substances.

4.12 Indications for a gushing mechanism

In the experiments, gushing was induced in beer and carbonated water after addition of class II hydrophobins FcHyd5p or Hfb2. According to Draeger (1996) and Krause (1936), the existence of microbubbles is required for the occurrence of gushing. Since no surface-active substances are available in carbonated water, gushing-inducing microbubbles must be formed by hydrophobins alone. Therefore, the mechanism leading to gushing must be associated with the properties of the hydrophobin interface of the bubble. According to the theory as published by Fischer (2001) the induction of gushing by substances such like FcHyd5p has to be caused by its ability to stabilize bubbles at sizes, which enable them to grow after supersaturation on the one hand. On the other hand, since hydrophobins are able to form highly ordered and stable films in air-water interfaces (Kisko *et al.*, 2009; Wösten *et al.*, 1993) it is suggested that growth of the hydrophobin interface upon pressure release is limited in those bubbles. When a bottle is opened, hydrophobin covered bubbles grow until the surface tension of the hydrophobin layer reaches a point, where either

growth is stopped or the bubbles burst providing new condensation nuclei for generation of gushing. In beer, three different types of microbubbles are probable: bubbles with an interface composition identical to non-gushing beer (single-domain), bubbles with a homogenous hydrophobin interface (single-domain), and bubbles with a mixed interface composition containing both (multi-domain). Homogeneous hydrophobin bubbles are supposed to behave similar to those in carbonated water. Hydrophobin domains in multi-domain bubbles are supposed to disintegrate along the domain borders upon bubble growth, due to their limited ability to grow. In both cases the incorporation of hydrophobin molecules into the bubble skin would lead to a breakdown of the bubble, resulting in multitude of new bubble nuclei.

4.13 Concluding considerations

As shown above, addition of class II hydrophobins FcHyd5p and Hfb2 led to gushing in German lager beer and carbonated beverages. However, it could also be demonstrated that in some lots of German lager beer from different breweries no gushing was induced upon addition of the standard dosage of 2 mg FcHyd5p lyophilisate. An increase of the amounts added revealed a principally similar relationship between added quantities and gushing volume. Further, it was shown that the application of enriched hop compounds was able to reduce the gushing volumes, which resulted from addition of hydrophobins. Last but not least AfpA and nsLTP1 as non-hydrophobin surface-active proteins proved to be able to reduce the gushing volume in beer. These are findings, which indicate, that a manipulation of the process of beer production towards an increased gushing stability is possible. Those manipulations require a deliberate choice of hop for beer production, analysis of the potential to influence the content of surface-active proteins and gushing-inhibiting substances in the finished beer.

Based on the findings of this study new questions arose concerning the role of surface-active proteins in the formation of gushing:

1. What influences do Z-proteins as major beer foam proteins from barley have on the occurrence and the extent of gushing?
2. What contents of nsLTP1, Z-proteins and class II hydrophobin FcHyd5p can be found in each step throughout the entire value chain of beer production? Can the contents be determined by immunochemical methods?
3. Does *F. tricinctum* produce a class II hydrophobin with a gushing potential comparable to FcHyd5p from *F. culmorum*? Since in 2008 a significant increase of

the occurrence of this fungus on kernels of brewing grains coincided with an increased occurrence of gushing.

4. How do FcHyd5p or Hfb2 differ from a class II hydrophobin from *F. verticilloides?* This fungus is not known as a gushing inducer, and therefore differences in structure and amino acid composition of such a class II hydrophobin could help to identify gushing relevant characteristics in hydrophobins.
5. Is it possible to visualize the incorporation of hydrophobins or surface-active proteins into the bubble skin, e. g. by scanning electron microscopy?

5 Summary

The phenomenon of gushing, i.e. the spontaneous over-foaming of beer and other carbonated beverages immediately upon opening of a bottle, is a highly unwanted condition. It causes severe economic losses to breweries and producers of carbonated drinks, but the mechanisms and inducing agents involved have not been fully identified up to now. Already in the middle of the last century it was recognized that the possible causes of gushing in beer can be referred to two types: The primary gushing, which is a malt related problem, and the secondary gushing, the reasons for which can regularly be related to technological failure during the brewing process. This splitting provided a cause-oriented research leading to feasible solutions for secondary gushing. However, the research on molecular causes and preventive measures for primary gushing is still on its way. In present research activities surface-active proteins are in the focus as gushing inducers, among them hydrophobins, extremely surface-active from filamentous fungi and non-specific lipid transfer proteins from brewing barley, which can be found as major foam protein in beer. The aim of the present study was, to establish a system that provides the possibility to evaluate the contribution of single surface-active proteins to gushing. It should also enable to assess the influence of process parameter, such as heat treatment, or the influence of other substances present in beer, such as hop compounds or surface-active proteins, on gushing-inducing agents. This should be accomplished by heterologous expression of surface-active proteins from fungi, which are related to the occurrence of gushing, and nsLTP1 from barley.

Based on this situation, the class II hydrophobin FcHyd5p from *F. culmorum*, a plant pathogenic fungus related to the occurrence of gushing in beer, was heterologously expressed in the yeast *P. pastoris* and an isolation protocol was developed. This protocol provided access to quantities sufficient for application of FcHyd5p in gushing experiments. During the tests it was found that the class II hydrophobin FcHyd5p is able to induce gushing in all beverages tested comprising beer, carbonated water and several carbonated fruit juices. Investigations on the heat stability of FcHyd5p revealed that it retains its gushing potential after treatment under conditions as prevailing at wort boiling. It was shown that the presence of sugars and acids as characteristic for wort during heat treatment leads to a slight decrease in its gushing potential, whereas heat treatment in deionized water had no effects on the gushing volume. Ultrasonication treatment, which was applied to break down assemblies of hydrophobin molecules, also had no influence on the potential of FcHyd5p to induce gushing. The previously reported finding, according to which hop components are able to inhibit gushing in beer, could be confirmed for gushing

induced by FcHyd5p. In this context, hop oils proved to be more efficient than modified iso-α acid products.

Next to FcHyd5p another class II hydrophobin, Hfb2 from *T. reesei*, the class I hydrophobin FcHyd3p from *F. culmorum* as well as the non-hydrophobin surface-active proteins AfpA from *F. graminearum* and nsLTP1 from barley were tested for their influence on the gas release in carbonated beverages. The corresponding genes were transferred into clones of *P. pastoris*, the proteins heterologously expressed, isolated and added to carbonated beverages. Isolation procedures were based on the protocol as described for the preparation of FcHyd5p.

The class II hydrophobin Hfb2, from *T. reesei*, which is not plant pathogenic, was used, since it is one of best chemically and physically characterized hydrophobins of this class. For this protein also a strong gushing-inducing potential was found. However, higher levels were needed to achieve the same extent of gushing in beer and carbonated water as compared to FcHyd5p. Hfb2 proved to retain its gushing potential after heat treatment, but it was more susceptible to the presence of wort sugars and wort acids during heat treatment than FcHyd5p. A main difference between both class II hydrophobins was found by application of hop products. Hop oil and modified iso-α acid-products were able to reduce the over-foaming volume of Hfb2 conditioned beer. However, the gushing potential of Hfb2 proved to be almost unaffected by linalool. This hop oil component was very effective in suppression of gushing when added to beer pre-treated with FcHyd5p.

The class I hydrophobin FcHyd3p was found to be able to induce a moderate form of gushing in carbonated water, but no over-foaming was observed, when it was added to beer. Heat treatment further reduced the gushing volumes found in carbonated water. Therefore, the class I hydrophobin FcHyd3p could be excluded as a relevant substance for induction of gushing in beer.

The non-hydrophobin proteins AfpA and nsLTP1 proved unable to induce gushing in beer or carbonated water. Heat treatment of these proteins did not change this finding. When AfpA or nsLTP1 was added to beer or carbonated water pre-conditioned with a class II hydrophobin a gushing inhibiting character was found for both surface-active proteins.

In tests, in which the foam stability of the recombinant proteins was examined over several days, it could be shown that these properties were closely related to the gushing potential. The class II hydrophobins showed a strong stabilizing potential, whereas for the class I hydrophobin this property was very limited. For AfpA and nsLTP1 foam was broken down already after one hour.

The findings of this study provide experimental evidence that the class II hydrophobin FcHyd5p also leads to induction of beer gushing under natural conditions, since this protein is naturally produced as a hyphal surface protein by *F. culmorum* and the closely related *F. graminearum*, both of which are fungi commonly found on malts leading to primary gushing of beer. The class I hydrophobin FcHyd3p, the fungispumin AfpA as well as nsLTP1 could be excluded as gushing inducers. It was indicated that the presence of surface-active proteins, such as AfpA or nsLTP1, as well as hop components are able to reduce or even completely prevent gushing. Based on this work it is possible now to screen compounds for their ability to inhibit gushing in beer. New starting points for process-aided ways for minimizing the risk of gushing in beer concerning the content of surface-active substances are given.

6 Zusammenfassung

Das Auftreten von Gushing, dem spontanen Überschäumen von Bier oder anderen kohlensäurehaltigen Getränken nach sachgerechter Öffnung, ist ein in hohem Maße unerwünschtes Ereignis sowohl für Verbraucher als auch für Getränkehersteller. Denn Gushing trägt zu Imageschädigungen und hohen wirtschaftlichen Verlusten der Getränkeindustrie bei. Die Mechanismen und ursächlichen Substanzen für dieses Problem aber konnten bis jetzt nur zum Teil aufgeklärt werden. Seit Mitte des letzten Jahrhunderts unterscheidet man zwei Typen von Gushing: Das primäre oder auch malzverursachte Gushing, das durch von Schimmelpilzbefall qualitativ verändertes Braugetreide verursacht wird, und das sekundäre Gushing, das auf technologische Fehler während der Bierherstellung zurückgeführt wird. Während die Aufteilung des Gushingproblems eine Ursachen-orientierte Forschung ermöglichte und zu Lösungen für den sekundären Typ führte, sind die Umstände, die zu primärem Gushing führen noch immer nicht geklärt. Im Fokus gegenwärtiger Forschungsaktivitäten zum malzverursachten Gushing stehen oberflächenaktiven Proteinen und darunter vor allem Hydrophobine, extrem amphiphile Proteine, die beim Wachstum von Schimmelpilzen produziert und sekretiert werden, als auch nicht-spezifische Lipid-Transfer Proteine (nsLTPs), pflanzliche Proteine, die in Bier den Hauptanteil der Schaumproteine ausmachen. Das Ziel der vorliegenden Arbeit war es, ein System zu etablieren, mit dem es möglich ist, den Beitrag einzelner oberflächenaktiver Proteine beim Auftreten von Gushing zu untersuchen. Daneben sollte dieses System die Möglichkeit bieten, den Einfluss von Prozessparameter, wie zum Beispiel den einer Hitzebehandlung, als auch den Einfluss anderer im Bier vorhandenen Substanzen, wie zum Beispiel den von Hopfenbestandteilen oder oberflächenaktiver Proteinen, auf die Gushingneigung zu bestimmen. Umgesetzt werden sollte dies durch die heterologe Expression von oberflächenaktiven Proteinen aus Schimmelpilze, die mit dem Auftreten von Gushing in Verbindung gebracht wurden, und von nsLTP1 aus Gerste.

Basierend auf den oben genannten Erkenntnissen, wurde das Klasse II Hydrophobin FcHyd5p aus *F. culmorum*, einem pflanzenpathogenen Schimmelpilz, der seit Längerem mit dem Auftreten von Gushing in Bier in Verbindung gebracht wird, heterolog in der Hefe *P. pastoris* exprimiert und eine Methode zur Isolierung dieses Proteins entwickelt. Dies ermöglichte es, ausreichende Mengen an FcHyd5p herzustellen, um mit diesem Gushingexperimente in kohlensäurehaltigen Getränken durchzuführen. In den Versuchen zeigte sich, dass durch Zugabe des Klasse II Hydrophobins FcHyd5p in allen untersuchten karbonisierten Getränken Gushing hervorgerufen werden kann. In diesem Zusammenhang wurden Bier, Mineralwasser und verschiedene kohlensäurehaltige Fruchtsaftschorlen getestet. Bei Untersuchungen zur Hitzestabilität von FcHyd5p stellte sich heraus, dass das

Hydrophobin sein Gushing induzierendes Potential durch eine der Würzekochung von Bier gleichwertige Hitzebehandlung nicht verliert. In Anwesenheit von Würzezuckern und Würzesäuren in deren charakteristischen Konzentrationen wurde eine leichte Reduzierung des Gushingpotentials gemessen. In Abwesenheit dieser Substanzen blieb eine Verminderung des erzeugten Gushingvolumens nach Erhitzen jedoch aus. Ultraschallbehandlungen, die angewendet wurden, um aggregierte Hydrophobin-Moleküle zu vereinzeln, zeigten keinen Einfluss auf das Gushingpotential von FcHyd5p. Die seit Langem bekannte Gushing inhibierende Wirkung verschiedener Hopfenkomponenten konnte auch für FcHyd5p verursachtes Gushing bestätigt werden. In diesem Zusammenhang erwiesen sich Hopfenöle effektiver als die modifizierten Iso-α-säuren.

Neben FcHyd5p wurden Hfb2 aus *T. reesei*, ebenfalls ein Klasse II Hydrophobin, das Klasse I Hydrophobin FcHyd3p aus *F. culmorum* als auch die beiden oberflächenaktiven Proteine AfpA, ein Fungispumin aus *F. graminearum*, und nsLTP1 aus Gerste auf ihren Einfluss auf die Gasentbindung in karbonisierten Getränken getestet. Dafür wurden die entsprechenden Gene in Klone der Hefe *P. pastoris* transferiert. Nach heterologer Expression und Isolierung der Proteine, basierend auf dem für FcHyd5p erarbeiteten Protokoll, wurden diese zu Bier und kohlensäurehaltigem Wasser zugegeben.

Das zweite Klasse II Hydrophobin, Hfb2 aus *T. reesei,* ein Schimmelpilz, der nicht Pflanzen-pathogen ist, wurde verwendet, da es eines der am besten chemisch und physikalisch charakterisierten Hydrophobine dieser Klasse ist. Wie FcHyd5p zeigte auch dieses Protein, ein starkes Gushing induzierendes Potential. Es waren jedoch größere Mengen dieses Proteins nötig, um dieselben Überschäumvolumina in Bier oder Wasser zu erreichen wie im Vergleich zu FcHyd5p. Auch in Bezug auf seine Hitzestabilität war Hfb2 sehr ähnlich zu FcHyd5p. Hfb2 behielt sein Gushingpotential nach einer der Würzekochung nachempfundenen Hitzebehandlung. Ebenso führte die Anwesenheit von Würzezuckern und Würzesäuren bei diesen Behandlungen zu reduzierten Gushingvolumina. Diese Reduzierungen waren aber stärker als bei FcHyd5p. In Abwesenheit der Würzekomponenten konnten nach Erhitzung keine Veränderungen festgestellt werden. Ein Hauptunterschied zwischen beiden Klasse II Hydrophobinen wurde in der Reaktion auf Hopfenkomponenten gefunden. Zwar konnten auch bei Hfb2 die Überschäumvolumina durch Zugabe von Hopfenöl- und modifizierten Iso-α Säure-Produkten reduziert werden, jedoch wurde das Gushingpotential von Hfb2 durch Linalool beinahe nicht beeinflusst. Diese Hopfenölkomponente zeigte bei FcHyd5p eine sehr starke Gushing inhibierende Wirkung.

Das Klasse I Hydrophobin FcHyd3 konnte nach Zugabe nur in karbonisiertem Wasser eine gemäßigte Form von Gushing erzeugen. In Bier konnte damit in keiner Weise

Überschäumen hervorgerufen werden. Eine Hitzebehandlung reduzierte das geringe Gushingpotential in Wasser nochmals. Damit konnte das Klasse I Hydrophobin FcHyd3p als relevante Gushing induzierende Substanz in Bier ausgeschlossen werden.

Mit den beiden oberflächenaktiven Proteinen AfpA und nsLTP1 konnte weder in Bier noch in karbonisiertem Wasser Gushing erzeugt werden. Dies wurde auch durch eine Hitzebehandlung nicht verändert. Es zeigte sich jedoch eine Gushing reduzierende Wirkung, wenn AfpA oder nsLTP1 zu Bier oder kohlensäurehaltigem Wasser gegeben wurde, das bereits mit einem der beiden Klasse II Hydrophobine versetzt war.

In Versuchen, bei denen die Schaumstabilität der rekombinanten Proteine über mehrere Tage untersucht wurde, konnte gezeigt werden, dass die Schaum stabilisierenden Eigenschaften eng mit dem Gushingpotential verknüpft waren. So wiesen die beiden Klasse II Hydrophobine ein sehr hohes Stabilisierungsvermögen auf, wohingegen dies beim Klasse I Hydrophobin nur sehr gering ausgeprägt war. Bei den Proteinen AfpA und nsLTP1 war der Schaum bereits nach einer Stunde verschwunden.

Die Ergebnisse dieser Arbeit legen in sehr deutlicher Weise nahe, dass das Klasse II Hydrophobin FcHyd5p auch unter realen Umständen zu Gushing in Bier führt, da dieses Protein natürlicherweise von *F. culmorum* und dem nahe verwandten Pilz *F. graminearum*, die sehr häufig auf Gushingmalz gefunden werden, als Oberflächenprotein produziert wird. Das Klasse I Hydrophobin FcHyd3p, das Fungispumin AfpA und nsLTP1 aus Gerste konnten als relevante Gushing induzierende Substanzen ausgeschlossen werden. Vielmehr deuten die Resultate darauf hin, dass die Anwesenheit von oberflächenaktiven Substanzen, wie AfpA und nsLTP1, und die Anwesenheit von Hopfenkomponenten Gushing reduzieren und sogar aufheben können. Basierend auf den Ergebnissen dieser Arbeit ist es nun möglich, Substanzen auf Gushing inhibierende Eigenschaften zu untersuchen. Im Hinblick auf den Gehalt an oberflächenaktiven Substanzen in Bier geben die Erkenntnisse neue Ansatzpunkte für Prozess-gesteuerte Wege, mit denen das Risiko für das Auftreten von Gushing minimiert werden kann.

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92. Zapf, M.W., Theisen, S., Rohde, S., Rabenstein, F., Vogel, R. F., Niessen, L., (2007). Characterization of AfpA, an alkaline foam protein from cultures of *Fusarium culmorum* and its identification in infected malt. Journal of Applied Microbiology, 103, 36-52.

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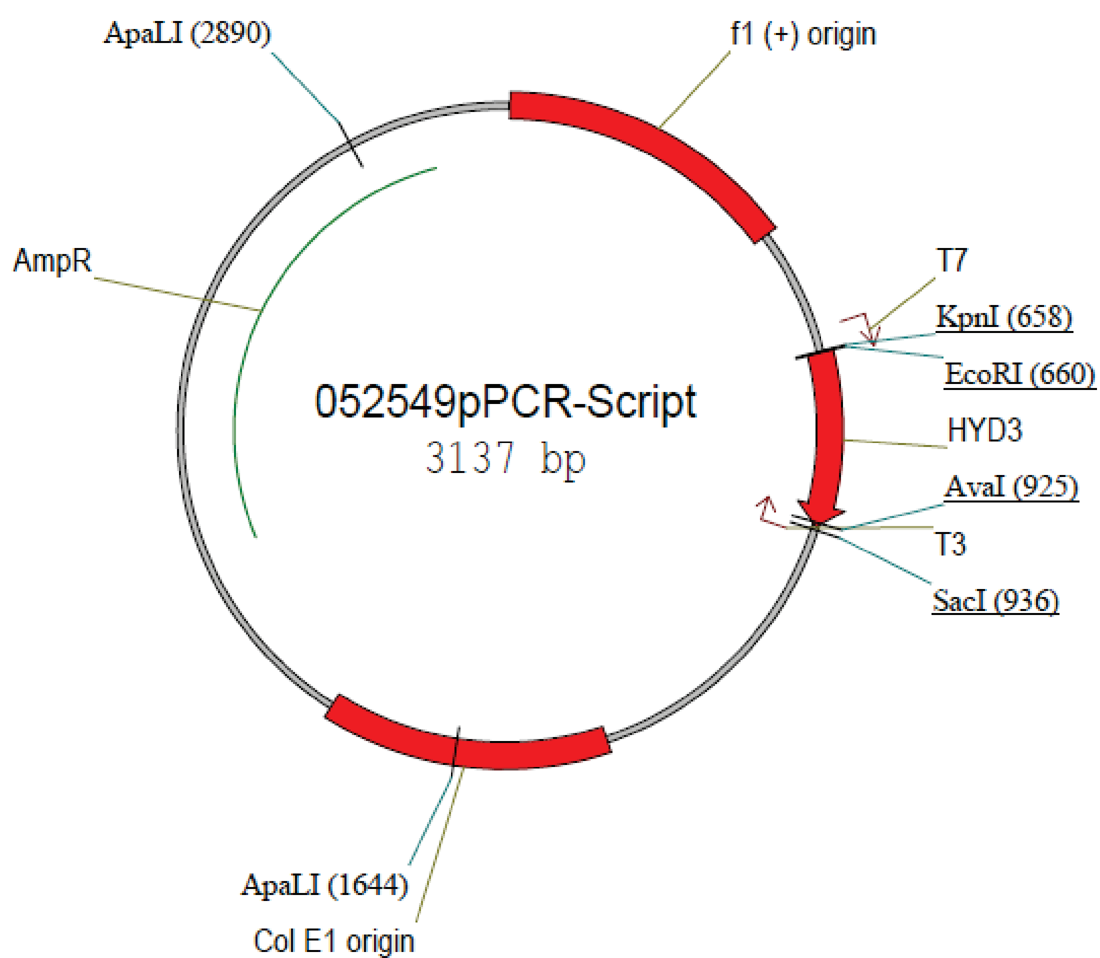
8 Appendix

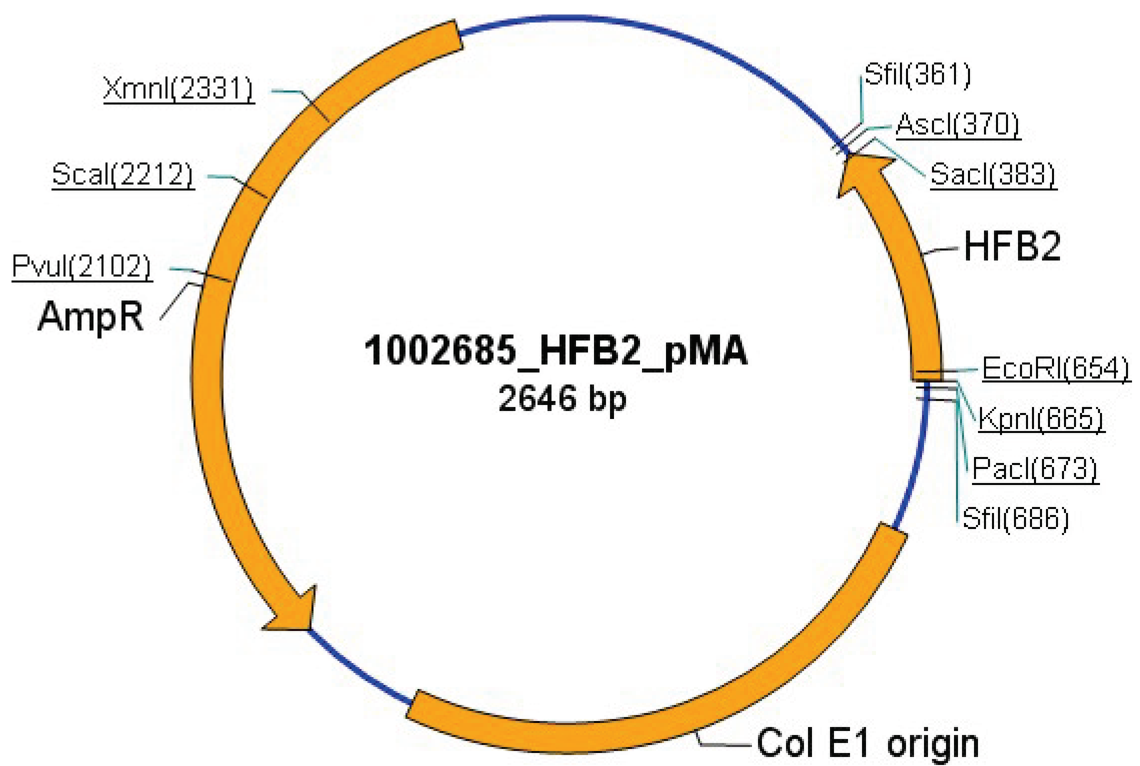
8.1 Plasmid 052549pPCR-Script

Plasmid 052549pPCR-Script containing the nucleotide sequence of the class I hydrophobin FcHyd3p from *F. culmorum* adapted to the codon-usage of *S. cerevisiae*.



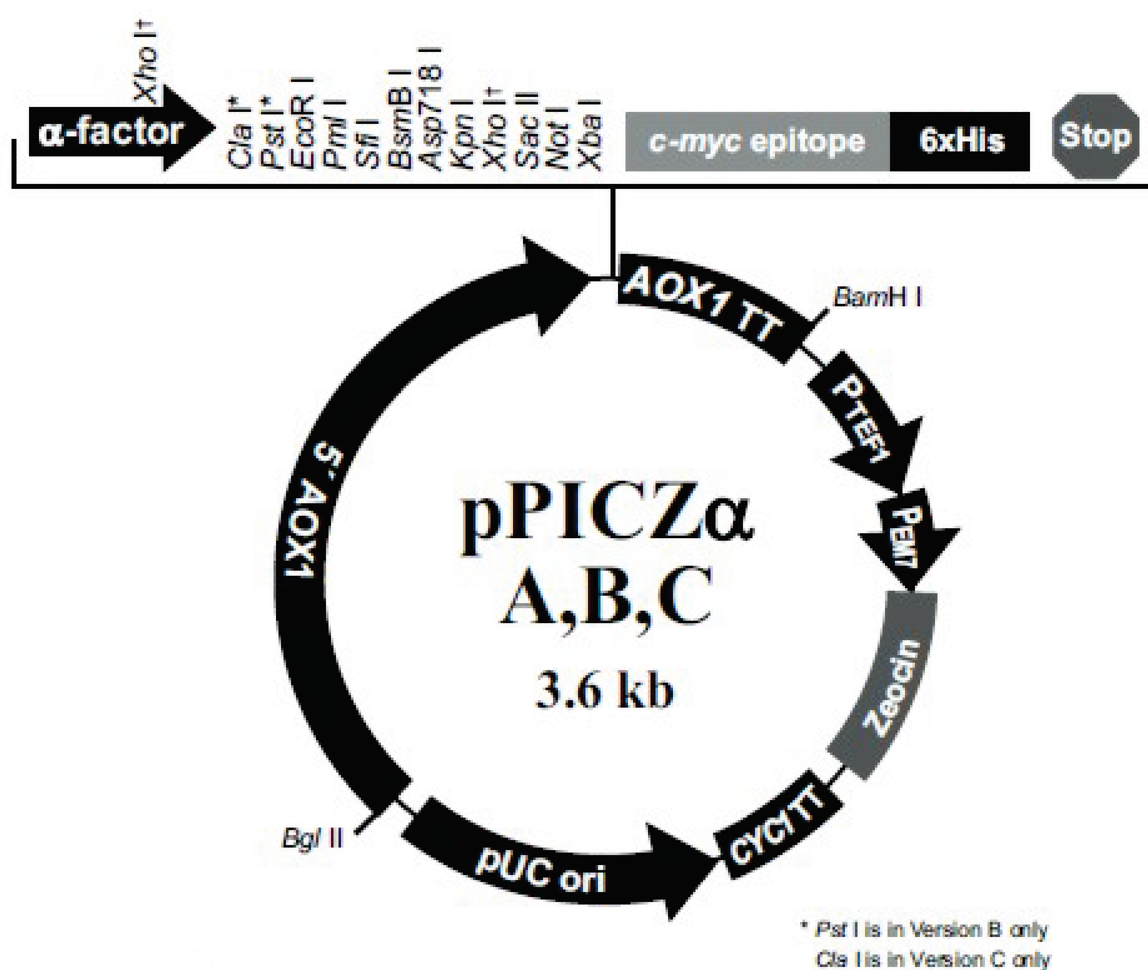
8.2 Plasmid 1002685_HFB2_pMA

Plasmid 1002685_HFB2_pMA containing the nucleotide sequence of the class II hydrophobin Hfb2 from *T. reesei* adapted to the codon-usage of *S. cerevisiae*.



8.3 Plasmid pPICZα

Plasmid pPICZα as used for transformations of *P. pastoris* X33 (EasySelect™ *Pichia* Expression Kit, Invitrogen, Carlsbad, USA).



8.4 Publications

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

Lutterschmid, G., Stübner, M., Vogel R. F., Niessen, L. (2010). Induction of gushing with recombinant class II hydrophobin FcHyd5p from *Fusarium culmorum* and the impact of hop compounds on its gushing potential. Journal of the Institute of Brewing, 116 (4), 339–347.

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

Lutterschmid, G., Vogel, R. F., Niessen, L., (2010). Influence of hop compounds on gushing induced by hydrophobin FcHyd5p from *Fusarium culmorum*. 2nd International Symposium for Young Scientists and Technologists in Brewing, Malting and Distilling, Freising, oral presentation.

Lutterschmid, G., Vogel, R. F., Niessen, L., (2010). The class II hydrophobin FcHyd5p from *Fusarium culmorum* as gushing inducer and impact of hop compounds on its gushing potential. International Commission on Food Mycology (ICFM) Workshop, Freising, oral presentation.

Lutterschmid, G., Stübner, M., Vogel, R. F., Niessen, L., (2009). Expression of hydrophobin FcHyd5p from *Fusarium culmorum* in *Pichia pastoris* and evaluation of its contribution to gushing. European Brewery Convention Congress, Hamburg, poster presentation.

8.5 Curriculum vitae

Personal data

Name	Georg Lutterschmid
Date of birth	1979-04-15
Place of birth	Aichach, Bavaria, Germany
Nationality	German
Religion	Roman Catholic
Marital status	unmarried

Education

10/2004 – 10/2006	Technology and biotechnology of food (Master), TU-München **Master's thesis**: „Identification of new alcohol dehydrogenases from lactobacilli"
11/1999 – 04/2004	Food technology (FH), TU-München, specialization biotechnology **Diploma thesis**: „Influence of different protein pretreatments on the cross linking of casein by transglutaminase"
07/1998 – 04/1999	Military service
09/1989 – 07/1998	Gymnasium bei St. Stephan, Augsburg, Germany

International experience

07/2004 – 10/2004	Teaching and technical assistant at the King Mongkuts Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand (internship)