# Detection and characterisation of *Fusarium* hydrophobins inducing gushing in beer

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**VTT**

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# Abstract

Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously foams out from its container immediately on opening. Gushing has a marked negative effect on the overall image of beer. Numerous factors causing and contributing to gushing have been reported. Two types of gushing can be distinguished based on the origin of gushing-inducing substances. Non-malt related gushing, i.e. secondary gushing, is due to faults in the beer production process or to incorrect treatment of packaged beer. Primary gushing is induced by fungal metabolites, so-called gushing factors, which are present in malt or in other cereal raw materials of beer. Particularly species of the genus *Fusarium* have been linked to primary gushing. Although gushing factors produced by fungi have been studied for decades, none of them have hitherto been fully characterised.

The hypothesis of this dissertation was that small fungal proteins called hydrophobins are one of the gushing factors inducing primary gushing. Hydrophobins are secreted, highly surface active, moderately hydrophobic proteins produced by filamentous fungi. Hydrophobins play key roles in the development and in the interactions of fungi with their environment and other organisms, particularly plants. The aim of this thesis was to isolate and characterise hydrophobins from gushing active fungi, especially from *Fusarium* species, and to demonstrate that these hydrophobins are able to induce gushing in beer. Currently, there is no practical, reliable and commercially available method for the prediction of beer gushing from large numbers of samples. The main goal of the work was to develop a test for detection of gushing potential of barley and malt by analysing the hydrophobin levels in samples. Moreover, the occurrence and fate of hydrophobins at different stages of the beer production chain were studied.

This study revealed numerous effects of *Fusarium* fungi on the quality of barley grown under Finnish field conditions and of the corresponding malt. In particular, *Fusarium* infection increased the gushing potential of malt. The results of the study indicated that the extent of the impacts is species-dependent, *F. graminearum* having more severe detrimental effects on barley and malt quality than *F. culmorum* and particularly *F. poae*.

We demonstrated that hydrophobins isolated from strains of the genera *Fusarium*, *Nigrospora* and *Trichoderma* induced beer gushing when added to bottled beer. Hydrophobin concentrations at the ppm level were sufficient for gushing induction. The gushing-inducing capabilities of the isolated hydrophobins varied probably due to their structural differences.

We generated profile hidden Markov models for the different hydrophobin classes and searched the *F. graminearum* genome database for predicted proteins with these models. The search revealed five putative hydrophobin genes belonging to both the hydrophobin classes I and II. The best matching sequences and the corresponding genes were isolated from *F. graminearum* as well as from the related species *F. culmorum* and *F. poae* by PCR and were characterized by sequencing. One each of the putative *F. graminearum* and *F. poae* hydrophobin genes were expressed in the heterologous host *Trichoderma reesei*. The proteins corresponding to the genes were purified and identified as hydrophobins and named GzHYD5 and FpHYD5, respectively. Concentrations of 0.003 ppm of these hydrophobins were observed to induce vigorous beer gushing.

An enzyme-linked immunosorbent assay (ELISA) was developed for determination of hydrophobin levels in barley and malt. A connection was found between the hydrophobin level and the gushing potential of malt, suggesting that the developed hydrophobin ELISA can be used for prediction of the gushing risk in malt.

*Fusarium* fungi were observed to produce hydrophobins during the growing period of barley in the field as well as during the malting process, especially during the steeping and germination steps. A small portion of hydrophobins originating from *Fusarium*-infected malt was shown to pass through the brewing process, ending up in the final beer where they induced gushing when present in sufficiently high levels. Addition of a selected antagonistic starter culture, the yeast strain *Pichia anomala* VTT C-04565, into the steeping water of barley was shown to suppress hydrophobin production in malting, which in turn decreased the gushing potential of the corresponding malt.

**Oluen ylikuohuntaa aiheuttavien *Fusarium*-hydrofobiinien osoittaminen ja karakterisointi**

# Tiivistelmä

Oluen ylikuohunta on ilmiö, jossa olut spontaanisti ja hallitsemattomasti vaahtoaa ulos pulloa avattaessa. Ylikuohunta jaetaan primääriin ja sekundääriin ylikuohuntaan. Sekundääriä ylikuohuntaa aiheuttavat erilaiset oluenvalmistuksen prosessitekniset ongelmat tai valmiin oluen virheellinen käsittely. Primäärin ylikuohunnan saavat aikaan homeiden tuottamat ylikuohuntatekijät, joita saattaa esiintyä maltaassa tai muissa oluen viljapohjaisissa raaka-aineissa. Yleisimmin primääriä ylikuohuntaa aiheuttavat *Fusarium*-homeet. Homeiden tuottamia ylikuohuntatekijöitä on tutkittu vuosikymmenien ajan, mutta niitä ei ole onnistuttu tarkasti identifioimaan.

Tämän tutkimuksen hypoteesina oli, että pienet pinta-aktiiviset homeproteiinit, hydrofobiinit, ovat oluen primääriä ylikuohuntaa aiheuttavia ylikuohuntatekijöitä. Hydrofobiineilla on tunnetusti useita merkittäviä tehtäviä homeiden kasvussa ja kehityksessä. Tutkimuksen tavoitteena oli eristää ja karakterisoida hydrofobiineja ylikuohuntaa aiheuttavista homeista, etenkin *Fusarium*-suvun lajeista, sekä osoittaa, että nämä hydrofobiinit aiheuttavat oluen ylikuohuntaa. Lisäksi tavoitteena oli kehittää luotettava ja teollisuuden laadunvalvontaan soveltuva menetelmä ylikuohunta-taipumuksen määrittämiseksi ohrasta ja maltaasta. Tutkimuksen tarkoituksena oli hankkia tietoa hydrofobiinien käyttäytymisestä ja kulkeutumisesta "ohrasta olueksi" -tuotantoketjussa.

Peltokokeet osoittivat, että myös Suomen oloissa *Fusarium*-homeet kykenivät lisääntymään haitallisesti, mikä heikensi ohran ja siitä valmistetun maltaan laatua. Etenkin ylikuohuntataipumus lisääntyi. Tutkimuksessa havaittiin eroja siinä, miten haitallisesti eri *Fusarium*-lajit vaikuttivat laatuparametreihin; *F. graminearum* heikensi laatua voimakkaammin kuin *F. culmorum* ja etenkin *F. poae*.

Tutkimus osoitti, että hydrofobiinit saavat aikaan ylikuohuntaa olueen lisättyinä. *Fusarium*-, *Nigrospora*- ja *Trichoderma*-suvun homeista eristetyt hydrofobiinit aiheuttivat oluen ylikuohuntaa jo hyvin pieninä pitoisuuksina (< 1 ppm). Eristettyjen hydrofobiinien ylikuohunta-aktiivisuudet erosivat toisistaan todennäköisesti rakenne-erojen seurauksena.

Käytimme tunnettujen hydrofobiinien aminohapposekvensseistä luomiamme todennäköisyysmalleja (profile HMMs) etsiessämme hydrofobiinien kaltaisia aminohapposekvenssejä *Fusarium graminearum* -homeen julkisesta genomitieto-pankista. Löysimme viisi hydrofobiinikandidaattia, joista valitsimme yhden toden-näköisimmän jatkotutkimuksiin. Eristimme ja karakterisoimme tätä hydrofobiinia vastaavat geenit *F. graminearum* -kannan lisäksi *F. culmorum*- ja *F. poae* -kannoista. Eristetyt *F. graminearum*- ja *F. poae* -hydrofobiinigeenit siirrettiin *Trichoderma*

*reesei* -tuottokantaan. Tuoteut ja puhdistetut proteiinit tunnistettiin hydrofobiineiksi ja nimettiin GzHYD5 (*F. graminearum*) ja FpHYD5 (*F. poae*). Molemmat hydrofobiinit aiheuttivat oluen ylikuohuntaa pitoisuutena 0,003 ppm.

Hydrofobiinivasta-aineisiin perustuva immunologinen ELISA-testi kehitettiin hydrofobiinitasojen määrittämiseen ohra- ja mallasnäytteistä. Testin tulosten todettiin indikoivan maltaan ylikuohuntataipumusta. Testin avulla tutkittiin hydrofobiinitasoja "ohrasta olueksi" -tuotantoketjussa. *Fusarium*-homeiden todettiin tuottavan hydrofobiineja ohraan kasvukauden aikana pellolla sekä mallastuksessa, etenkin liotuksen ja idätyksen aikana. Pieni osa maltaan sisältämistä hydrofobiineista kulkeutui oluenvalmistusprosessin läpi päätyen valmiiseen olueen. Lisäämällä mallastuksen luontaiseen mikrobiyhteisöön kuuluvaa *Pichia anomala* VTT C-04565 -hiivaa ohran liotusveteen saatiin vähennettyä hydrofobiinien muodostumista mallastuksen aikana ja samalla valmiin maltaan ylikuohuntapotentiaalia.

# Preface

This thesis is based on studies carried out at the VTT Technical Research Centre of Finland during the years 1998–2010. The research was part of the research programs conducted by the Finnish Malting and Brewing Research Laboratory (Oy Panimolaboratorio Ab) and was funded by the Finnish Malting and Brewing Industry and Tekes – the Finnish Funding Agency for Technology and Innovation. Financial support was also obtained from VTT, from the Raisio Group Research Foundation and from the Professor T.-M. Enari Foundation. This financial support is greatly appreciated.

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# Academic dissertation

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# List of orginal publications

This thesis is based on the following original publications which are referred to in the text as I–V (Appendix A). The publications are reproduced with kind permission from the publishers. In addition, some unpublished data is presented.

I    Sarlin, T., Laitila, A., Pekkarinen, A. and Haikara, A. 2005. Effects of three *Fusarium* species on the quality of barley and malt. J. Am. Soc. Brew. Chem. 63, pp. 43–49.

II    Sarlin, T., Nakari-Setälä, T., Linder, M., Penttilä, M. and Haikara, A. 2005. Fungal hydrophobins as predictors of the gushing activity of malt. J. Inst. Brew. 111(2), pp. 105–111.

III    Sarlin, T., Kivioja, T., Kalkkinen, N., Linder, M. and Nakari-Setälä, T. 2012. Identification and characterization of gushing-active hydrophobins from *Fusarium graminearum* and related species. J. Basic Microbiol. 52, pp. 184–194.

IV    Sarlin, T., Vilpola, A., Kotaviita, E., Olkku, J. and Haikara, A. 2007. Fungal hydrophobins in the barley-to-beer chain. J. Inst. Brew. 113(2), pp. 147–153.

V    Laitila, A., Sarlin, T., Kotaviita, E, Huttunen, T., Home, S. and Wilhelmson, A. 2007. Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors. J. Ind. Microbiol. Biotechnol. 34, pp. 701–713.

# Author's contributions

I   Tuija Sarlin had the main responsibility for preparing and writing the article and is the corresponding author. She participated in planning of the study and was mainly responsible for the experimental work and interpretation of the results, except that the evaluation of barley and malt quality was performed together with Arja Laitila and Auli Haikara.

II  Tuija Sarlin had the main responsibility for preparing and writing the article and is the corresponding author. She was mainly responsible for planning of the study, design of the experiments, the experimental work and interpretation of the results, except that the isolation, purification and characterisation of hydrophobins were performed together with Tiina Nakari-Setälä and Markus Linder.

III Tuija Sarlin had the main responsibility for preparing and writing the article and is the corresponding author. She planned the study together with the co-authors. She was responsible for the experimental work and interpretation of the results, except that the generation and utilisation of the profile HMMs were carried out by Teemu Kivioja, the structural characterisation of the hydrophobins was mainly performed by Nisse Kalkkinen and the purification of hydrophobins was performed together with Markus Linder. Tiina Nakari-Setälä supervised the cloning and expression work of the hydrophobin genes.

IV  Tuija Sarlin had the main responsibility for preparing and writing the article and is the corresponding author. She planned the study, was responsible for the experimental work and interpreted the results except that the malting and brewing trials were carried out together with Arvi Vilpola.

V   Tuija Sarlin was responsible for the determination of hydrophobins and gushing potential of malt. She interpreted the results together with Arja Laitila.

# Contents

# List of symbols

| | |
|---|---|
| AfpA | Alkaline foam protein A |
| ANOVA | Analysis of variance |
| AP | Alkaline phosphatase |
| ATU | Atmospheric pressure |
| BLAST | Basic Local Alignment Search Tool |
| CZID | Czapek-Dox Iprodione Dichloran |
| Da | Dalton, a unit for molecular mass |
| DNA | Deoxyribonucleic acid |
| DON | Deoxynivalenol |
| EBC | European Brewery Convention |
| ELISA | Enzyme-linked immunosorbent assay |
| EMBL | European Molecular Biology Laboratory |
| FAN | Free amino nitrogen |
| FHB | *Fusarium* Head Blight |
| GC-MS | Gas chromatography- mass spectrometry |
| GF | Gushing factor |
| His | Histidine |
| HSD | Honestly significant difference |
| kDa | Kilodalton |
| NCBI | National Centre for Biotechnology Information |
| mRNA | Messenger ribonucleic acid |
| ns-LTP1 | Non-specific lipid transfer protein 1 |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reation |
| ppb | Part per billion |
| ppm | Part per million |
| profile HMMs | Profile hidden Markov models |
| qPCR | Real-time quantitative PCR |
| RP-HPLC | Reversed phase high performance liquid chromatography |
| rpm | Round per minute |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| STDEV | Standard deviation |

# 1. Introduction

Beer is one of the oldest as well as one of the most widespread beverages in the world. The main raw materials of beer are malted cereal, most often barley, and water. Hops are used as aroma components. Yeast is needed for the fermentation process to convert sugars derived from the malt into ethanol and flavour compounds. Yeast is not able to utilise starch and other grain macromolecules as such. During the malting process, various enzymes are produced in barley which enable degradation of the grain macromolecules into more soluble, fermentable compounds.

The malting process consists of three steps: steeping, germination and kilning. During steeping, the moisture content of barley is increased up to 46% by alternating immersion periods in water and air rest periods. After steeping, the barley is allowed to germinate for 3–6 days. Germination is terminated and the barley is dried by kilning in hot air.

The degradation reactions of macromolecules continue during the first step of brewing, wort production, during which the milled malt is mixed with water, mashed, filtered and boiled with hops. Clarified, cooled and oxygenated wort is then inoculated with brewer's yeast and the main fermentation starts. After the main fermentation, the so-called green beer still requires maturation (secondary fermentation) before the typical aroma, flavour and carbonation level of beer are achieved. Figure 1 shows a simplified scheme of the malting and brewing processes. For a more detailed description of these processes see Bamforth and Barclay 1993, Lewis and Young 1995, and Briggs *et al.* 2004. The quality of beer is greatly dependent on the quality of raw materials. In addition, optimised and quality controlled malting and brewing processes are essential for the production of good quality beer.
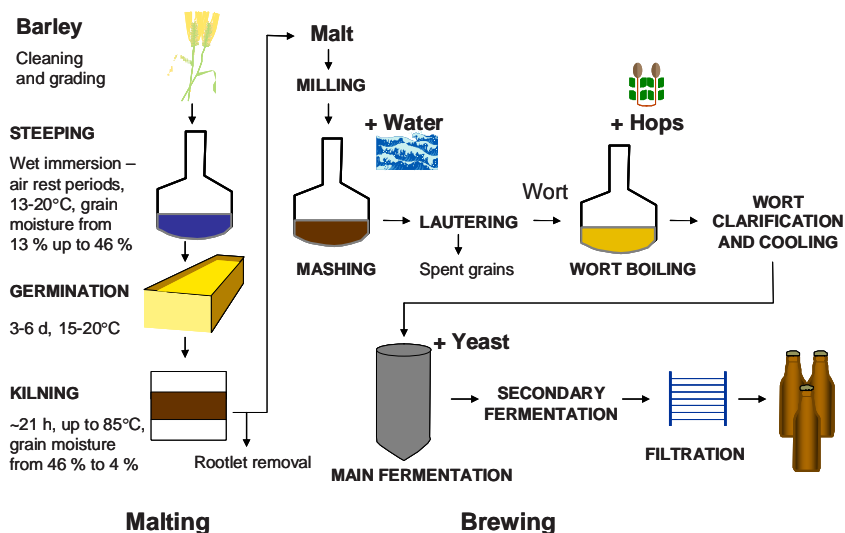
**Figure 1.** Outline of the malting and brewing processes.

## 1.1 Gushing of beer

One of the beer quality faults associated with the quality of raw materials and process conditions is gushing of beer. Gushing is a phenomenon in which beer spontaneously overfoams out from its container immediately on opening (Gjertsen 1967). The gushing phenomenon has been known for a very long time, and research articles on gushing have been published since the beginning of the 20th century. However, the mechanism of gushing is still not fully understood.

Gushing affects the image of beer negatively. The loss of a beer brand's image in cases of gushing may have significant economic impacts. Based on a German survey, over 50% of breweries have experienced gushing at least once (Niessen *et al.* 2007). An increasing percentage of European malt samples analysed during the beginning of the 21st century showed gushing tendency (Aastrup 2003). The summer of 2007 was exceptionally rainy in Central Europe, resulting in a higher number of malt samples with gushing propensity (Rath 2009). In the USA, severe epidemics of *Fusarium* head blight, a fungal disease, occurred on barley in the 1990s, lowering the quality of the crop and increasing the risk of beer gushing and mycotoxin formation (Schwarz *et al.* 1996, McMullen *et al.* 1997, Steffenson 1998).

Gushing is a complex phenomenon. Numerous factors causing and contributing to gushing have been reported (Brenner 1957a and 1957b, Thorne 1964, Gardner 1973). Two types of gushing can be distinguished based on the origin of gushing-inducing substances (Amaha and Kitabatake 1981, Casey 1996). Primary gushing is due to abnormalities in the raw materials of beer and is induced by fungal metabolites which are present in malt or in other cereal raw materials of beer. Non-malt related gushing, i.e. secondary gushing, is due to faults in the beer production process or to incorrect

treatments of packaged beer. The occurrence of secondary gushing is sporadic and limited to a certain brewery or even to a certain batch of beer, whereas primary gushing is more epidemic and often affects several breweries at the same time over an extended period (Thorne 1964, Amaha and Kitabatake 1981).

A unifying feature of the gushing phenomenon is the formation of a large number of tiny gas bubbles throughout the beer at the moment of bottle opening. The sudden expansion of these bubbles causes uncontrolled release of carbon dioxide, converting the beer into a stream of foam (Thorne 1964). According to Krause's theory, spontaneous bubble formation is unlikely in beer with a normal carbon dioxide content at moderate temperatures and the presence of pre-formed nuclei, seeds, is needed to induce gushing (Thorne 1964, Gardner 1973). The crucial question is why these nuclei are formed only in gushing beer and not in normal beer. Gardner (1973) classified three possible types of nuclei which might facilitate gas release from solutions:

- Type I: solid hydrophobic particles
- Type II: gas residues sorbed on a solid "support"
- Type III: stabilized microbubbles.

Type I nuclei require *de novo* formation of a bubble, whereas Types II and III originate from embryonic bubbles (Gardner 1973). Although Type I hydrophobic materials have been speculated to lower the energy required for bubble formation, in practice the *de novo* gas nuclei formation is unlikely in beer as already stated above (Gardner 1973, Pellaud 2002). This means that the presence of gas nuclei of Type II or Type III in beer is a prerequisite for bubble formation and thus for beer gushing.

It has been widely agreed that stable microbubbles are major contributors to the gushing phenomenon (Pellaud 2002). According to the Laplace equation (1) these microbubbles must have a certain critical radius which determines their size in order to be stable in liquid (Draeger 1996). If the radius of the bubble is smaller than the critical radius, the bubble will collapse due to the higher external pressure caused by surface tension. If the radius exceeds the critical radius, the internal pressure of the bubble will be higher and the bubble will expand until it is lifted to the surface by buoyancy.

$$p_B = p_F + p_L = p_F + 2\sigma/r_c \tag{1}$$

where   $p_B$ = internal pressure of the bubble
$p_F$ = pressure of the surrounding liquid
$p_L$ = the Laplace pressure resulting from surface tension
$\sigma$ = surface tension of the liquid
$r_c$ = critical radius of the bubble.

Upon beer bottle opening the external pressure drops and the microbubbles will expand, causing a decrease of microbubble internal pressure (Pellaud 2002). Those bubbles reaching a new internal pressure low enough to enable dissolved carbon dioxide to diffuse inside the microbubble will grow vigorously. Other bubbles will either shrink because of gas diffusion outside the bubble or remain stable (Pellaud 2002). Pellaud (2002) stated that in a normal beer most gas nuclei will

remain inactive upon bottle opening. The difference between normal and gushing beer resides in the number of active nuclei rather than in the total number of nuclei.

Pellaud (2002) reclassified gushing factors (GFs) according to their effect on microbubbles in packaged beer as follows:

- Primary GFs: factors increasing the stability of microbubbles (e.g. surface active compounds)
- Secondary GFs: factors increasing the initial amount of stable microbubbles (e.g. solid particles with entrapped gas pockets)
- Tertiary GFs: external factors influencing the effect (potency) of stable microbubbles at the time of beer opening (e.g. temperature, carbonation level of beer).

In this dissertation the traditional classification between primary and secondary gushing is mainly used.

### 1.1.1 Secondary gushing

Factors commonly associated with secondary, non-malt related gushing are listed in Table 1.

**Table 1.** Factors contributing to secondary gushing of beer.

| Factor | Reference |
|---|---|
| Calcium oxalate crystals | Brenner 1957a and 1957b, Zepf and Geiger 2000a |
| Haze | Casey 1996 |
| Metal ions (iron and heavy metals) | Brenner 1957a, Rudin and Hudson 1958, Thorne 1964, Gardner 1972, Kieninger 1976 |
| Iso-$\alpha$-dimers and isomerized hop extracts | Rudin and Hudson 1958, Gardner 1972, Kieninger 1976, Amaha and Kitabatake 1981 |
| Polyphenols originating frorm hop pellets | Müller *et al.* 2010 |
| Filter aid particles such as Kieselguhr | Casey 1996, Pellaud 2002 |
| Foreign small particles e.g. impurities from bottles | Brenner 1957a and 1957b, Thorne 1964, Kieninger 1976 |
| Cleaning agent residues | Dachs and Nitschke 1977 |
| Surfactants forming micelles | Christian *et al.* 2009a |
| High air headspace in bottle | Amaha and Kitabatake 1981 |
| Rough bottle internal surface | Brenner 1957b |
| High carbonation level | Amaha and Kitabatake 1981, Ilberg *et al.* 2009 |
| High level of oxygen and nitrogen | Brenner 1957a and 1957b |
| Turbulent bottle filling, cavitation | Pellaud 2002 |
| Temperature and time of beer storage | Brenner 1957a, Thorne 1964, Amaha and Kitabatake 1981 |
| Temperature at opening of packaged beer | Amaha and Kitabatake 1981 |
| Bottle position and mechanical stress during storage and transportation | Brenner 1957a, Amaha and Kitabatake 1981 |

A common cause of secondary gushing is the formation of calcium oxalate precipitations in bottled beer (Brenner 1957a, Zepf and Geiger 2000a). Wort contains oxalic acid originating from malt and to a lesser extent from hops. Calcium oxalate forms when oxalic acid reacts with calcium ions entering the brewing process e.g. with hard brewing water or with filter aids such as Kieselguhr. Insoluble calcium oxalate precipitates in many crystalline and amorphous forms. These precipitations may entrap gas bubbles to form gas nuclei inducing beer gushing (Brenner 1957b). Like calcium oxalate crystals, haze, cleaning agent residues, filter aid particles and small foreign particles act as gushing inducers (Brenner 1957a and 1957b, Pellaud 2002). Small bubbles can also be trapped in the cracks of rough internal bottle surfaces (Brenner 1957b). Metal ions can react with beer components such as isohumulone originating from hops to form chelates or other insoluble complexes which provoke gushing (Brenner 1957a, Rudin and Hudson 1958, Gardner 1972). Christian *et al.* (2009a) observed that addition of a pure aliphatic surfactant, hexadecyltrimethylammonium chloride (CTAC) above the critical micelle concentration in beer induced gushing even without shaking. They concluded that the formed micelles begin to grow upon diffusion of carbon dioxide into the hydrophobic inner part of micelles, forming stabilized microbubbles which then induce gushing.

Müller *et al.* (2010) studied impacts of different hop compounds on gushing. They brewed beers from gushing-positive malt using different types of hop pellets. They reported that the higher the amounts of polyphenols orginated from hop pellets in beers, the higher were the overfoaming volumes.

High air headspace in the bottle neck of packaged beer increases the possibilities for gas nuclei formation (Brenner 1957b, Amaha and Kitabatake 1981). Oxygen and nitrogen have lower diffusion rates than carbon dioxide, which may explain the persistence of microbubbles formed from these two gases in beer (Brenner 1957b). In addition, high oxygen level in headspace may induce oxidation reactions of some beer components, resulting in compounds provoking gushing (Thorne 1964, Amaha and Kitabatake 1981). In beer with a gushing tendency, increasing carbon dioxide content increases the gushing volumes observed and vice versa (Amaha and Kitabatake 1981, Ilberg *et al.* 2009). This can be explained by Fick's diffusion law which states that the higher the gas saturation in liquid, the higher the mass transfer into rising bubbles. Turbulent bottle filling introduces more air and microbubbles into beer (Pellaud 2002).

Low temperatures and prolonged storage of beer increase the formation of precipitations such as calcium oxalate crystals which provoke gushing (Thorne 1964). It has been demonstrated that the higher the temperature at opening of beer bottles after agitation, the more intense the gushing (Amaha and Kitabatake 1981). This can be explained by increased transformation of dissolved carbon dioxide into gaseous form and by the increased diffusion rate of carbon dioxide into rising bubbles at higher temperatures. By contrast, Ilberg *et al.* (2009) were able to suppress gushing by increasing the opening temperature of gushing beer from 15 °C to 28 °C.

Mechanical disturbance of beer, i.e. agitation, produces or activates gas nuclei in the beer. The effect of mechanical disturbance during transportation is more pronounced in beer bottles lying horizontally than in bottles standing vertically.

It can be concluded that the factors inducing secondary gushing increase the amount of microbubbles in beer or influence the violence of beer gushing. Thus, they can be classified as secondary or tertiary gushing factors based on the classification of Pellaud (2002). Of all the secondary and tertiary factors only the introduction of an unreasonably high amount of microbubbles (for example via Kieselguhr particles or poor filling conditions) can trigger beer gushing in the absence of primary gushing factors (Pellaud 2002). This kind of secondary gushing will typically concern only certain individual beer bottles rather than the whole beer batch, which is often observed with primary gushing factors.

### 1.1.2 Primary gushing

Primary, malt-related gushing is commonly reported to be caused by *Fusarium* fungi (Gjertsen *et al.* 1965, Haikara 1980, Schwarz *et al.* 1996, Munar and Sebree 1997). Other genera such as *Alternaria*, *Aspergillus*, *Nigrospora*, *Penicillium* and *Stemphylium* have also been reported to induce gushing (Prentice and Sloey 1960, Gjertsen *et al.* 1965, Amaha *et al.* 1973, Gyllang and Martinson 1976, Haikara 1980). *Fusarium graminearum* (teleomorph *Gibberella zeae*) is the most common *Fusarium* head blight (FHB) pathogen of cereals worldwide, as well as a well-documented gushing inducer (Sloey and Prentice 1962, Gjertsen *et al.* 1965, Niessen *et al.* 1992, Noots et al. 1998, Salas *et al.* 1999). *Fusarium culmorum*, another common FHB pathogen, is the most harmful species in temperate zones such as Northern Europe and has also been reported to induce gushing (Haikara 1983, Niessen *et al.* 1992, Schildbach 1995). Several other *Fusarium* species such as *F. avenaceum*, *F. moniliforme*, *F. poae* and *F. sporotrichioides* have been reported to induce gushing (Sloey and Prentice 1962, Haikara 1983, Vaag *et al.* 1993, Laible and Geiger 2003, Wang *et al.* 2010). However, there are differences between the *Fusarium* species in terms of how severely they affect the gushing potential of malt. Haikara (1983) observed that *F. culmorum* isolates induced more vigorous gushing than *F. avenaceum* isolates.

Exceptionally wet and rainy weather during the growing season, especially during flowering, early kernel-fill and harvest periods of barley, promotes heavy fungal infection of the crop (Gjertsen *et al.* 1965, Schildbach 1995, McMullen *et al.* 1997). In addition, improper storage of harvested barley may favour fungal growth (Noots *et al.* 1998). Moreover, high moisture content of barley and moderate temperatures enable fungal growth during malting. It is known that gushing factors produced by fungi can be present in barley or they can be formed during malting (Munar and Sebree 1997, Aastrup 2003).

Fungal gushing factors are assumed to be surface active molecules which stabilize gas nuclei in beer by forming a layer, skin, around the microbubbles (Gardner 1973, Draeger 1996, Pellaud 2002). This layer may prevent breakdown of the microbubbles in stored beer by hindering the gas diffusion outside the bubbles but may enable

dissolved carbon dioxide to diffuse inside the bubbles upon bottle opening, leading to expansion of the microbubbles and overfoaming of beer. The layer also provides a high mechanical resistance towards external stresses. In fact, Yount et al. (1984) detected a membrane of surface active material around stable microbubbles in aqueous media in their microscopic studies. In addition to stabilisation, the surface active molecules may decrease the surface tension of beer, lowering the pressure difference between the inside and outside of microbubbles according to the Laplace equation (1) and preventing the microbubbles from collapsing (Pellaud 2002). However, the effect of a surface tension drop observed with classical surfactants is so small that the first assumption of a protective layer is more likely.

Fungal gushing factors have been studied for decades, especially during the 1970s. As shown in Table 2, they have been reported to be polypeptides or peptide-containing substances. Very small amounts of these substances, quantities in the ppm range or lower, have been reported to induce gushing of beer (Amaha *et al.* 1973, Kitabatake and Amaha 1977, Kitabatake *et al.* 1980, Kitabatake 1978). The gushing activity of these gushing factors has been demonstrated to be retained at acid and neutral pH values after heating for two hours at 100 °C (Amaha *et al.* 1973), suggesting that these factors would survive the wort boiling step in brewing. Aastrup *et al.* (1996) reported that addition of proteolytic enzymes to gushing-inducing malt extract significantly reduced gushing tendency, suggesting that the gushing-inducing factors present in malt were proteins or polypeptides.

Research on gushing factors activated again in the beginning of 21st century. Recently, two types of proteins, non-specific lipid transfer protein 1 (ns-LTP1) and fungispumins, have been associated with the gushing phenomenon (Hecht and Hippeli 2007, Zapf *et al.* 2007). Plant based ns-LTP1, a prominent protein in barley grain, malt and beer, is well known for its role in foam stability and its synthesis by plants has been demonstrated to increase in response to fungal invasion (Stanislava 2007). Hecht and Hippeli (2007) reported that not ns-LTP1 itself, but rather glycated peptides generated during proteolytic fragmentation of modified ns-LTP1 species by heat-stable *Fusarium* proteases may initiate beer gushing. They concluded that the heat-stable *Fusarium* proteases maintain their activity during mashing and wort boiling, whereas a protease inhibitor naturally present in grains is inactivated by heat, resulting in the breakdown of ns-LTP1 and presumably also other proteins present in beer. Zapf *et al.* (2007) demonstrated that a fungispumin, the alkaline foam protein A (AfpA) produced by *Fusarium*, enhances but does not induce beer gushing, indicating that fungispumins may contribute to the gushing phenomenon but that they are not the primary cause of beer gushing. Lutterschmid *et al.* (2011) also studied the gushing-inducing potential of ns-LTP1 and AfpA. No gushing was observed after addition of these proteins to beer in milligram amounts. On the contrary, these proteins showed a gushing reducing potential. Lutterschmid *et al.* (2011) speculated that low levels of ns-LTP1 in gushing beers observed by Hecht and Hippeli (2007) may contribute to gushing by changing the relative concentrations of the gushing-inducers and -inhibitors in beer towards gushing-inducers. At the turn of the millennium the idea of hydrophobins as fungal gushing factors was also introduced (Haikara *et al.* 2000).

**Table 2.** Properties of the gushing factors produced by different fungi.

| Isolated from | Chemical structure | Molecular weight, kDa | Concentration needed for gushing, ppm | Remarks | Reference |
|---|---|---|---|---|---|
| *Nigrospora* sp. | Polypeptide | 16.5 | 0.05 | Hydrophobic, contains 16 cysteine residues per mole of protein and 8 disulphide bonds which were essential for gushing activity | Amaha *et al.* 1973, Kitabatake and Amaha 1977 |
| *Stemphylium* sp. | Peptidoglycan | nd[1] | 4 | Composed of 85 % glucosamine and 10 % peptide | Amaha *et al.* 1973 |
| *Fusarium graminearum* | Hexapeptide | nd | 0.4 | | Amaha *et al.* 1973, personal communication |
| *Penicillium crysogenum* | Cyclic tetrapeptide | nd | 0.3 | | Kitabatake *et al.* 1980 |
| Northern European malt | Peptide-like | 10 | 0.5 | Acidic, contains carbohydrates and polyphenols | Kitabatake 1978 |

[1] Not determined.

## 1.2 Hydrophobins as possible gushing factors of beer

Hydrophobins are secreted, highly surface active, moderately hydrophobic proteins produced by filamentous and dimorphic (i.e. capable of both filamentous and yeast-like growth) fungi of the Ascomycota and the Basidiomycota (Wessels 1994 and 1996, Linder *et al.* 2005). A characteristic feature of these proteins is their eight conserved cysteine residues forming four disulphide bridges in the molecule made up of approximately 100 amino acids. The consensus cysteine spacing of fungal hydrophobins according to Wessels (1996) is presented in Figure 2. Based on sequence comparison, hydrophobins are divided into two different classes, I and II (Wessels 1996). Class II hydrophobins have been observed thus far only in ascomycetes, whereas class I hydrophobins have been observed in both ascomycetes and basidiomycetes (Linder *et al.* 2005). The class I hydrophobins can further be divided into two sub-groups Ia and Ib, which represent the class I hydrophobins of ascomycetes and basidiomycetes, respectively. The overall nucleotide sequence homology of hydrophobins between the two classes and between species is usually poor (Wessels 1994 and 1997, Linder *et al.* 2005). However, the similarity of amino acid sequences is more pronounced although still rather low.

Currently, there are 1327 entries for hydrophobins in the protein database of National Centre for Biotechnology Information (NCBI)[1]. When the hypothetical and partial sequences are excluded, the number of entries reduces to 571. Hydrophobins have been found in 84 species of the Ascomycota and in 23 species of the Basidiomycota. In many cases, more than just one hydrophobin are found in one species. Only a few studies have been reported on *Fusarium* hydrophobins. Fuchs *et al.* (2004) identified five hydrophobin genes encoding both class I and II hydrophobins in *F. verticillioides* (teleom. *Gibberella moniliformis*). In addition, two hydrophobin genes of *F. culmorum* have been identified (Zapf *et al.* 2006). Moreover, two hypothetical proteins of *F. oxysporum* found from the NCBI protein database have sequence similarities with the known hydrophobins.

$$X_{2-38} - C - X_{5-9} - C - C - X_{11-39} - C - X_{8-23} - C - X_{5-9} - C - C - X_{6-18} - C - X_{2-13}$$

where   C = cysteine,
         X = any other amino acid

**Figure 2.** Consensus cysteine spacing of hydrophobins (Wessels 1996).

Hydrophobins self-assemble at their hydrophilic-hydrophobic interfaces such as between water and air to form amphipathic membranes of which one side is hydrophilic and the other side is hydrophobic (Wösten *et al.* 1993). This property allows hydrophobins to fulfil a broad spectrum of functions in fungal growth and

[1] Search strategy: hydrophobin, hydrophobin not hypothetical or partial

development. Hydrophobins are present in fungal cell walls, where they are involved in the formation of mycelium and spores (Wessels 1996). Fungi secrete hydrophobins into their surroundings, where the proteins can decrease the surface tension of water or change the nature of a surface from hydrophilic to hydrophobic or vice versa (Wösten and Wessels 1997). These properties are useful when the fungus penetrates the air-water interface or attaches to hydrophobic host surfaces such as cuticular waxes of plant tissues. Hydrophobins have been shown to play roles in pathogenic and mutualistic interactions (Templeton *et al.* 1994, Wessels 1996, Wösten and Wessels 1997).

Both class I and class II hydrophobins form aggregates (Wessels 1994). Aggregates of the class I hydrophobins are highly insoluble and can be dissociated with reagents such as formic acid and trifluoroacetic acid (Wessels 1994). Class II hydrophobin aggregates dissociate more readily and are soluble in e.g. 60% ethanol and 2% sodium dodecyl sulphate (SDS) (Wessels 1996). Hydrophobins have been observed to self-assemble around air bubbles and oil droplets in water, which enables the stabilisation of these structures (Wessels 1996). The hydrophobin membranes formed at hydrophilic-hydrophobic interfaces have exceptionally high surface elasticity (Cox *et al.* 2007). This property is associated with the tendency of hydrophobins to form very stable foams.

Gushing factors produced by fungi have been studied for decades, but none of them have been fully characterised (see Table 2). The known properties of fungal gushing factors and hydrophobins are compared in Table 3. Interestingly, the properties of hydrophobins are in good congruence with those of fungal gushing factors. With respect to gushing theory, the most important properties of the primary gushing factors are their surface activity and their ability to form a stabilizing layer around the gas nuclei in beer. As indicated above, hydrophobins have been described as the most surface active proteins known. They are able to self-assemble into stable, elastic protein films at hydrophilic-hydrophobic interfaces in aqueous solutions. In fact, the elasticity of hydrophobin membranes is orders of magnitude higher than observed for any other surface active proteins (Cox *et al.* 2007). In addition, the amounts of hydrophobins produced by wild-type fungi in culture medium (e.g. up to 60 ppm of the hydrophobin SC3 by *Schizophyllum commune*) are in agreement with the amount of gushing factors required to induce gushing (Pellaud 2002).

Deckers *et al.* (2010) suggested that hydrophobin molecules self-assemble at the surface of $CO_2$ bubbles in beer. This hydrophobin layer may prevent the dissolution of $CO_2$ and, thus, stabilize the $CO_2$ bubbles. Drop in pressure at opening of the container causes these nanobubbles to expand and finally explode. This explosion releases energy required for the simultaneous nucleation of many new bubbles, which leads to gushing.

The capability of hydrophobins to induce beer gushing needs to be investigated in practice. Gushing factors must survive throughout the brewing process and end up in the final beer. There is no information about the occurrence and fate of hydrophobins at different stages of the beer production chain. However, many of the reported characteristics of hydrophobins appear to match with the properties of fungal gushing factors (Table 3).

**Table 3.** Comparison of the properties of fungal gushing factors and hydrophobins.

| Fungal gushing factors | Hydrophobins |
|---|---|
| Produced by fungi | Produced by fungi |
| Small proteins or compounds containing peptides | Small proteins |
| Secreted | Present in fungal cell walls or secreted into the surroundings |
| Some are hydrophobic | Moderately hydrophobic |
| Some contain a considerably high amount of cysteine residues | Characteristic pattern of 8 cysteine residues |
| Assumed to be surface active compounds | Highly surface active proteins |
| Quantities in ppm range or lower induce beer gushing | Quantities in ppm range produced by fungi |
| Gushing activity survives at acid and neutral pH and at high temperatures | Form stable, insoluble aggregates |
| Assumed to stabilize gas nuclei in beer by forming a protein layer around microbubbles | Self-assemble into amphipathic layers between phases e.g. on gas-liquid interphases causing the stabilization of bubbles |
| The stabilizing layer must resist external stresses | Form stable, highly elastic membranes |

## 1.3   Estimation of the gushing risk from barley and malt

### 1.3.1   Measurement of *Fusarium* fungi

Currently the gushing risk of barley and malt is predicted by quantifying the presence of *Fusarium* fungi by conventional culturing methods (EBC Analytica-Microbiologica II, 2005). In addition, novel molecular biological methods such as real time PCR can be applied for quantification of the *Fusarium*-group or different *Fusarium* species (Niessen *et al.* 1999, Waalwijk *et al.* 2004, Sarlin *et al.* 2006, Vogeser and Dahmen 2007, Rath 2009). Immunological methods for quantification of *Fusarium* antigens have been developed for estimating the level of *Fusarium* contamination (Vaag 1991, Manke and Rath 1997). Schwarz *et al.* (1996) observed a significant correlation between the intensity of gushing and the levels of deoxynivalenol (DON, a mycotoxin) and ergosterol (a fungal cell wall component) in barley and corresponding malt grown in North America. Although DON and ergosterol themselves do not induce gushing, the authors suggested that these metabolites could be used for prediction of the gushing risk.

The principle weakness inherent to the methods presented above lies in the fact that they do not directly detect the actual gushing-inducing factors. The risk of producing gushing active malt from barley depends not only on the extent and composition of the *Fusarium* community in the barley but also on the activity and viability of gushing active *Fusarium* spp. present in the grain as well as in the malting process (Munar and Sebree 1997). Thus, examination of the gushing potential by quantifying total *Fusarium* contamination in barley cannot be regarded as sufficiently reliable. In fact, some studies have shown that the actual *Fusarium*

level of barley and malt is a poor predictor of gushing propensity (Schwarz *et al.* 1996, Munar and Sebree 1997). This could be explained by the fact that the viability of fungi declines during storage, although the gushing factors produced are still present and active. In addition, the gushing-inducing capabilities of *Fusarium* species differ (Haikara 1983). Not all fungal species associated with beer gushing are able to produce DON, which compromises the use of DON content as an indication of gushing risk (Casey 1996). Rath (2009) compared the gushing potential and the DON concentration of European malt samples harvested in 2007 and did not observe a correlation between these two parameters. The correlation observed by Schwarz *et al.* (1996) is probably due to the fact that *F. graminearum*, a strong DON producer and gushing inducer, is the most prevalent *Fusarium* species of barley in North America (McMullen *et al.* 1997, Steffenson 1998). A more complex *Fusarium* community is associated with FHB in small-grain cereals in Europe (Bottalico and Perrone 2002). European FHB complex consists mainly of *F. graminearum, F. culmorum, F. avenaceum* and *F. poae.*

### 1.3.2 Measurement of the gushing propensity of raw materials

Many different procedures have been developed for determining the gushing propensity of barley, malt, wort and beer (Table 4) (Haikara 1980, Amaha and Kitabatake 1981, Schildbach 1988, Donhauser *et al.* 1991, Casey 1996, Vaag *et al.* 1993, Anger 2006). Agitation is a critical component of every assay. As can be seen in Table 4, there are differences e.g. in the direction, frequency and duration of agitation between the assays. The most commonly used methods are the Weihenstephan gushing test and the Carlsberg gushing test with or without modifications. In the Weihenstephan test, the malt samples are mashed to produce wort, followed by wort carbonation (2.8 bar overpressure at 4 °C) and bottling. The bottles are left to stand for 2 days before turning them four times around (360° vertically with a frequency of 0.66 Hz) and opening. In the Carlsberg gushing test described by Vaag *et al.* (1993) and modified for barley by Aastrup (2003), an aqueous extract of barley or malt is prepared in a laboratory blender and added to commercial beer. After pasteurization, the beer bottles are shaken horizontally with a movement parallel to the longitudinal direction of the bottles (16 strokes per min) for three days. After shaking, the bottles are kept still for 10 minutes, inverted three times and opened after 30 seconds (Figure 3). The amount of gushing is usually determined from the change in weight of the bottle (g) or the volume of overfomed beer is recorded (ml). Both tests described above are labour-intensive and time-consuming, and are impractical for screening large numbers of samples. In addition, the result of the Carlsberg test is greatly influenced by the beer to which the malt extract is added. To overcome this problem, the Carlsberg gushing test has been modified by replacing beer with carbonated mineral water (Anger 2006, Rath 2009). However, there are still reproducibility problems with the modified test, indicating a need for further optimisation of the test procedure (Rath 2009).

**Table 4.** Different procedures developed for determining the gushing propensity of barley, malt, wort and beer.

| Test name | Sample preparation | Agitation | Operations before opening | Duration of the test | Reference |
|---|---|---|---|---|---|
| Gushing test using a microbrewing procedure | Mashing of malt sample to produce wort followed by wort boiling with hops, fermentation, stabilization, carbonation and bottling. | Bottles are rocked for 2 days at 150 rpm in a horizontal rotating shaker. | Bottles are allowed to stand for 10 min, inverted three times and opened after 30 s. | About 10 days | Haikara 1980 |
| VLB's gushing test for malt | Congress wort is prepared from malt, fermented for 3 days, carbonated, bottled and pasteurized. | After storage for 2-4 weeks, bottles are rocked for 24 h by rotating 2 x 180° at 30 s intervals. | Bottles are allowed to stand for 5 min. | 3-5 weeks | Schildbach 1988, Casey 1996 |
| Weihenstephan test for malt, wort and beer | Mashing of malt sample to produce wort followed by wort clarification and carbonation and bottling after 48 h at 4°C. | After storage for 48 h at 20°C, bottles are turned four times through 360° in the vertical plane. | Bottles are allowed to stand for 5 min. | About 6 days | Donhauser *et al.* 1991, Anger 2006 |
| Carlsberg gushing test for malt and barley | Grain sample is mixed with water in a blender and centrifuged. Clear supernatant is boiled and filtered. In the case of barley the supernatant is treated with Termamyl. 50 ml of commercial bottled beer is replaced with the filtrate and pasteurized. | Bottles are rocked for 3 days horizontally with movement parallel to the longitudinal direction of the bottles (16 strokes per min). A horizontal rotating shaker at 50 rpm is also used in some laboratories. | Bottles are allowed to stand for 10 min, inverted three times in 10 s and opened after 30 s. | About 4 days | Vaag *et al* 1993, Aastrup 2003, personal communications |
| Modified Carlsberg gushing test for malt | As described above but using 50 ml of the malt filtrate is added to carbonated mineral water instead of beer and sodium azide is used instead of pasteurization. | As described above but using a longitudinal movement with a frequency of 75 rpm. | As described above. | About 4 days | Anger 2006, Rath 2009 |

**Figure 3.** Gushing positive sample determined with the Carlsberg gushing test (Linder *et al.* 2005).

In addition to the weaknesses of the gushing tests described above, these tests provide mostly qualitative information about the gushing potential of barley and malt. Christian *et al.* (2009c) presented new ideas about how to quantify more precisely the gushing potential of malt. They suggested that, in the modified Carlsberg gushing test, instead of measuring the amount of overfoaming the smallest amount (concentration) of added wort or malt extract capable of inducing gushing in mineral water should be recorded. They also demonstrated that an addition of hop extract into the wort or malt extract samples can suppress gushing, and that the amount of hop extract needed for suppression can be used for quantifying the gushing potential of malt.

Currently, there is no practical, reliable and commercially available method for the prediction of beer gushing from large numbers of samples. Every year, labour-intensive and costly analyses are used to evaluate the gushing risk of raw materials. There is a need for rapid analyses detecting precisely those factors responsible for gushing in beer.

## 1.4 Prevention strategies for gushing

### 1.4.1 Measures for preventing secondary gushing

Secondary gushing occurs due to faults in the beer production process or due to incorrect treatments of packaged beer, and therefore the strict process control and proper handling of packaged beer help to overcome this problem. Precipitation of

calcium oxalate is known to induce beer gushing, as stated above (Brenner 1957a and b, Amaha and Kitabatake 1981, Zepf and Geiger 2000a, Gastl *et al.* 2009). In order to prevent the formation of calcium oxalate precipitations in finished beer, as high an amount of oxalic acid as possible should be allowed to react with calcium prior to beer filtration and bottling (Brenner 1957b, Zepf and Geiger 2000a). Since low temperatures favour separation of insoluble calcium oxalate, it is suggested that beer is stored at a temperature as close to its freezing point as possible in order to undergo cold maturation before filtration (Brenner 1957b, Zepf and Geiger 2000b). Subsequent introduction of calcium e.g. through filter aids should be avoided (Brenner 1957b). Blending of filtered beer with any liquor, wort or beer may upset the balance between calcium and oxalic acid, leading to the formation of calcium oxalate precipitations (Zepf and Geiger 2000b). It is therefore recommended that the blending liquor used to dilute high gravity brewed beer to its final strength should have a calcium concentration lower than or equal to that of the undiluted beer.

In addition to the higher precipitation of calcium oxalate, higher solubility of gases is achieved at cold temperatures. Cold maturation followed by beer chilling will redissolve microbubbles to a certain extent, resulting in a decrease in the amount of gas nuclei in beer (Pellaud 2002).

Pasteurization increases the external pressure on microbubbles in beer bottles, resulting in partial gas nuclei collapse which in turn decreases the gushing risk of beer (Pellaud 2002). Repasteurisation has been reported to eliminate the gushing activity of packaged beer (Brenner 1957a). In addition to high pressure, higher temperature during pasteurization may enhance the solubility of precipitates such as calcium oxalate.

Unusual levels of iron and heavy metals in brewing liquors as well as the presence of foreign particles should be avoided (Brenner 1957b, Amaha and Kitabatake 1981, Gastl *et al.* 2009).

All efforts that reduce the air content of packaged beer minimize the gushing risk (Brenner 1957b, Amaha and Kitabatake 1981). The use of deaerated water combined with careful evacuation of the bottles prior to gentle filling without cavitation and turbulence will minimize the amount of gas nuclei in beer (Pellaud 2002). In addition, an appropriate filling level and the vertical position of the bottles during transportation will also decrease the beer gushing risk (Pellaud 2002). It has been observed that the larger the neckspace air the more intense is the gushing of beer. On the other hand, bottles completely filled with beer do not show gushing under conditions of agitation in which normally filled bottles do gush (Amaha and Kitabatake 1981). Reduction of the carbon dioxide content in beer also reduces the gushing risk (Gastl *et al.* 2009, Ilberg *et al.* 2009).

In addition, the avoidance of too intensive protein modification during malting and mashing, extensive protein secretion during mash and wort boiling as well as an extensive hot and cold break separation have been associated with the reduction of gushing risk (Gastl *et al.* 2009). Moreover, the use of vital yeast without autolysis and proteinase activity is recommended in order to minimize the gushing risk (Gastl *et al.* 2009).

The measures which decrease the amount of gas nuclei in finished beer will only provide a temporary cure if the primary gushing factors are present. Any subsequent introduction of gas bubbles into the beer will re-increase the gushing risk because the formation of stabilized microbubbles will re-occur due to the self-assembly of primary gushing factors around the bubbles (Brenner 1957a, Pellaud 2002). The only way to avoid gushing is to eliminate primary gushing factors.

### 1.4.2 Measures for preventing primary gushing

The avoidance of fungal compromised, especially *Fusarium*-infected barley and malt in the beer production chain is the best current practice to prevent gushing (Pellaud 2002, Wolf-Hall 2007). This requires strict control of barley and malt microbiota. Fungi including *Fusarium* species are a natural component of the diverse microbial community of barley, which have not only negative but also some positive effects on the quality of barley and malt (Noots *et al.* 1998, Flannigan 2003, Laitila 2007). After an exceptionally wet growing season, deleterious fungal growth may occur in barley. In addition to restriction of the amount of certain fungal species in barley, reduction of the vitality and the activity of the fungi is also important because vital fungi can proliferate and produce gushing factors during malting (Noots *et al.* 1998, Munar and Sebree 1997). There are various chemical, physical and biological methods to control the microbial quality of barley and malt (Haikara *et al.* 1993, Noots *et al.* 1998, Laitila 2007, Wolf-Hall 2007).

Cultural practices such as the use of *Fusarium*-resistant barley cultivars, crop rotation and the use of fungicides can be applied in the field in order to reduce the *Fusarium* contamination of barley (McMullen *et al.* 1997). In addition, the introduction of biological control agents such as antagonistic bacteria (*Pseudomonas*, lactic acid bacteria etc.) or fungi (*Cryptococcus*, *Trichoderma* etc.) in the field can protect grains against *Fusarium* infection (Khan and Doohan 2009, Reinikainen *et al.* 1999, Schisler *et al.* 2002, Schisler and Boehm 2011). Proper drying and storage of barley restrict the fungal growth after harvest (Noots *et al.* 1998).

Various common malting practices such as barley cleaning and grading before malting, change of steeping water and temperature control in steeping and in germination decrease the fungal load and proliferation during malting (Kunze 1999, Briggs and McGuinness 1993). It has been shown that the *Fusarium* contamination level of a barley lot can be significantly reduced by rejecting the smallest sized kernels (< 2.5 mm) (Perkowski 1998).

Some fungi including *Fusarium* species are known to be sensitive to heat. Heat treatments of barley with hot water and hot steam have been reported to reduce significantly the *Fusarium* contamination and the gushing potential of malt (Olkku *et al.* 2000, Kottaballi *et al.* 2003, Briggs *et al.* 2004). Moreover, irradiation of barley can reduce the viability of fungi (Noots *et al.* 1998, Kottaballi *et al.* 2003 and 2006). Furthermore, the prolonged storage of harvested barley has been observed to reduce the viability of fungi, especially at elevated temperatures (Beattie *et al.* 1998).

Munar and Sebree (1997) reported that storage of barley decreased the gushing potential of the corresponding malt produced.

The addition of different additives in malting such as acids, bases and disinfectants, e.g. hypochloric acid, hop beta-acids, sodium hypochlorite, formaldehyde, hydrogen peroxide and ozone, most often in the steeping water, has been reported to prevent or inhibit fungal growth during malting (Haikara 1980, Papadopoulou *et al.* 2000, Wolf-Hall 2007). Precautions must be taken with the use of chemical means because the treatments may even stimulate the production of harmful fungal metabolites such as mycotoxins and gushing factors (Havlova *et al.* 2006, Wolf-Hall 2007). In addition, the use of chemicals is often restricted by legislation. Furthermore, the general attitude of consumers and of the industry favours processing with minimal amounts of additives.

Starter cultures can be used as a biological control method in malting. Starter cultures compete with the indigenous microbes for space and nutrients, and/or they produce antimicrobial substances (Lowe and Arendt 2004, Laitila 2007, Wolf-Hall 2007). The addition of starter cultures such as lactic acid bacteria and ascomycetous yeasts such as *Geotrichum candidum* and *Pichia anomala* (synonym *Wickerhamomyces anomalus*) in malting has been reported to restrict the growth of *Fusarium* fungi, hence decreasing the gushing risk (Haikara *et al.* 1993, Haikara and Laitila 1995, Boivin and Malanda 1997, Laitila *et al.* 2002, Laitila 2007). Barakat *et al.* (2010) reported that addition of the antifungal protein AFP from *Aspergillus giganteus* in malting prevented the growth of different *Fusarium* species. Importantly, AFP treatment did not compromise the quality of the final malt or of the corresponding wort.

The use of a combination of various control methods in the beer production chain to impose a hurdle effect on fungal growth and gushing factor production is recommended in order to minimize the gushing risk (Leistner 2000, Laitila 2007, Wolf-Hall 2007).

In addition to strict control of barley and malt microbiota, some preventive actions against gushing can also be applied during the brewing process. Hops contain both gushing promoting and suppressing substances (Amaha and Kitabatake 1981, Hanke *et al.* 2009). Additions of hop components such as α-acids, β-acids, humulone and linalool to gushing beer have been demonstrated to reduce or even inhibit gushing. In addition, unsaturated fatty acids present in hops such as palmitoleic, oleic, linoleic and linolenic acids have also been shown to suppress gushing (Amaha and Kitabatake 1981). Hence, the use of hop products with high gushing suppressing properties in brewing could be a tool to minimize the gushing risk.

Some fungal metabolites can also inhibit beer gushing. Laible and Geiger (2003) observed that addition of extracellular polar lipids produced by some non-gushing *Fusarium* species to gushing beer inhibited the gushing phenomenon. They concluded that mono- or polyunsaturated fatty acids such as linoleic acid bound in complex fusarious lipids appear to be responsible for gushing inhibition.

Christian *et al.* (2009b) demonstrated that the temperature during the mashing process has a significant effect on the gushing potential of the produced wort. They were able to produce both gushing and non-gushing worts from the same

malt by altering the mashing procedure. They observed that only those worts produced using a mash temperature higher than 80 °C induced beer gushing. In addition to the separated wort the malt-grist also had to be exposed to temperatures above 80 °C in order to produce gushing wort. Christian *et al.* (2009b) also showed that gushing could be suppressed by adding small amounts of non-gushing wort (10% v/v) prepared with mash temperatures ≤ 80 °C into the gushing wort. This result indicated that the wort originally contained gushing-suppressing substances, which lost their ability to suppress gushing in high temperatures (Christian *et al.* 2009b). In addition, the separation of particles with sizes larger than 200 nm from non-gushing wort decreased but did not eliminate the gushing suppressing ability of this wort. This observation indicated that particles with sizes smaller and larger than 200 nm contribute to gushing suppression (Christian *et al.* 2009b).

Various treatments to decrease the gushing tendency of beer have been studied. For example the addition of pepsin or some other proteases to beer before pasteurization, or treatments with absorbents such as charcoal, fuller's earth or kaolin as well as with nylon powder reduced gushing tendency (Amaha and Kitabatake 1981, Aastrup *et al.* 1996). However, the practical use of proteases and of most absorbents is limited because they may impair the flavour and foam stability of the beer. Gjertsen (1967) concluded that the most practical correction measure for gushing beer is the appropriate blending of gushing beer with normal, non-gushing beer. This is in accordance with the finding that a minimum concentration of gushing-inducing substances is required for gushing induction (Christian *et al.* 2009b).

# 2.    Aims of the study

Although gushing factors produced by fungi have been studied for decades, none of them have been fully characterised. They have been reported to be polypeptides or peptide-containing substances. The hypothesis of this dissertation was that small fungal proteins called hydrophobins are one of the gushing factors inducing primary gushing of beer. The aim of this study was to isolate and characterize hydrophobins from gushing active fungi, especially from *Fusarium* species, and to demonstrate that these hydrophobins are able to induce gushing in beer. Currently, there is no practical, reliable and commercially available method for the prediction of beer gushing from large numbers of samples. The main goal of the work was to develop a test for detection of gushing potential of barley and malt by analysing the hydrophobin levels in samples. Another aim was to study the occurrence and fate of hydrophobins at different stages of the beer production chain by analysing the hydrophobin levels of samples taken throughout the barley-to-beer chain. In addition, means for preventing the formation of gushing factors were investigated.

# 3.  Materials and methods

Materials and methods used in this study are described only briefly in this section. For more detailed information see the original publications (Papers I–V).

## 3.1  Microbial strains

Microbial strains used in this study were obtained from the VTT Culture Collection. The strain information and the cultivation practices are presented in the Papers I–V.

## 3.2  Barley and malt samples

Non-inoculated and articifially *Fusarium*-inoculated barley and malt samples were obtained from the field trials as well as from the malting trials described below (Papers I, II and IV). Naturally *Fusarium*-infected two-row barley (Scarlett, crop year 2005) was used in the biocontrol studies (Paper V). The gushing negative two-rowed Scarlett malt (crop year 2001), with a low *Fusarium* contamination rate, was produced for the brewing studies in the pilot malting house at VTT Bio and Process Technology. A heavily *Fusarium* infected, gushing positive malt (six-row malting variety Robust, crop year 2002) for the brewing studies was kindly provided by Prof. Paul Schwarz, North Dakota State University, the USA. The gushing positive and negative standard malt samples were purchased from Carlsberg Research Laboratory, Denmark. The gushing positive standard malt contained 15% of artificially *Fusarium culmorum* infected kernels and was shown to induce vigorous beer gushing when analysed using the Carlsberg gushing test. In addition, natural barley and malt samples for evaluation of the developed hydrophobin ELISA were obtained from different European countries and from the USA.

### 3.2.1  Field trials

Field trials were carried out as described in Paper I. During the summer of 1998, two different two-row barley cultivars, Cv. A and Cv. B, were grown and artificially inoculated with three *Fusarium* species, namely *F. culmorum*, *F. graminearum* and

*F. poae*, at two experimental farms located in Hauho and Jokioinen in southern Finland. The field trial was repeated with one *Fusarium* species (*F. culmorum*) and one barley cultivar (Cv. A) at the Hauho experimental farm during the summer of 2000. Spiked samples were collected during the growing periods and analysed for *Fusarium* infection, moisture content and hydrophobin level (Papers I and IV). Total precipitation and temperature data were collected daily. The harvested barley samples were analysed for microbes, mycotoxins and hydrophobin levels (Papers I and IV).

### 3.2.2 Inoculation of barley samples with *Fusarium* species prior to malting

Barley samples (Scarlett, crop year 2005) were inoculated with 9 different *Fusarium* strains belonging to the species *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. sporotrichioides* and *F. tricictum* prior to malting (see Table 11 for strain information). Before inoculation, barley samples were heat treated in a hot water bath (80 °C, 5 s) in order to suppress the growth of indigenous fungi. The heat-treated barleys with moisture contents adjusted to 30% were inoculated with the selected *Fusarium* strains by adding $10^7$ spores/kg barley. The spores were harvested from mycelia grown on potato dextrose agar plates (Difco Laboratories, the USA). The inoculated samples were incubated for 2.5 days at 20 °C. After incubation, the barley samples were malted immediately as described below.

## 3.3 Malting trials

The malting trials were carried out in 1 kg scale in a Seeger laboratory malting unit (Seeger GmbH, Germany) or in a specially designed computer controlled micromalting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). The malting procedures used in this study are described in Papers I, IV and V. Samples for the microbial and hydrophobin analyses were taken from barley, and from barley after steeping, germination and kilning (after rootlet removal). In addition, the malt analyses described below were conducted.

## 3.4 Brewing experiments

To study the effects of different brewing steps on the hydrophobin content migrating in the brewing process, the gushing positive and negative malt samples were brewed in 10 litre scale (Paper IV). Samples of malt grist, spent grains, wort before and after boiling, cold break, yeast after the main fermentation and beer before and after filtration were taken for hydrophobin analyses. In addition, laboratory scale high gravity mashing was performed using the gushing positive and negative malt samples in order to study the effects of mashing on hydrophobins (Paper IV). The effects of the wort boiling and the beer filtration steps on hydrophobins were

also studied using pure hydrophobin samples added to wort or to distilled water (Paper IV).

## 3.5 Barley, malt, wort and beer analyses

### 3.5.1 Microbial analyses

Methods applied to study the microbiota of the barley and malt samples taken throughout the barley-malt production chain are presented in Table 5.

**Table 5.** Microbial analyses of the barley and malt samples including conventional culturing methods as well as one molecular biological method.

| Analysis | Paper | Ref. |
|---|---|---|
| *Fusarium* fungi: | | |
|     Direct plating of kernels | I, V | 1, 2 |
|     Plating of homogenized samples | I | |
| Other field fungi (direct plating) | I, V | 1 |
| *Pseudomonas* spp. | I, V | |
| Lactic acid bacteria | I, V | |
| Total aerobic heterotrophic bacteria | I, V | |
| Yeasts | I, V | |
| qPCR for trichothecene producing fusaria | | 3 |

References: 1) EBC Analytica-Microbiologica II 2005, 2) Abildgren *et al.* 1987, 3) Sarlin *et al.* 2006.

### 3.5.2 Gushing test

Gushing potentials of the barley and malt samples were determined according to the Carlsberg gushing test described by Vaag *et al.* (1993) (Papers I, II, IV and V). An aqueous extract of ground barley and malt was added to a Finnish commercial beer and agitated with a horizontally rotating shaker (50 rpm) for three days. After agitation the bottles were kept still for 10 min, inverted three times and opened after 30 sec. The amount of gushing was determined from the change in weight of the bottle. The beers brewed from the gushing positive and negative malt samples in the brewing experiments were agitated and the gushing propensity was determined as described above (Paper IV). In addition, the gushing-inducing capability of hydrophobins was studied by adding purified hydrophobins into the bottles of beer or carbonated mineral water and agitating the bottles as described above (Papers II and III).

### 3.5.3 Method for analysing hydrophobin content in samples

A competitive ELISA (Enzyme-Linked ImmunoSorbent Assay) method was developed for detection of hydrophobins in barley and malt as well as in samples taken from the malting and brewing processes (Paper II). Briefly, 5 g of the ground sample was extracted with PBS buffer in the proportion of 1:10. Liquid samples, such as wort and beer, were tested directly without the extraction. The extract was centrifuged and the supernatant was transferred to a clean tube, and antibodies against the hydrophobin FpGUSH isolated from *F. poae* were added. After incubation, the sample-antibody mixture was transferred to at least three replicate wells of an immunoplate coated with a hydrophobin extract of *F. poae* and the immunological reactions were allowed to proceed. After the sequential incubations of the secondary antibody – alkaline phosphatase (AP) conjugate and the substrate for AP detection, the absorbance was read at 405 nm using a microtitre plate reader and the mean of the absorbance values of the replicate wells was calculated. Due to the nature of the competitive ELISA, a lower absorbance value corresponds to a higher amount of hydrophobins in the sample. Hence, the hydrophobin level is expressed as the inverse of the mean absorbance value (1/Abs). This competitive ELISA method for hydrophobins is referred to as the hydrophobin ELISA in this dissertation.

### 3.5.4 Other analyses

The methods applied for barley, malt, wort and beer analyses are presented in Table 6.

**Table 6.** Methods applied for barley, malt, wort and beer analyses.

| Analysis | Method | Paper | Ref. |
|---|---|---|---|
| **Barley analyses** | | | |
| Moisture content | EBC 3.2 | I, V | 1 |
| Crop yield | | I | |
| Kernel size | | I | |
| Germination capacity | EBC 3.5.1 | I, V | 1 |
| Germination energy | EBC 3.6.2 | I, V | 1 |
| Protein content | EBC 3.3.1 | I, V | 1 |
| **Malt analyses** | | | |
| Malt friability | EBC 4.15 | V | 1 |
| Malt modification, Calcofluor | EBC 4.14 | I, V | 1 |
| $\alpha$-Amylase | | I | 2 |
| Endogenous $\beta$-glucanase | Megazyme MBG | I, V | |
| Microbial $\beta$-glucanase | Megazyme MGB | I, V | |
| Xylanase | Megazyme XYL | I, V | |

Table 6. Continued…

| Wort analyses | | | |
|---|---|---|---|
| *Congress mashing:* | | | |
| Extract content | EBC 4.5.1 | I | 1 |
| Wort colour | EBC 4.7.2 | I | 1 |
| Free amino nitrogen (FAN) | EBC 4.10 | I | 1 |
| Soluble nitrogen, Kjeldahl method | EBC 4.9.1 | I | 1 |
| Wort β-glucan content, fluorimetric | EBC 4.16.2 | I | 1 |
| Filtration rate | | I | 3 |
| **Foam stability** | NIBEM | II | |
| **Mycotoxins** | GC-MS or HPLC | I | |
| | DON Test Kit | II | |

References: 1) EBC Analytica 1998, 2) McCleary and Sheehan 1987, 3) Sjöholm *et al.* 1994.

## 3.6 Isolation and characterisation of hydrophobins

### 3.6.1 Isolation, characterisation and expression of hydrophobin genes of *Fusarium* fungi

Profile hidden Markov models (profile HMMs) were generated for the hydrophobin classes Ia, Ib and II from the multiple sequence alignments of their known members available in public domain databases (Paper III). Profile HMMs are statistical models of multiple sequence alignments (Krogh *et al.* 1994). They capture position-specific information about how well conserved each column of the alignment is, and which amino acid residues are likely in that column. The published *Fusarium graminearum* genome database of predicted proteins (http://www.broad institute.org) was searched with the profile HMMs. The best matching sequences and the corresponding genes were isolated from *F. graminearum* and related species by PCR, cloned in *Escherichia coli* DH5α cells and characterized by sequencing. The putative hydrophobin genes were transformed and expressed in the heterologous host *Trichoderma reesei* (Paper III).

### 3.6.2 Isolation of hydrophobins from mycelium and from culture filtrate

Hydrophobins produced by fungi during cultivation in liquid culture media were isolated as described by Nakari-Setälä *et al.* (1996) using the sequential extraction of mycelium or by bubbling air through culture filtrates and then collecting the foam produced (Papers II and III). In addition, an extraction of mycelium with 4 M guanidine hydrochloride was applied (Paper III). Aqueous two-phase extraction using a surfactant was used for hydrophobin isolation from culture filtrates and mycelium extracts of the hydrophobin transformants (Paper III).

### 3.6.3 Purification of hydrophobins

The hydrophobin samples were purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) using a Vydac C4 column with the Äkta Explorer chromatographic system (Pharmacia Biotech, Sweden) (Papers II and III). Elution was performed with a 0–100% gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions containing hydrophobins were collected.

### 3.6.4 Protein analytical methods

The methods applied for protein analyses are presented in Table 7.

**Table 7.** The methods applied for protein analyses.

| Analysis | Paper | Ref. |
|---|---|---|
| SDS-PAGE with silver staining | II, III | 1 |
| Western blotting | II | |
| Protein concentration | II, III | |
| N-terminal amino acid sequencing | II, III | |
| Molecular weight | II, III | |
| Amino acid analysis | III | |
| Structural characterisation by alkylation and enzymatic digestion with trypsin | III | 2 |
| Basic Local Alignment Search Tool, BLAST | | |
| ClustalW version 2.1 | | |

Reference: 1) Laemmli 1970, 2) Selinheimo *et al.* 2006.

The Basic Local Alignment Search Tool, BLAST, is a bioinformatics software tool for comparing primary biological sequence information such as the amino acid sequences of different proteins. A BLAST search (version: WU-BLAST2, http://www.ebi.ac.uk/Tools/sss/ wublast/) was used to find the most similar protein sequences from protein databases compared to the putative hydrophobins isolated in this study. ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), a general purpose multiple sequence alignment program for DNA and proteins, was used to align the hydrophobin sequences.

## 3.7   Statistical analyses

Regression analysis was used for determining the relationship between known hydrophobin concentrations and hydrophobin levels measured using the hydrophobin ELISA (Paper II). The analysis was performed using the Statistical Toolpak of Microsoft Excel. In addition, one-way analysis of variance (ANOVA) with Tukey's

Honestly Significant Difference (HSD) test was used for evaluation of the significance of *P. anomala* treatment for hydrophobin levels detected in malts ($P < 0.05$) using the software SPSS 14.0 for Windows (Paper V).

# 4. Results and discussion

## 4.1 Effect of artificial *Fusarium* infection of barley grown under Finnish field conditions on gushing potential (Paper I)

It is well known that *Fusarium* infection may reduce the quality of barley, which in turn affects malting and brewing performance (Haikara 1983, Flannigan 2003, Schwarz *et al.* 1997 and 2001). This study revealed numerous effects of *Fusarium* fungi on the quality of barley grown under Finnish field conditions and on the corresponding malt (Table 8). In particular, *Fusarium* infection increased the gushing potential of malt, providing an excellent research material for further studies. Moreover, this study indicated that the extent of the impacts is species-dependent. The *F. graminearum* strain originating from the United States of America had more pronounced effects on the quality of barley and malt than the Finnish *F. culmorum* strain and especially the German *F. poae* strain (Paper I). *F. graminearum*, associated with warm and humid regions, is not a common cereal pathogen in Finland (Sarlin *et al.* 2006), but the study showed that this highly virulent and toxic pathogen is able to proliferate under Finnish field conditions. *Fusarium* infection has not only negative but to a certain extent also positive effects on the quality of barley and malt (Noots *et al.* 1998, Flanningan 2003, Laitila 2007). For example the increased malt enzyme activities also observed in this study may decrease the β-glucan content of wort, thus enhanceing its filtration rate.

Cultivation analyses showed that growth of the *F. graminearum* and *F. culmorum* strains in barley in the field was faster and more intense than that of the *F. poae* strain, resulting in reduced crop yield and quality (Paper I). The DNA level of trichothecene-producing *Fusarium* species from the infected barley samples was quantified using the specific TMTRI real-time quantitative PCR assay targeted for amplification of the trichodiene synthase gene *tri5* (Sarlin *et al.* 2006). The results revealed that the barley samples infected with *F. graminearum* contained approximately 10 and 100 times more trichothecene-producing *Fusarium* DNA compared to the *F. culmorum*- and *F. poae*-infected barley samples, respectively. These findings are consistent with the results of previous studies which have indicated that *F. graminearum* is more virulent than *F. poae* in terms of causing

*Fusarium* Head Blight in barley (Salas *et al.* 1999, Schwarz *et al.* 2001). In general terms, *F. poae* is regarded as a relatively weak pathogen compared to F. graminearum and *F. culmorum* (Stenglein 2009).

Analogously to infection rates, the *F. graminearum* strain was observed to produce higher amounts of mycotoxins both in the field and during malting than the *F. culmorum* strain and particularly the *F. poae* strain (Paper I, Table II and IV). Moreover, higher proteinase, xylanase and exogenous β-glucanase activities were measured from the samples inoculated with *F. graminearum* and *F. culmorum* compared to the activities measured from the non-inoculated and the *F. poae* -inoculated samples (Paper I, Table V). *F. graminearum* and *F. culmorum* infection decreased the filtration rate of wort. *F. graminearum* infection also had the most pronounced effect on wort colour, soluble nitrogen and FAN. These findings are consistent with the results of Schwarz *et al.* (2001 and 2002), who also reported that *F. graminearum* infection had more pronounced effects on barley and malt quality than *F. poae* infection. Haikara (1983) also observed that *F. culmorum* infection reduced barley and malt quality more intensely than did *F. avenaceum* infection.

**Table 8.** Effects of *Fusarium* infection on barley and malt quality parameters.

| Increase | Decrease |
|---|---|
| Mycotoxin content | Crop yield |
| Protein content of barley | Kernel plumpness |
| Total nitrogen content of barley | Germination capacity of barley |
| Proteinase activities in barley | Endogenous β-glucanase activity in malt |
| Microbial β-glucanase activity in malt | β-glucan content of malt |
| Xylanase activity in malt | Filtration rate of wort |
| Wort colour | |
| Soluble nitrogen content in wort | |
| FAN in wort | |
| Gushing | |

All the strains studied induced gushing of beer, but the *F. poae* strain induced the weakest gushing propensity (Figure 4). The malt samples made from the *F. graminearum* and *F. culmorum* -inoculated barleys grown under Finnish field conditions frequently induced pronounced beer gushing in the gushing test, whereas the *F. poae* -infected malt samples showed variable gushing potentials varying from gushing negative to slightly gushing positive. The lower *F. poae* infection rates in barleys and the corresponding malts probably contributed to the lower gushing potentials detected in the *F. poae* -infected malts (Figure 4). The results of Haikara (1983) supported the conclusion that different Fusarium species have different gushing-inducing activities. She reported that the *F. culmorum* strains studied were more active gushing inducers than *F. avenaceum* strains.
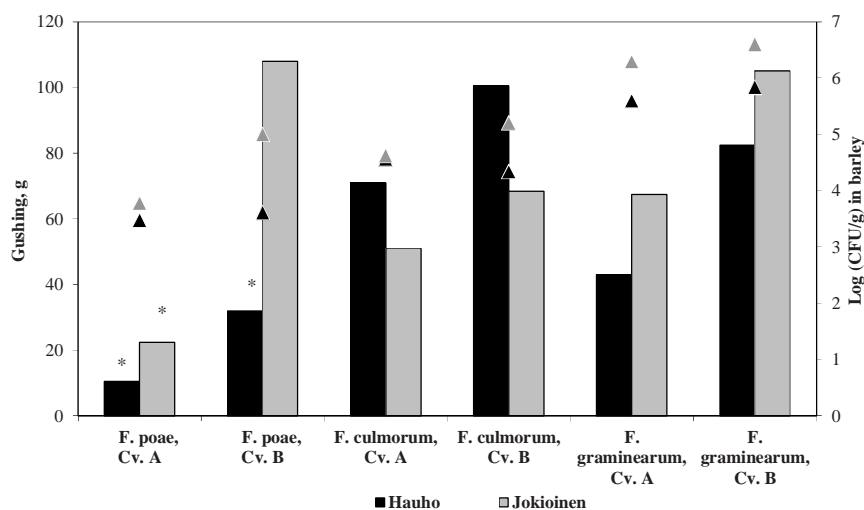
**Figure 4.** Gushing potentials of the malt samples (columns) produced from barleys artificially inoculated with *F. poae*, *F. culmorum* and *F. graminearum* in the field. In addition, *Fusarium* counts of the barleys (triangles) are also shown. The results are from two barley cultivars, Cv. A and Cv. B, grown at the Hauho and Jokioinen Experimental farms in southern Finland. The gushing results are averages from two successive gushing tests. *: variable gushing.

Gushing potential was followed throughout the malting process by analyzing the samples of barley, steeped barley, green malt and kilned malt. The gushing activities of the malting samples varied during the process depending on the initial *Fusarium* contamination level in barley (Paper I, Table III). The gushing potential of barley, particularly in the case of non-inoculated samples, decreased during steeping. However, it could again increase during germination or kilning, whereas the gushing propensity of heavily *Fusarium*-infected barley remained high throughout the entire malting process (Paper I, Table III). The more intense *Fusarium* contamination in most of the samples originating from the Jokioinen experimental farm obviously resulted in a more pronounced increase in gushing propensity during germination and kilning compared to the samples from Hauho. Commercial malting barley lots from the same crop year had low *Fusarium* contamination rates and did not induce gushing at any stage of malting (data not shown). A similar impact of malting on gushing potentials of *Fusarium*-infected barleys was reported by Munar and Sebree (1997).

The effect of barley storage time on the gushing potential of the corresponding malt was studied by malting the same barley samples 3–4 times during storage of 17 months. The effect of barley storage time on the gushing potential depended on the initial *Fusarium* contamination level in barley. The gushing potentials of the malt samples made from the non-inoculated barleys with low *Fusarium* contamination levels decreased during the storage period whereas the gushing potentials of the

malt samples made from the heavily *Fusarium*-infected barleys remained high (Figure 5). Reduction in gushing potential of malt as a result of prolonged storage time of the corresponding barley was also observed by Munar and Sebree (1997). Moreover, Beattie *et al.* (1998) reported that the viability of fusaria declined significantly over 7 months of storage, which may reduce the production of gushing factors during malting.
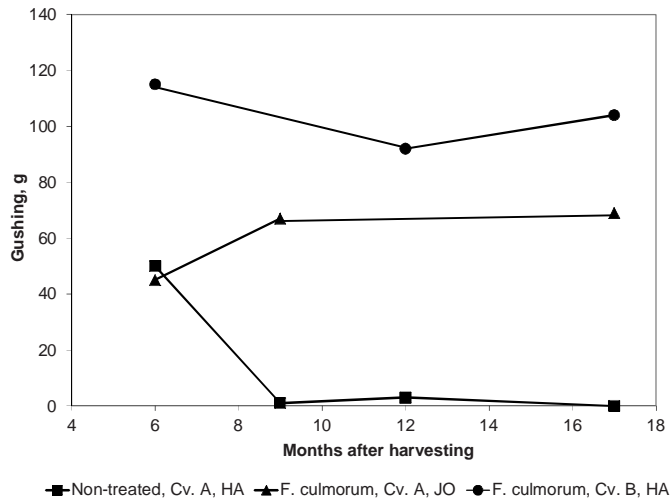


**Figure 5.** Effect of storage time of barley on the gushing potential of corresponding malt. Non-treated and *F. culmorum* -inoculated barley samples (cultivars A and B) grown at the Hauho (HA) and Jokioinen (JO) experimental farms were malted 3–4 times during 17 months of storage and the gushing potential of the corresponding malts was analysed.

This study and the previous studies of Haikara (1983) and Schwarz *et al.* (2001 and 2002) indicated that the extent of the effect of different *Fusarium* species on barley and malt quality, like on the gushing potential, varies. This is most probably due to the differences in aggressiveness of the pathogens towards barley (Stenglein 2009). Hence, the determination of *Fusarium* contamination level in barley and malt does not necessarily provide sufficient information concerning the quality of the sample. Identification to the species level and subsequent quantification of different *Fusarium* species would improve the quality assessment. Traditional *Fusarium* species identification requires expert level knowledge and is time-consuming. Molecular biological techniques such as real-time quantitative PCR offer more practical means for the species determination. However, the presence of a certain species in a sample does not necessarily mean that the species has produced unwanted metabolites. The most accurate prediction of the quality risks can only be achieved by analysing the actual unwanted metabolites, in the case of gushing risk the known gushing-inducing factors.

## 4.2 Hydrophobins and beer gushing (Papers II, III)

Hydrophobins are produced ubiquitously by filamentous fungi. Hydrophobins have been identified in many ascomycetes and basidiomycetes including some *Fusarium* species (Fuchs *et al.* 2004, Linder *et al.* 2005, Zapf *et al.* 2006). Most species produce more than just one hydrophobin. The unique properties of fungal hydrophobins such as their hydrophobicity, high surface activity and ability to form stable amphipathic membranes between the liquid and gas phases, made them potential candidates for fungal gushing factors. The aim of this study was to isolate and characterise hydrophobins from gushing-active fungi and to demonstrate that these hydrophobins induce beer gushing.

### 4.2.1 Isolation and characterisation of hydrophobins from gushing-active fungi

In this study two different approaches were applied to isolate hydrophobins from gushing-active fungi. Firstly, gushing-active fungi were cultivated in different culture media and hydrophobins were isolated from culture filtrates or from fungal mycelia (Paper II). Secondly, statistical profile hidden Markov models (profile HMMs) were generated for the hydrophobin classes Ia, Ib and II, and these models were used to search putative hydrophobin genes from the published genome database of *F. graminearum* (Paper III). This fungus is a well-documented gushing inducer (Gjertsen *et al.* 1965, Amaha *et al.* 1973, Schwarz *et al.* 1996). The corresponding genes of the best matching hydrophobin sequences were amplified from *F. graminearum* and the related species *F. culmorum* and *F. poae* by PCR and characterised by sequencing. The selected hydrophobin genes were then expressed in *Trichoderma reesei* and the produced hydrophobins were purified (Paper III).

Hydrophobins were isolated from liquid culture medium of two gushing-active fungi, *F. poae* and *Nigrospora* sp. (Paper II). The typical conserved cysteine pattern of hydrophobins was found in the partial amino acid sequences of the isolated proteins (Paper II, Figure 2). In addition, alkylation with 4-vinylpyridine indicated that the protein isolated from *F. poae* contained 8 cysteine residues (unpublished data), which is a characteristic feature of hydrophobins. Both proteins had a molecular mass of 8.5 kDa, which agrees with the molecular masses of other hydrophobins (Bowden *et al.* 1994, Mackay *et al.* 2001, Nakari-Setälä *et al.* 1996 and 1997). The BLAST search revealed that the isolated proteins of *F. poae* and *Nigrospora* sp. were the most closely related to the hydrophobins of several fungal species. The putative *F. poae* hydrophobin, named FpGUSH, was the most closely related to the hydrophobin of *Trichoderma virens* and the trihydrophobin CFTH1 of *Claviceps fusiformis*, with the partial sequence identities of 58% and 53%, respectively. CFTH1 has been proposed to have a role in the formation of aerial hyphae (de Vries *et al.* 1999). The putative *Nigrospora sp.* hydrophobin, named NGUSH, had the best matches with the Cerato-ulmin hydrophobin (CU) of *Ophiostoma himal-ulmi* and CU of *Ophiostoma ulmi* as well as with the Cryparin hydrophobin (CRP) of *Cryphonectria parasitica*, with partial sequence identities of 63%, 61% and 52%, respectively.

The CU hydrophobin has been suggested to be a parasitic fitness factor of the causal agents of Dutch elm disease (Temple *et al.* 1997). CRP has been observed to affect hydrophobicity of hyphae and to facilitate the eruption of fungal fruiting bodies through the bark of the host tree (Kazmierczak *et al.* 2005). The partial amino acid sequences of the FpGUSH and NGUSH are compared with those of several known class II hydrophobins found in databases in Figure 6. Interestingly, Kitabatake and Amaha (1977) reported that the gushing-inducing factor of *Nigrospora*, called NGF, had a molecular mass of 16.5 kDa, consisted of 166 amino acid residues and carried approximately 16 cysteine residues. If NGF is assumed to be a dimer, then the molecular mass as well as the number of amino acid and cysteine residues of the monomeric protein corresponds to those of hydrophobins.

The search for hydrophobins from the *F. graminearum* genome database revealed five previously uncharacterized genes showing similarity to known hydrophobin sequences (Paper III). Four of them, the locus tags FG01763.1, FG01764.1, FG03960.1 and FG09066.1, were classified as genes encoding class I hydrophobins and one, the locus tag FG01831.1, as a gene encoding a class II hydrophobin. The deduced amino acid sequence alignments of these putative hydrophobins and several known hydrophobins found in databases are shown in Figure 6. The BLAST search revealed that the putative hydrophobins of FG01763.1 and FG01764.1 are the most closely related to the class Ia hydrophobins of *Trichoderma asperellum* (a biocontrol agent), *Ajellomyces capsulata* (a dimorphic fungus existing as a saprophytic mycelial form in the soil and as a pathogenic yeast form in the lungs of its host, causing histoplasmosis), *Ajellomyces dermatitidis* (a cause of blastomycosis) and *Paracoccidioides brasiliensis* (a human pathogenic, thermally dimorphic fungus). The TasHYD1 hydrophobin of *T. asperellum* has been observed to be involved in plant root colonization (Viterbo and Chet 2006). The study of Albuquerque *et al.* (2004) revealed that mRNAs of both hydrophobin genes (Pbhyd1 and Pbhyd2) of *P. brasiliensis* are mycelium-specific and are highly accumulated during the first 24 h of the mycelium-to-yeast transition. The putative hydrophobin of FG03960 has similarities with both class Ia and class Ib hydrophobins (Figure 6). It is the most closely related to hydrophobins of basidiomycetes, such as those of *Coprinopsis cinerea* (COH), *Flammulina velutipes* (FVH1), *Lentinula edodes* (Le.HYD2) and *Schizophyllum commune* (SC3). Ng *et al.* (2000) proposed that Le.HYD2 mediates attachment of the dikaryotic mycelia to the hydrophobic surface of the substrate. SC3 of *S. commune* has been shown to enable aerial hyphal formation by lowering the water surface tension, and to mediate attachment to hydrophobic surfaces (van Wetter *et al.* 2000, Wösten *et al.* 1994 and 1999). The putative hydrophobin of FG09066.1 is highly similar to the hydrophobins Hyd3p from *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) and FcHyd3p from *Fusarium culmorum*. The functions of Hyd3p and FcHyd3p are not known. The putative Class II hydrophobin of FG01831.1 was found to be closely related to the *F. verticillioides* hydrophobin Hyd5p, to the *F. oxysporum* hydrophobin FOXB_00066 and especially to the *F. culmorum* hydrophobin FcHyd5p. The functions of these three deduced hydrophobins are also not known. The hydrophobin MPH1 of *Magnaporthe grisea*, which is also closely related to the putative hydrophobin of FG01831.1, is known to be expressed during invasive growth

within the host (Kim *et al.* 2001). Our findings were in accordance with those of Fuchs *et al.* 2004 and Zapf *et al.* 2006, who also reported high sequence similarities between the deduced hydrophobins of *F. culmorum*, *F. graminearum* and *F. verticillioides*. All the putative hydrophobins identified in this study were predicted to have a signal sequence, and thus they should be secreted proteins.

**Figure 6.** Sequence alignments from deduced hydrophobin protein sequences found in databases. Clustal W was used to align the hydrophobin sequences belonging to the classes Ia, Ib and II. Only sequences after the first cysteine (C) were used in the alignments. Putative hydrophobins identified in this study are shown in red.

Figure 6. Continued…

**Class Ib**

| Name | Species |
|------|---------|
| Le.HYD1 | *Lentinula edodes* |
| Le.HYD2 | *L. edodes* |
| COH2 | *Coprinopsis cinea* |
| FVH1 | *Flammulina velutipes* |
| POH2 | *Pleurotus ostreatus* |
| COH1 | *Co. cinea* |
| DGH3 | *Dictyonema glabratum* |
| DGH2 | *D. glabratum* |
| DGH1 | *D. glabratum* |
| ABH3 | *Agaricus bisporus* |
| POH3 | *Pl. ostreatus* |
| F4HYD1 | *Fi. velutipes* |
| HYPA/ABH1 | *Ag. bisporus* |
| HYPC/ABH2 | *Ag. bisporus* |
| AaPRI2 | *Agrocybe aegerita* |
| VMH1 | *Pl. ostreatus* |
| POH1 | *Pl. ostreatus* |
| FBH1 | *Pl. ostreatus* |
| PNH2 | *Pholiota nameko* |
| PNH1 | *Ph. nameko* |
| PNH3 | *Ph. nameko* |
| HYP8_A.bisporus | *Ag. bisporus* |
| HYDPH-1 | *Pisolithus tinctorius* |
| HYDPH-2 | *Pi. tinctorius* |
| HYDPH-3 | *Pi. tinctorius* |
| HYD1_T.terreum | *Tridroboma terreum* |
| AF331452 | *Pl. ostreatus* |
| SC4 | *Schizophyllum commune* |
| SC1 | *S. commune* |
| SC6 | *S. commune* |
| VMH3 | *Pl. ostreatus* |
| SC3 | *S. commune* |
| FG03960.1 | *F. graminearum* |

**Class Ib continued**

| Name | Species | |
|------|---------|---|
| Le.HYD1 | *Lentinula edodes* | 75 |
| Le.HYD2 | *L. edodes* | 80 |
| COH2 | *Coprinopsis cinea* | 83 |
| FVH1 | *Flammulina velutipes* | 80 |
| POH2 | *Pleurotus ostreatus* | 82 |
| COH1 | *Co. cinea* | 82 |
| DGH3 | *Dictyonema glabratum* | 82 |
| DGH2 | *D. glabratum* | 82 |
| DGH1 | *D. glabratum* | 84 |
| ABH3 | *Agaricus bisporus* | 82 |
| POH3 | *Pl. ostreatus* | 88 |
| F4HYD1 | *Fi. velutipes* | 81 |
| HYPA/ABH1 | *Ag. bisporus* | 80 |
| HYPC/ABH2 | *Ag. bisporus* | 83 |
| AaPRI2 | *Agrocybe aegerita* | 93 |
| VMH1 | *Pl. ostreatus* | 83 |
| POH1 | *Pl. ostreatus* | 82 |
| FBH1 | *Pl. ostreatus* | 81 |
| PNH2 | *Pholiota nameko* | 82 |
| PNH1 | *Ph. nameko* | 82 |
| PNH3 | *Ph. nameko* | 86 |
| HYP8_A.bisporus | *Ag. bisporus* | 82 |
| HYDPH-1 | *Pisolithus tinctorius* | 83 |
| HYDPH-2 | *Pi. tinctorius* | 83 |
| HYDPH-3 | *Pi. tinctorius* | 83 |
| HYD1_T.terreum | *Tridroboma terreum* | 81 |
| AF331452 | *Pl. ostreatus* | 76 |
| SC4 | *Schizophyllum commune* | 82 |
| SC1 | *S. commune* | 83 |
| SC6 | *S. commune* | 83 |
| VMH3 | *Pl. ostreatus* | 81 |
| SC3 | *S. commune* | 81 |
| FG03960.1 | *F. graminearum* | 77 |

Figure 6. Continued…

| Class II Name | Species | Sequence | |
|---|---|---|---|
| Hyd5p | *Fusarium verticillioides* | CSG--LYGTSQCCATDVLGVADLDCGNPPSTPANATDFSAVCSAIGQR-ARCCVLP-ILDQGILCNTPTGVQD | 69 |
| FOXB_00066 | *F. oxysporum* | CSG--LYGTSQCCATDVLGVADLDCGNPPSTPANATDFSAVCSAIGQR-ARCCVLP-ILDQGILCNTPTGVQD | 69 |
| **FpHYD5** | ***F. poae*** | CTG--LYGSSQCCATDVLGVANLDCGTPPSVPANATDFSAVCAEIGQR-ARCCVLP-ILDQGILCNTPTGVQD | 69 |
| **FG1831.1 = GzHYD5** | ***F. graminearum*** | CSG--LYGTSQCCATDVLGVADLDCGNPPSSPTDADNFSAVCAEIGQR-ARCCVLP-ILDQGILCNTPTGVQD | 69 |
| FcHYD5p | *F. culmorum* | CSG--LYGTSQCCATDVLGVADLDCGNPPSSPTDADNFSAVCAEIGQR-ARCCVLP-ILDQGILCNTPTGVQD | 69 |
| MPH1 | *Magnaporthe grisea* | CSG--LYGSAQCCATDILGLANLDCGQPSDAPVDADNFSEICAAIGQR-ARCCVLP-ILDQGILCNTPAGVTP | 69 |
| NCU08192.1 | *Neurospora crassa* | CSG--LYGTAQCCATDVLGVADLDCANPPATLANATHFESTCAAIGQR-ARCCVLP-IVSY---LSYPD---- | 62 |
| HCF-5 | *Cladosporium fulvum* | CP----ALDTPLCCQADVLGVLDLTCEAPSDD-TSVSNFEAACATTG-LTARCCTLP-LLGEALLCTTTP---- | 62 |
| HCF-6 | *C. fulvum* | CP----ANRVPQCCQLSVLGVADVTCASPSSGLTSVSAFEADCANDG-TTAQCCLIP-VLGLGLFCSNP---- | 63 |
| CU | *Ophiostoma ulmi* | CT--GLLQKSPQQCNTDILGVANLDCHGPPSVPTSPSQFQASCVADGGRSARCCTLS-LLGLALVCTDPVGI-- | 69 |
| **NGUSH** | ***Nigrospora sp.*** | CANNGVLFSAPNCCATDVLGLADLDCT-PPKVPTSPXDFQ------------------- | 39 |
| CRP | *Cryphonectria parasitica* | CS--STLYSEAQCCATDVLGVADLDCETVPETPTSASSFESICATSG-RDAKCCTIP-LLGQALLCQDPVGL- | 68 |
| ABS59375 | *Trichoderma virens* | CPTL--LYSNPQQCCSASVLNIADLDCEPPRKRPSRKHDFKQICAAQGSD-AKCCVLP-LLGLGILCTDAIV-- | 67 |
| **FpGUSH** | ***F. poae*** | CPGA--LYSQTQCCSAGVGDIVDV--------------------------------- | 22 |
| CFTH1_I | *Claviceps fusiformis* | CPAG--LYSNPQQCCATDVLGVADLDCKNPSSAPMSGDNFKSICNAVGQQ-AKCCVLP-VAGQAVLCQQDSIN-- | 67 |
| CFTH1_II | *Cl. fusiformis* | CPAGL-LYSNPQQCCSTGVLGVADLDCKNPSSAAPTSGDDFQKICANGGQQ-AQCCSIP-VAGQAVLCQPAI--- | 67 |
| CPPH1_III | *Cl. purpurea* | CPSG--LYSNPQQCSTDVLGVASLDCVNPSSKPRSGDNFKSTCAAAEKH-ASCCVIP-VAGQAVLCQPAM--- | 66 |
| CPPH1_IV | *Cl. purpurea* | CPSG--LYSNPQQCSTDVLGVASLDCVNPSSKPRSGDNFKSTCAAAGKH-ASCCVIP-VAGQGVLCQPAM--- | 66 |
| CPPH1_II | *Cl. purpurea* | CPSG--LYSNPQQCCATDVLGVASLDCVNPSSKPRSGDDFKSTCGAEGKH-ASCCAIP-VAGQGVLCQPAM--- | 66 |
| CPPH1_I | *Cl. Purpurea* | CPSG--LYSNPQQCCSTNVLGVAALNCKTPSFAPTSFQSFFKSAQS--GGQ-PQCCVVP-AAGQGVLCQQAPI--- | 64 |
| HFBI | *T. reesei* | CPPG--LFSNPQQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQ-PLCCVAP-VAGQALLCQTAVGA- | 68 |
| HFBII | *T. reesei* | CPTG--LFSNPLCCATNVLDLIGVDCKTPTIAVDTGAIFQAHCASKGSK-PLCCVVP-VADQALLCQKAIGTF- | 69 |
| SRH1 | *T. hartzianum* | CPNG--LYSNPQQCGANVLGVAALDCHTPRVDVLTGPIFQAVCAAEGGKQPLCCVVP-VAGQDLLCEEAQGTF- | 70 |
| CFTH1_III | *Cl. fusiformis* | CPSG--LYSVPQQCCATDVLGVADLDCGNPSRQPTDSSDFASVCAAKG-QRARCCVLP-LLGQAVLCTGA---- | 65 |
| CPPH1_V | *Cl. purpurea* | CPSG--LYSVPQQCATGLLGVLDLDCQSPSRTSFSSSDFKSTCRSEG-RKARCCVLP-VAGQDVLCTNPL--- | 66 |
| QID3 | *T. hartzianum* | CPAG--LYSNPQSCATDVLGLADLDCAVPSTTPHDGPNFQSICVANGGKRARCCVLP-VLGLGVLCQNPVGTN- | 70 |
| HFBIII | *T. reesei* | CPEGL-LYTNPLCCDLDVLGVADVDCCVVPPAKPSSCKSFGSVCASIGRK-PRCCAVP-VAGVALLCTDPIPAI- | 70 |
| Hyd4p | *F. verticillioides* | CPDGG-LIGTPQQCCSLDLVGVLSGECSSPSKTPNSAKEFQEICAASGQK-ARCCFLSEVFTLGAFCQKPVGVTA | 72 |
| FOXB_09104 | *F. oxysporum* | CPSGG-LFGNPQQCCSLNLVGVLSGDCRAPTKTPNSAKEFQAICAESGQK-ARCCGLSEILELGAFCQKPVGVSA | 72 |

The most significant hydrophobin hit found with the Marcov models was the gene FG01831.1, which was chosen for further studies. Amplification of the gene FG01831.1 from genomic *F. graminearum* DNA by PCR revealed an amplicon with an expected size and DNA sequence (Paper III). The isolated hydrophobin gene was referred to as *Gibberella zeae hyd5* gene with an EMBL accession number FN66863. 16 different *Fusarium* strains representing 10 different *Fusarium* species as well as one strain each of *Cochliobolus sativus*, *Alternaria alternata*, *Aspergillus ochraceus* and *Penicillium chrysogenum* (Paper III, Table 1) were screened for the presence of a *G. zeae hyd5* homologue in their genomes. The results obtained indicated that all five isolates of *F. graminearum* had the gene (Figure 7, some data not shown). Moreover, an amplicon was also produced with the strains of *F. culmorum*, *F. equiseti*, *F. poae* and *F. sporotrichioides* (Figure 7, some data not shown). No amplicon was produced with the *F. oxysporum* strains studied, even though a highly similar hypothetical hydrophobin FOXB_00066 is expected to be produced by *F. oxysporum* according to the protein database of NCBI. The amplicons produced from *F. culmorum* (VTT D-80148) and *F. poae* (VTT D-82182) were cloned and sequenced. The DNA sequences obtained revealed that the *G. zeae hyd5* gene shared 96% and 88% identity with the corresponding genes of *F. culmorum* and *F. poae*, respectively (introns excluded). Our study confirmed the results of Zapf *et al.* (2006) with respect to the identification of FcHyd5 hydrophobin gene from *F. culmorum*. In addition, we identified the *G. zeae hyd5* homologue in *F. poae* referred to as *Fusarium poae hyd5* gene with an EMBL accession number FN669508.

The *G. zeae hyd5* and the *F. poae hyd5* genes were chosen for expression studies in *T. reesei* (Paper III). Both genes were transformed into a strain of *T. reesei* from which the cellulose- and lactose-inducible HFBII hydrophobin gene had been deleted. The transformants with a correct expression cassette were cultivated in shake flasks containing *Trichoderma* minimal medium supplemented with 3% lactose in order to promote expression of the heterologous hydrophobins. The culture filtrates of the transformants were subjected to aqueous two-phase extraction and the extracts were further purified by RP-HPLC. The purified proteins were subjected to detailed characterization. The putative hydrophobin produced by the *G. zeae hyd5* transformant had an average molecular mass of 7 571 Da and contained 8 cysteine residues, as predicted. In addition, tryptic digestion, internal sequencing, and amino acid composition confirmed that the purified protein was the correct hydrophobin encoded by the *G. zeae hyd5* gene (Paper III). The hydrophobin was named GzHYD5. The amino acid sequence identities between GzHYD5 and several selected *Fusarium* hydrophobins are shown in Table 9. The putative hydrophobin produced by the *F. poae hyd5* transformant was determined to have an average molecular mass of 9 213 Da, which was greater than the calculated mass of the predicted mature protein (7 517.6 Da). However, the amino acid analysis performed showed a very good fit with the expected composition (Paper III, Table 2). Because the amino acid composition
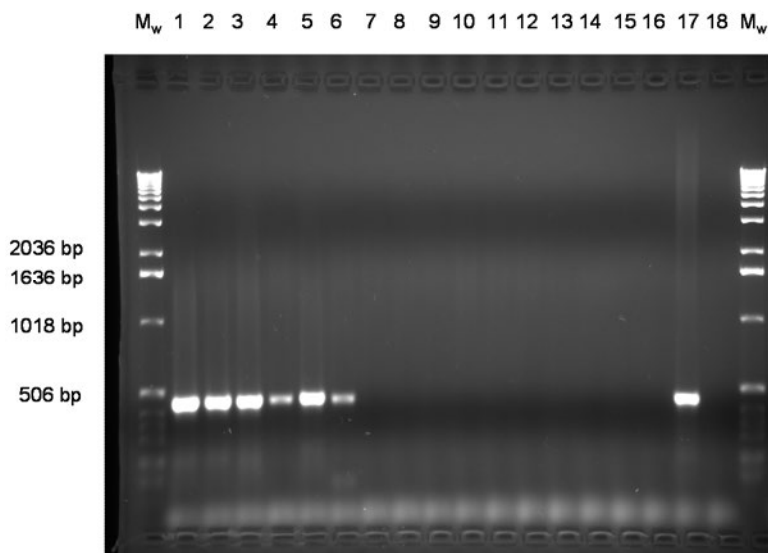
**Figure 7.** Screening of a *G. zeae hyd5* homologue in the genome of selected fungal strains by PCR (Paper III). The produced amplicons of different fungal strains are visualized in the 1% agarose gel. Lanes: M$_w$) Molecular weight marker, 1 Kb DNA Ladder (Invitrogen), 1) *F. graminearum* D-82082, 2) *F. graminearum* D-82169, 3) *F. graminearum* D-95472, 4) *F. poae* D-76038, 5) *F. sporotrichioides* D-72014, 6) *F. equiseti* D-82087. 7) *F. sambucinum* D-77056, 8) *F. solani* D-77057, 9) *F. avenaceum* D-80141, 10) *F. oxysporum* D-80134, 11) *F. oxysporum* D-98690, 12) *F. tricinctum* D-96600, 13) *Cochliobolus sativus* D-76039, 14) *Alternaria alternata* D-76024, 15) *Aspergillus ochraceus* D-00808, 16) *Penicillium chrysogenum* D-96661, 17) Positive control, *F. graminearum* D-051036 18) Negative control, H$_2$O.

was correct but the mass was too large, we concluded that the protein was glycosylated. Consistently with this, a typical N-glycosylation site, NATD, was found in the putative sequence. Additionally, the amino acid analysis also revealed that the sample contained glucosamine (unlike the GzHYD5). Glycosylation of proteins has been shown to occur in *T. reesei* (Selinheimo *et al.* 2006). Looking at the structures of the homologous proteins HFBI and HFBII from *T. reesei* (Hakanpää *et al.* 2004 and 2006), we noted that the predicted glycosylation site was positioned in a surface-exposed part of the protein on its hydrophilic side (Paper III, Picture 4). This positioning would not interfere with the surface binding function of the hydrophobin. Thus, we concluded that all data support the conclusion that the isolated protein is the correct hydrophobin encoded by the *F. poae hyd5* gene and that it is post-translationally modified by glycosylation corresponding to a mass difference of 1 695 Da observed between the determined and calculated molecular masses of the protein. The hydrophobin encoded by the *F. poae hyd5* gene was named FpHYD5. The FpHYD5 was found to be closely related to the *Fusarium* hydrophobins Hyd5p, FcHyd5p, GzHYD5 and FOXB_00066 (Figure 6

and Table 9). The yields of GzHYD5 and FpHYD5 were 1 mg/l and 18 mg/l, respectively. In laboratory fermenter cultivations of the wild-type *T. reesei* strains, maximum production of 500 mg/l and 30 mg/l of *T. reesei* HFBI and HFBII have been observed, respectively (Bailey *et al.* 2002). *Schizophyllum commune* has been reported to secrete the SC3 and SC4 hydrophobins in quantities up to 60 mg/l and 10 mg/l, respectively (Wösten *et al.* 1999, van Wetter *et al.* 2000).

**Table 9.** Amino acid sequence identities between selected mature *Fusarium* hydrophobins. Signal peptides were cleaved between residues arginine-24 and glutamine-25 analogously as was seen for GzHYD5 and FpHYD5 (Paper III).

| | | Identity (%) compared to | |
|---|---|---|---|
| **Hydrophobin** | **Species** | GzHYD5 | FpHYD5 |
| GzHYD5 | *F. graminearum* | | 86 |
| FpHYD5 | *F. poae* | 86 | |
| FcHyd5p | *F. culmorum* | 100 | 86 |
| Hyd5p | *F. verticillioides* | 90 | 89 |
| FOXB_00066 | *F. oxysporum* | 90 | 89 |

### 4.2.2 Gushing-inducing ability of hydrophobins

In order to demonstrate that hydrophobins are able to induce beer gushing, the RP-HPLC -purified hydrophobins were added into bottled beer and the bottles were shaken gently for three days before opening according to the Carlsberg gushing test (Section 3.6.2). All the class II hydrophobins studied induced beer gushing but the amounts of hydrophobins needed for gushing induction varied (Table 10). The most gushing active hydrophobins were HFBI and HFBII from *T. reesei*, GzHYD5 from *F. graminearum* and FpHYD5 from *F. poae*. An amount of 0.003 ppm of these hydrophobins in beer was able to induce vigorous gushing, whereas about 10 and 50 times higher amounts of the NGUSH hydrophobin of *Nigrospora* and the FpGUSH hydrophobin of *F. poae* were needed for gushing induction, respectively (Table 10). The class II hydrophobin FcHyd5p from *F. culmorum* has also been reported to induce beer gushing (Zapf *et al.* 2006, Lutterschmid *et al.* 2010, Stübner *et al.* 2010). An amount of ca. 0.2 ppm of purified recombinant FcHyd5p with 6x His-tag was needed to induce beer gushing (Stübner *et al.* 2010). However, Lutterschmid *et al.* (2010) observed that the gushing induction by the same amount of FpHyd5p may vary between beers of different breweries and even between different beer lots of the same brewery, indicating the presence of substances in beer that are able to inhibit gushing. In addition, the hydrophobin-like protein BGIP from *F. poae* has been shown to induce gushing (Wang *et al.* 2010). They reported that an amount of ca. 1.6 ppm of BGIP in beer induced vigorous gushing. Interestingly, the study of Zapf *et al.* (2006) and Lutterschmid *et al.* (2010) indicated that only the class II hydrophobins, not the class I hydrophobins such as FcHyd3p from *F. culmorum*, induce beer

gushing. Although it is cosidered that all filamentous fungi produce hydrophobins, only a few genera have been reported to be gushing active. This could be due to the different gushing-inducing capabilities of hydrophobins, as indicated in this study and in the studies of Zapf *et al.* (2006) and Lutterschmid *et al.* (2010).

**Table 10.** Amounts of different hydrophobins of *T. reesei*, *Nigrospora*, *F. poae* and *F. graminearum* needed to induce gushing in beer.

| Hydrophobin | Amount of beer gushed, g (n = 2) | | | | | |
|---|---|---|---|---|---|---|
| µg/0.33 l beer | *T. reesei* HFBI | *T. reesei* HFBII | *F. poae* FpGUSH | *Nigrospora* NGUSH | *F. graminearum* GzHYD5 | *F. poae* FpHYD5 |
| 0.1 | 0 | 0 | - | - | - | - |
| 1 | 10 | 12 | 0 | 0 | 106 | 34 |
| 10 | 189 | 192 | 0 | 66 | 178 | 167 |
| 50 | - | 206 | 16 | 183 | 207 | 207 |

-: not determined.

The gushing phenomenon is not only restricted to beer; other carbonated beverages such as sparkling wine, champagnes, ciders, fruit spritzers etc. may gush (Christian *et al.* 2010, personal communications). The presence of fungal hydrophobins in infected raw materials such as grapes and apples may lead to overfoaming of these products. In this study hydrophobins were demonstrated to induce overfoaming of carbonated mineral water (Papers II and III). As in the case of beer, an amount of 0.003 ppm of hydrophobins GzHYD5 and FpHYD5 in carbonated mineral water was able to induce gushing (Paper III, Table 3). Our findings are consistent with those of Stübner *et al.* (2010), who reported that as well as in beer a concentration of 0.3 ppm of FcHyd5p preparation also induced gushing in carbonated water. In addition, FcHyd5p has been observed to induce gushing in other carbonated beverages such as black currant juice, apple juice, elder juice and apple/grape juice (Lutterschmid *et al.* 2010).

The results obtained in this study and in several other recent studies (Zapf *et al.* 2006, Lutterschmid *et al.* 2010, Stübner *et al.* 2010, Wang *et al.* 2010) confirmed that hydrophobins are able to induce beer gushing. Deckers *et al.* (2011) studied the hypothesis that hydrophobins stabilize $CO_2$ bubbles by preventing the dissolution of $CO_2$. These nanobubbles contain energy (overpressure) that is released when the beverage container is opened, causing the simultaneous nucleation of many bubbles, which leads to primary gushing. Comparison of a commercial gushing and non-gushing beer using an analytical method based on dynamic light scattering (DLS) revealed the presence of particles with a diameter of ca. 100 nm in the gushing beer at atmospheric pressure (Deckers *et al.* 2011). Similar sized particles were observed when hydrophobins were added to non-gushing beers or to sparkling water, indicating that these 100 nm particles are nanobubbles stabilized by hydrophobin assemblages. Addition of ethanol to a concentration of 60%, which is known to destabilize hydrophobin structures, led to

the disappearance of the 100 nm particles in previously gushing samples, suggesting that these particles were indeed $CO_2$ bubbles stabilized by hydrophobins (Deckers *et al.* 2011).

## 4.3 Development and evaluation of ELISA for hydrophobins in barley and malt (Paper II)

Enzyme Linked ImmunoSorbent Assay (ELISA) is an immunological detection method based on the specific binding reactions between antigens and corresponding antibodies. The number of these reactions is proportional to the amount of antigens in a sample and is quantified by enzymatic color reaction. Polyclonal antibodies against the RP-HPLC -purified FpGUSH hydrophobin from *F. poae* were raised in rabbits by injecting the hydrophobin preparation into a muscle of the animal four times during the immunization period of four months. The specificities of the antibodies were tested using a Western blotting technique (Paper II). In this immunoblot analysis the polyclonal antibodies of the FpGUSH hydrophobin reacted only with its own antigen and not with the hydrophobins isolated from *T. reesei* and *Nigrospora*. This result indicated that the hydrophobins of *T. reesei* and *Nigrospora* differed from the FpGUSH hydrophobin to such an extent that they could not be detected with the same polyclonal antibodies. However, gushing of beer is considered to be most commonly caused by *Fusarium* species (Haikara 1980, Schwarz *et al.* 1996), which justifies the use of the *F. poae* hydrophobin antibodies in the ELISA for prediction of the gushing potential of barley and malt.

In addition to its own antigen, the antibodies raised against the FpGUSH hydrophobin were observed to react with other proteins present in barley and malt (data not shown). In order to decrease these unspecific reactions of the hydrophobin antibodies, a so-called competitive ELISA was developed for detection of hydrophobins in barley and malt. In competitive ELISA, antigens present in a sample compete with the standard antigens added in the assay for the binding sites of antibodies. In the developed hydrophobin ELISA, hydrophobins in barley and malt extracts compete with the FpGUSH hydrophobins attached to the wells of an immunoplate for the binding sites of polyclonal antibodies of the FpGUSH hydrophobin. A schematic picture of the principle of the developed hydrophobin ELISA is presented in Figure 8. In the case of a gushing-positive sample, the antibodies of the FpGUSH hydrophobin react with hydrophobins present in the sample instead of the FpGUSH hydrophobins attached to an immunoplate. The higher the amount of hydrophobins in a sample, the smaller is the amount of the primary *F. poae* hydrophobin antibodies, as well as the amount of the secondary antibody – enzyme conjugates, bound to the FpGUSH hydrophobins attached to an immunoplate, which leads to weaker colour development of the substrate. This means that a lower absorbance value corresponds to a higher amount of hydrophobins in a sample. In order to make interpretation of the results easier, the hydrophobin level was expressed as the mean of the inverse absorbance values (1/Abs).
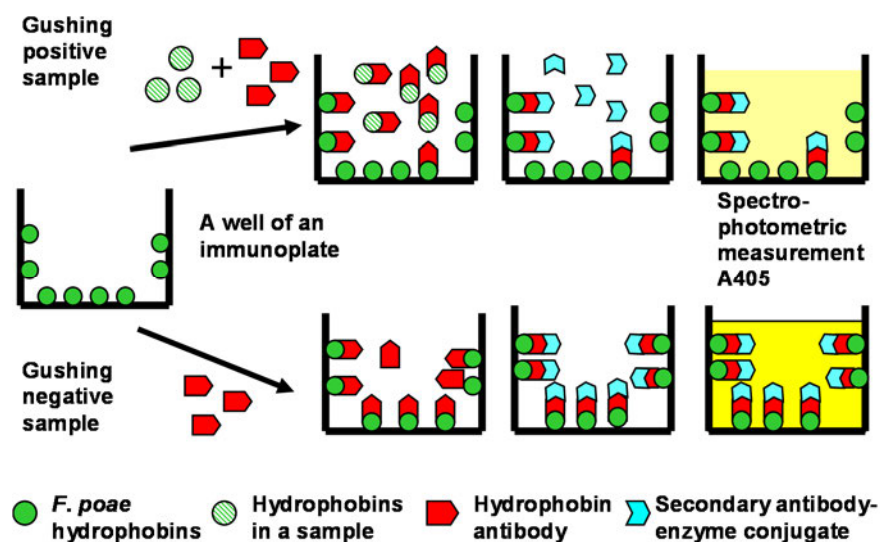
**Figure 8.** A schematic picture of the principle of the hydrophobin ELISA developed for detection of hydrophobins in barley and malt samples. A lower absorbance value (A405) corresponds to a higher amount of hydrophobins in the sample. The hydrophobin levels are expressed as the means of the inverse absorbance values (1/Abs).

Validation of the hydrophobin ELISA was performed by analyzing standards with known FpGUSH hydrophobin concentrations ranging from 5 to 200 µg/ml in an aqueous extract of a gushing-negative malt (Paper II). A linear correlation ($r^2 \approx 0.95$) was found between the logarithm of hydrophobin concentrations below 100 µg/ml and the results of the hydrophobin ELISA (A405 nm) (Paper II, Figure 3). The hydrophobin ELISA could not distinguish between hydrophobin concentrations above 100 µg/ml, which means that the assay can be used to estimate hydrophobin concentrations below 1000 µg/g of barley or malt without sample dilution.

The hydrophobin ELISA was also validated by analysing the malt samples made from artificially inoculated barleys. Barley samples were inoculated ($10^7$ spores/kg barley) with 9 different *Fusarium* strains belonging to the species of *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. sporotrichioides* and *F. tricictum* and malted at 1 kg scale. All the malt samples gave a positive reaction in the hydrophobin ELISA, indicating that the *Fusarium* strains studied produce hydrophobins during malting and that these hydrophobins can be detected with the hydrophobin ELISA developed (Table 11 and Sarlin *et al.* 2009). In addition, all the malt samples were gushing positive when tested with the Carlsberg gushing test (Table 11 and Sarlin *et al.* 2009). The result indicated that the hydrophobin ELISA could be used for gushing prediction.

When the hydrophobin level of the malt samples and the amount of overfoamed beer induced by the corresponding malt extracts in the gushing test were

compared, no clear correlation was found (Table 11). For example, the hydrophobin levels of the malts infected with *F. cereals*, *F. culmorum* and *F. sporotrichioides* were almost equal, whereas clear differences were observed in the results of the gushing test between these malts. This observation is in accordance with the previous conclusion that the gushing-inducing capability of hydrophobins may differ. However, the Carlsberg gushing test cannot be regarded as precisely quantitative, which makes comparison between the samples difficult.

**Table 11.** Gushing potentials and hydrophobin levels of malts produced from barleys inoculated prior to malting with different *Fusarium* species. Standard deviations (STDEV) are also shown (Sarlin *et al.* 2009).

| Inoculation | Strain code | Gushing potential g ±STDEV | Hydrophobin level 1/Abs. ±STDEV |
|---|---|---|---|
| Non-inoculated | | 0 ±0 | 1.2 ±0.1 |
| *F. avenaceum* | VTT D-80141 | 116 ±7 | 6.1 ±0.7 |
| *F. cerealis* | VTT D-96601 | 118 ±10 | 5.3 ±0.9 |
| *F. culmorum* | VTT D-80148 | 69 ±24 | 5.3 ±0.7 |
| *F. graminearum* | VTT D-82082 | 95 ±17 | 3.1 ±0.2 |
| *F. graminearum* | VTT D-95470 | 112 ±6 | 5.3 ±0.3 |
| *F. langsethiae* | VTT D-03931 | 46 ±13 | 2.8 ±0.1 |
| *F. poae* | VTT D-82182 | 84 ±17 | 5.7 ±0.3 |
| *F. sporotrichioides* | VTT D-72014 | 27 ±15 | 5.2 ±0.6 |
| *F. tricinctum* | VTT D-96600 | 31 ±17 | 6.5 ±0.3 |

## 4.4 Application of the developed hydrophobin ELISA to the estimation of hydrophobin levels in barley and malt (Paper II)

### 4.4.1 Hydrophobin level in barley and malt compared to gushing potential of malt

The hydrophobin ELISA was used to assess the levels of *Fusarium* hydrophobins in barleys infected both naturally and artificially with *Fusarium* fungi in the field and in the corresponding malts. Hydrophobin concentration in a sample was estimated using a standard curve prepared by analysing standards with known FpGUSH hydrophobin concentrations in an aqueous extract of a gushing-negative malt. The results of the hydrophobin ELISA were compared to the results of the gushing test. A connection was found between the hydrophobin level and the gushing potential of malt; the risk of gushing was observed to be increased if the 1/Abs value of the malt in the hydrophobin ELISA exceeded 1.7, corresponding to a hydrophobin concentration of ca. 250 µg/g malt (Figure 9). All the malt samples with 1/Abs values of 2.5 or higher, corresponding to hydrophobin concentrations of ca. 500 µg/g

malt, repeatedly induced vigorous gushing in the gushing test. The results suggested that a hydrophobin analysis could be used for the prediction of gushing risk in malt.
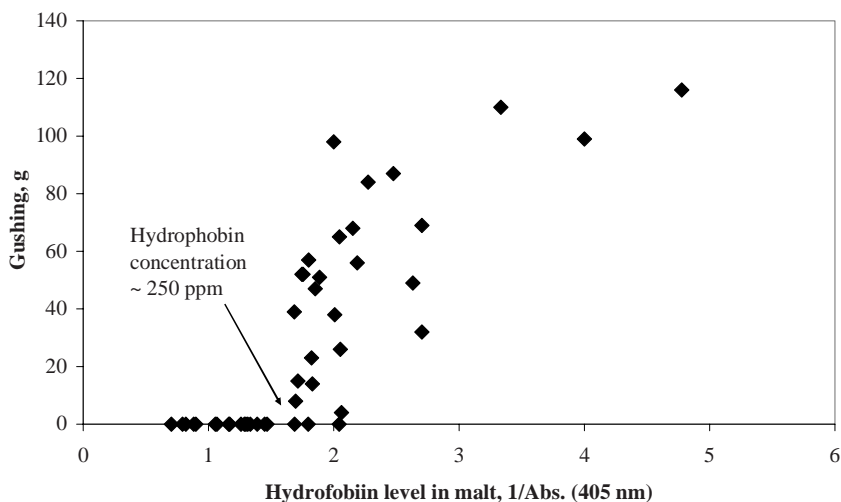


**Figure 9.** Comparison of gushing potentials and hydrophobin levels (1/Abs.) of malt samples made from barleys infected both naturally and artificially with *Fusarium* fungi (n = 44).

When the results of the hydrophobin ELISA of the barley samples were compared to the gushing potential of the corresponding malts, no clear correlation was found except in the cases where artificially or otherwise heavily *Fusarium*-infected barleys were studied (Table 12). This was probably due to the production of hydrophobins during the malting process, which could be concluded from the lower hydrophobin levels detected in the barleys compared to those of the corresponding malts (Table 12). Previous studies also support this conclusion because fungi, especially fusaria, have been shown to proliferate and to produce gushing factors in malting conditions (Haikara 1983, Munar and Sebree 1997, Schwarz *et al.* 1996).

**Table 12.** Hydrophobin levels in naturally (N) and artificially *Fusarium*-infected barleys and in the corresponding malts detected with the hydrophobin ELISA. Gushing potentials of the malts are also shown.

| Sample pair | Hydrophobin level | | Gushing potential of malt |
|---|---|---|---|
| | Barley | Malt | |
| | 1/Abs. | 1/Abs. | g |
| N1 | 1.1 | 1.4 | 0 |
| N2 | 1.1 | 1.3 | 0 |
| N3 | 1.1 | 2.0 | 1 |
| N4 | 1.2 | 1.5 | 0 |
| N5 | 1.2 | 2.5 | 30 |
| N6 | 1.2 | 2.6 | 110 |
| N7 | 1.4 | 2.5 | 20 |
| N8 | 1.4 | 3.1 | 7 |
| N9 | 1.6 | 3.1 | 100 |
| N10 | 2.0 | 5.0 | 110 |
| N11 | 2.1 | 3.0 | 2 |
| N12 | 2.3 | 2.8 | 1 |
| N13 | 3.5 | 7.2 | 73 |
| Non-inoculated 1 | 1.0 | 1.4 | 0 |
| Non-inoculated 2 | 1.0 | 2.2 | 1 |
| *F. poae* inoculated 1 | 1.1 | 2.3 | 13 |
| *F. poae* inoculated 2 | 1.2 | 3.2 | 21 |
| *F. culmorum* inoculated 1 | 3.2 | 4.9 | 92 |
| *F. culmorum* inoculated 2 | 3.6 | 5.6 | 62 |
| *F. graminearum* inoculated 1 | 3.6 | 7.9 | 62 |
| *F. graminearum* inoculated 2 | 3.8 | 6.7 | 76 |

### 4.4.2 Hydrophobin level and gushing potential of malt compared to the DON content of malt

Schwarz *et al.* (1996) found a strong correlation between the content of the *Fusarium* mycotoxin deoxynivalenol (DON) and the gushing potential of malts produced from North American barley samples, indicating that the DON analysis could be used for prediction of the gushing risk in malt. In this study the DON content of malt samples was also analysed and compared to the hydrophobin levels and to the gushing potentials detected from the samples. As can be seen from Figure 10, no correlation was found between the results of the hydrophobin ELISA and the DON contents of the malts. This observation implies that the accumulation of hydrophobin cannot be predicted from the DON content of the malt sample. Munar and Sebree (1997) also stated that the formation of DON and the gushing factors might be independent of each other. Moreover, we did not

observe a connection between the DON contents and gushing potentials of malts (Paper II, Figure 5). Most of the samples analysed in this study were grown in Finland, asopposed to the North American malt samples studied by Schwarz *et al.* (1996). The DON contents of Finnish barleys have been reported to be low (Eskola *et al.* 2001, Laitila and Haikara 2000). For example in the seasons of 1995–1999, all Finnish commercial malting barley samples studied contained less than 96 $\mu$g DON/kg barley (Laitila and Haikara 2000). The most common *Fusarium* species in Finnish grain has been reported to be *F.* avenaceum, which does not produce DON (Eskola *et al.* 2001, Yli-Mattila *et al.* 2002). By contrast, a strong DON producer and gushing inducer, *F. graminearum*, has been reported to be the predominating *Fusarium* species in North American barley (Schwarz *et al.* 1996, Steffenson 1998).
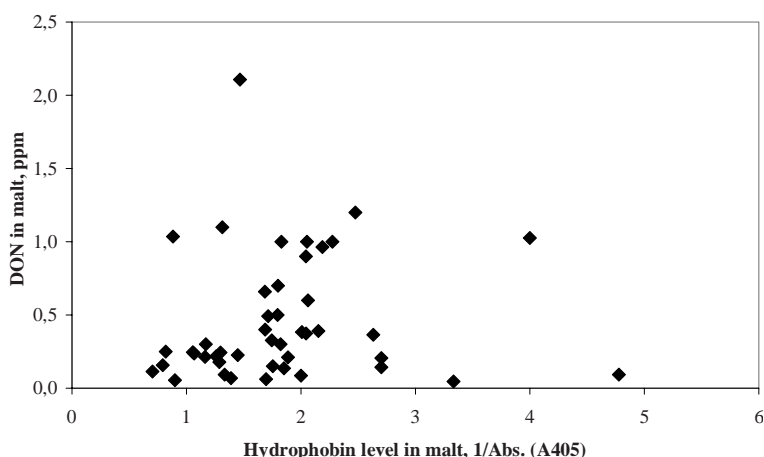


**Figure 10.** Hydrophobin levels (1/Abs.) of the malt samples analysed by the hydrophobin ELISA compared to DON contents of the malts (n = 44).

## 4.5 Hydrophobins in the barley-to-beer chain (Paper IV)

In order to be able to induce beer gushing hydrophobins must pass through the malting and brewing processes and enter the finished beer. This study surveyed the occurrence and fate of hydrophobins at different stages of the beer production chain in pilot scale by analysing the hydrophobin levels of the samples collected throughout the barley-to-beer chain with the hydrophobin ELISA (Paper IV).

### 4.5.1 Production of hydrophobins by fungi in the field

Fungi, especially *Fusarium* species, are known to be able to produce gushing factors during the growing period of barley (Aastrup 2003, Munar and Sebree 1997). The results of the hydrophobin ELISA of the barley head samples collected revealed

that hydrophobins were produced during barley grain development in the field (Paper IV). Hydrophobins were present in the *F. culmorum* -infected heads three weeks after inoculation and the formation of hydrophobins continued throughout the growing period when wet weather conditions favoured the proliferation of *Fusarium* species (Paper IV, Figure 1).

### 4.5.2 Production of hydrophobins by fungi during malting

*Fusarium* fungi are able to proliferate and to produce gushing factors during the malting process, as was shown in this study as well as in the studies of Munar and Sebree (1997) and Schwarz *et al.* (1996). The results of the hydrophobin ELISA of the non-inoculated and the *Fusarium*-inoculated barley samples collected during the lab-scale malting process revealed that hydrophobins were produced during malting, especially during the steeping and germination steps (Figure 11). Lower hydrophobin levels were detected in the final malt after removal of the rootlets. A comparison between the estimated amounts of hydrophobins detected in barley and in malt revealed that over tenfold higher amounts of hydrophobins could be found in malts compared to those in the corresponding barleys (data not shown). The highest hydrophobin levels (> 6.0 1/Abs.) were detected in the malts produced from barleys inoculated with *F. culmorum* and *F. graminearum*, compared to the hydrophobin levels of the malts produced from non-inoculated and *F. poae* -inoculated barleys (Figure 11). The former malts also induced the most vigorous gushing in the gushing test, as shown in Figure 11.
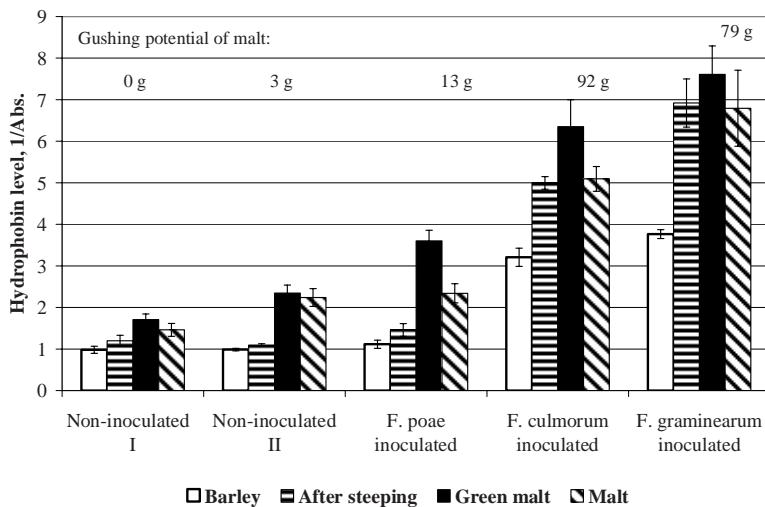


**Figure 11.** Changes in hydrophobin levels of the non-inoculated and *Fusarium*-inoculated barley samples taken at different stages of the laboratory scale malting process. Hydrophobin levels were analysed using the hydrophobin ELISA (n = 4). Standard deviation bars and the gushing potentials of malts are included.

Relatively high initial hydrophobin levels were detected in barleys inoculated with *F. culmorum* and *F. graminearum*, which made them easily distinguishable from the non-inoculated barley samples based on the results of the hydrophobin ELISA (Figure 11). However, the formation of hydrophobins during malting complicates the use of the hydrophobin analysis for prediction of the gushing potential directly from barley with a low initial hydrophobin level, as in the case of the non-inoculated II and *F. poae* -inoculated barley samples resulting in gushing positive malts as shown in Figure 11. In conclusion, the developed hydrophobin ELISA is suitable for prediction of gushing risk from malt but not from barley.

   This study revealed that the hydrophobin production activity of fungi can decrease as a result of long-term storage of barley (Table 13). This was true in the case of non-inoculated barleys, although no reduction in hydrophobin production was detected during malting of the *Fusarium*-inoculated barleys despite the prolonged storage time (Table 13). These results were in accordance with the gushing potentials of the malt samples made from barleys stored for up to 17 months; the gushing potentials of the malt samples made from the non-inoculated barleys decreased during barley storage, whereas no reduction was observed in the malt samples made from the heavily *Fusarium*-infected barleys (Table 13 and Figure 5, page 45).

**Table 13.** Effect of the storage time of barley on the hydrophobin level and on the gushing potential of the corresponding malt. Standard deviations (STDEV) are included.

| Sample | Storage of barley | Hydrophobin level | Gushing potential |
|---|---|---|---|
|  | months | 1/Abs. ±STDEV | g ±STDEV |
| Non-inoculated barley I |  | 0.7 ±0.0 | nd |
| Malt Ia | 5 | 1.5 ±0.0 | 0 ±0 |
| Malt Ib | 9 | 1.1 ±0.0 | 0 ±0 |
| Non-inoculated barley II |  | 0.9 ±0.0 | nd |
| Malt IIa | 6 | 4.4 ±0.3 | 49 ±11 |
| Malt IIb | 12 | 2.8 ±0.5 | 3 ±3 |
| Malt IIc | 17 | 2.0 ±0.1 | 0±0 |
| *F. culmorum* -inoculated barley |  | 2.8 ±0.6 | nd |
| Malt *F. culmorum* a | 6 | 3.7 ±0.1 | 101 ±19 |
| Malt *F. culmorum* b | 12 | 4.7 ±0.3 | 92 ±5 |

### 4.5.3   Fate of hydrophobins in the brewing process

In order to study the fate of hydrophobins in the brewing process, gushing positive and negative malt samples were processed first in laboratory scale and then in pilot scale, and analysed for hydrophobin levels in samples collected throughout the processes. In addition, pure hydrophobins were used in the studies of wort boiling and beer filtration (Paper IV).

The results of the hydrophobin ELISA indicated that hydrophobins were partly extracted into the wort during mashing, although hydrophobins were also detected in spent grains and cold break (Paper IV, Figure 3). When the estimated extractible hydrophobin content of malt grist was calculated and compared with the hydrophobin content of the corresponding wort (Table 14), a substantial loss of hydrophobins was observed to occur during mashing; at most 20% of the hydrophobins present in malt grist was found in wort. In this study we mashed the malt samples in laboratory scale according to one standard mashing procedure. Changes in the mashing procedure, such as in a proteolytic hold or in agitation speed, could affect the final hydrophobin levels in the wort. Moreover, as reported by Christian *et al.* (2009b) the temperature during the mashing process can have a significant effect on the gushing potential of the wort produced.

**Table 14.** Hydrophobin content of malt grist and of the corresponding wort estimated using the hydrophobin ELISA. One gushing-negative and one gushing-positive malt were mashed in laboratory scale. Gushing-positive malt was mashed in duplicate.

| Sample | Hydrophobins, $\mu$g | | Hydrophobins, % |
| --- | --- | --- | --- |
| | in malt grist | in wort | in wort |
| Gushing negative malt | 1 500 | 200 | 15 |
| Gushing positive malt 1 | 32 900 | 4 200 | 13 |
| Gushing positive malt 2 | 29 800 | 6 100 | 20 |

Some of the proteins are precipitated or inactivated during wort boiling. This study showed that hydrophobins were partly precipitated or inactivated during this stage (Paper IV, Table II). Boiling alone could not destroy or inactivate hydrophobins, because the gushing-inducing potential of the hydrophobin boiled in water was found to be comparable to that of the unboiled hydrophobin (Paper IV, Table II). The results indicated that wort contains substances which interact with hydrophobins in wort boiling, causing a reduction in the gushing activity of hydrophobins. Our results are consistent with the results of Lutterschmid *et al.* (2010), who reported that boiling of FcHyd5p (a hydrophobin of *F. culmorum*) in synthetic wort containing only acid and sugar components of beer resulted in an overfoaming volume reduction of 41% compared to the overfoaming volume measured with the unboild FcHyd5p. No reduction in gushing volume was observed after boiling the FcHyd5p hydrophobin in water. The authors suggested that the reduction of gushing potential by boiling the hydrophobin in synthetic wort could be caused by glycosylation of the protein during heat treatment in the presence of wort sugars. This could also occur during wort boiling in the brewing process. In addition, Lutterschmid *et al.* (2010) observed that addition of common hop compounds such as hop oils, especially linalool, as well as modified iso-α-acids to beers treated with FcHyd5p reduced gushing volumes. The effects observed could be caused by the foam negative properties of the hop oils. Interestingly, addition of the same hop products to hydrophobin-treated carbonated water resulted in an increase of the gushing

volume. These findings suggest that none of the studied hop products are capable of inhibiting gushing on their own. Other substances from beer may be needed to provide gushing inhibiting properties.

The results of the filtration studies with pure hydrophobins in water suggested that hydrophobins were partly removed by filtration (Paper IV). Our results are consistent with the results of Wang *et al.* (2010), who reported that some of the BGIP hydrophobins isolated from *F. poae* were removed by filtration with a 0.45 µm nitrocellulose filter. Based on the properties of hydrophobins, it could be assumed that hydrophobins adhered to the filter sheets or that they form aggregates large enough to be retained by the filters. However, the level of reduction was dependent on the initial hydrophobin concentration of the sample; with a hydrophobin concentration of 15 µg/ml, 60% of the hydrophobins was lost during the filtration, compared to a loss of only 15% with a hydrophobin concentration of 5 µg/ml.

The study of hydrophobin levels in the samples of gushing-negative and -positive malts collected throughout the pilot scale brewing process revealed that the beer brewed from the gushing-positive malt contained a higher amount of hydrophobins than the beer brewed from the gushing-negative malt (Paper IV, Figure 4). The former beer overfoamed when shaken according to the gushing test, indicating that a hydrophobin level high enough to induce gushing of beer was first extracted from the malt and then survived the brewing process. As indicated in the mashing studies, some of the hydrophobins were removed with spent grains and cold break (wort boiling), but also with surplus yeast (Paper IV, Figure 4). In addition, the beer filtration step reduced the hydrophobin levels (Paper IV, Figure 4). The changes in the relative levels of hydrophobins during brewing were calculated on the basis of the approximation of the total hydrophobin contents of the gushing-positive malt and the corresponding brewing liquors (Paper IV, Figure 5). The calculation revealed that most of the hydrophobins originating from the gushing-positive malt were removed with the spent grains (up to 70%). In addition, wort boiling, fermentation and beer filtration steps reduced the hydrophobin content to such an extent that only approximately 10% of the original hydrophobin content of the gushing-positive malt was present in the finished beer.

## 4.6 Prevention of hydrophobin production during malting (Paper V)

The use of biological control with well-characterized, antagonistic microbes such as cereal-derived lactic acid bacteria, yeasts and yeast-like fungi in malting has been reported to restrict the growth of *Fusarium* fungi (Haikara *et al.* 1993, Haikara and Laitila 1995, Boivin and Malanda 1997, Laitila *et al.* 2002 and 2011). Several biocontrol applications in malting have been developed to commercial scale. Laitila (2007) reported the antifungal potentials of several yeasts isolated from industrial maltings, *Pichia anomala* being the most effective species against *Fusarium* fungi. *P. anomala* occurs naturally in cereals and it has been used e.g. for biopreservation of moist feed grains (Druvefors *et al.* 2002, Druvefors 2004).

The species is classified as safe (biosafety level 1). This study revealed that *P. anomala* could suppress hydrophobin production during malting. *P.* anomala VTT C-04565 (C565) was added as a biocontrol agent in laboratory scale malting with naturally *Fusarium*-infected barley exhibiting gushing potential. *P.* anomala C565 restricted *Fusarium* growth and hydrophobin production during malting to such a degree that the subsequent malt did not induce beer gushing in the gushing test (Paper V, Figure 3 and Table 2). Antifungal action is often due to several mechanisms which are mainly poorly understood (Laitila 2007). Competition for nutrients and space has been regarded as the main mode of action. We concluded that as a fast growing organism, P. anomala C565 most probably competed for space with fusaria. In addition, ethyl acetate was detected in the malting process containing *P. anomala* C565. Ethyl acetate has been suggested to be one component of the antifungal action of *P. anomala* (Druvefors *et al.* 2002). Although fusaria are part of the normal malting process, to our knowledge gushing of beer occurs only rarely. Our results indicated that the indigenous yeast community of industrial malting processes may contribute to the restriction of *Fusarium* growth and hydrophobin production in malting, thus decreasing the gushing potential of malt.

*Fusarium* species are known to be sensitive to heat (Olkku *et al.* 2000, Kottaballi *et al.* 2003, Briggs 2004). Olkku *et al.* 2000 reported that heat treatment of barley (60–100 °C for 0.5–3 s) prior to malting notably decreased the *Fusarium* contamination of final malt without influencing grain germination. Moreover, it significantly reduced mycotoxin production during the malting process and alleviated the gushing tendency (Olkku *et al.* 2000). We also studied the effect of heat treatment (77 °C for 5–10 s) of three different barleys prior to a laboratory scale malting on the *Fusarium* contamination and on the hydrophobin level of the final malt. Our results confirmed the previous results of Olkku *et al.* (2000) according to which the activity of *Fusarium* fungi could be effectively reduced by exposing the grains to heat prior to the malting process (Laitila *et al.* 2008). We observed that heat treatment of barley reduced *Fusarium* growth and hydrophobin production during malting (Table 15 and Laitila *et al.* 2008). Moreover, heat treatment tended to decrease the gushing potential of final malts (Laitila *et al.* 2008).

**Table 15.** Effect of heat treatment of barley prior to malting on *Fusarium* contamination (analysed on CZID plates) and on the hydrophobin level of the final malt (Laitila *et al.* 2008).

| Sample | Heat treatment at 77°C | *Fusarium* contamination in malt, % | | Hydrophobin level in malt, 1/Abs. ±STDEV | |
|---|---|---|---|---|---|
| | | non-treated | heat treated | non-treated | heat treated |
| Barley 1 | 5 s | 59 | 9 | 1.0 ±0.0 | 0.9 ±0.0 |
| Barley 2 | 10 s | 91 | 9 | 2.3 ±0.1 | 1.5 ±0.1 |
| Barley 3 | 10 s | 100 | 14 | 3.9 ±0.3 | 1.2 ±0.1 |

# 5. Conclusions

Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously overfoams out from its container immediately on opening. Gushing has a marked negative effect on the overall image of beer. Every year, labour-intensive and costly methods are used to evaluate the gushing risk of raw materials, and yet these methods are poor predictors of gushing tendency in practice. This study revealed for the first time that small fungal proteins, hydrophobins, are one of the gushing factors inducing beer gushing. Fungi were observed to produce hydrophobins during the growing period of barley in the field and during malting. Hydrophobins were demonstrated to be able to pass through the brewing process, ending up in the final beer. With the hydrophobin ELISA developed the gushing risk in malt could be predicted. This test provides a faster and more reliable means for gushing prediction compared to the current practices because it is based on determination of the actual gushing factors, hydrophobins. In addition, this study showed that a selected antagonistic starter culture suppressed hydrophobin production in malting and thus prevented beer gushing.

The main findings of this thesis were as follows:

The *Fusarium culmorum* -, *F. graminearum* - and *F. poae* -strains studied had severe impacts on barley and malt quality, especially on the gushing potential, when artificial contamination was performed in Finnish field conditions. The extent of the effects was species-dependent, *F. graminearum* having more pronounced effects on the quality of barley and malt than *F. culmorum* and especially *F. poae*.

Hydrophobins were isolated from gushing-active fungi belonging to the species *F. graminearum*, *F. poae* and *Nigrospora* sp. All the hydrophobins studied induced beer gushing even at low concentrations (ppb). However, the amount needed for gushing induction varied between the hydrophobins. Our results indicated that hydrophobins are indeed one of the gushing factors produced by fungi. To our knowledge, this was the first study in which fully characterized and purified proteins were shown to induce beer gushing.

Searching of hydrophobins with the profile HMMs of the different hydrophobin classes from the *F. graminearum* genome database of predicted proteins revealed five uncharacterized genes showing similarity with known hydrophobin sequences. One of them, referred to as *Gibberella zeae hyd5* gene (EMBL accession number FN668637), was chosen for future studies. Screening of several fungal species for

the presence of a *G. zeae hyd5* homologue in their genomes suggested that in addition to *F. graminearum* the strains of *F. culmorum*, *F. equiseti*, *F. poae* and *F. sporotrichioides* had the same gene. Comparison of translated amino acid sequences without predicted signal peptides indicated 100 % and 88 % identity between the protein encoded by the *G. zeae hyd5* and the corresponding proteins of *F. culmorum* and *F. poae*, respectively. The *G. zeae hyd5* and the *G. zeae hyd5* homologue in *F. poae*, referred to as the *Fusarium poae hyd5* gene (EMBL accession number FN669508), were transformed and expressed in *T. reesei*. The hydrophobin produced by the *G. zeae hyd5* transformant had an average molecular mass of 7 571 Da and contained 8 cysteine residues, as predicted. In addition, tryptic digestion, internal sequencing and amino acid composition confirmed that the purified protein, named GzHYD5, was the correct hydrophobin encoded by the *G. zeae hyd5* gene. The hydrophobin produced by the *F. poae hyd5* transformant was determined to have an average molecular mass of 9 213 Da, which was greater than the calculated mass of the predicted mature protein (7517.6 Da). However, all the data obtained supported the conclusion that the isolated protein, named FpHYD5, was the correct hydrophobin encoded by the *F. poae hyd5* gene and that it was post-translationally modified by glycosylation. Both GzHYD5 and FpHYD5 induced vigorous gushing in beer as well as in carbonated mineral water.

An immunological detection method, hydrophobin ELISA, was developed for determination of hydrophobin levels in malt. A connection was found between the hydrophobin level and the gushing potential of malt, suggesting that the hydrophobin ELISA can be used for predicting the gushing risk in malt.

The results of the hydrophobin ELISA revealed that hydrophobins are produced by fungi during barley grain development in the field and especially during malting. Due to the decreased viability of *Fusarium* spp., the capability of fungi to produce hydrophobins was reduced during prolonged storage of barley. Hydrophobins were extracted during mashing and a portion survived the brewing process, ending up in the final beer where they induced gushing when present in sufficiently high levels. The estimation of hydrophobin content, based on the hydrophobin ELISA results of the samples collected during the pilot-scale brewing study, showed that only about 10–20% of the hydrophobin present in the gushing-positive malt was found in the final beer.

The use of *P.* anomala C565 as a biocontrol agent in laboratory scale malting suppressed *Fusarium* growth and hydrophobin production during malting to such a degree that the gushing potential of the final malt was reduced.

# 6.  Future outlook

The hydrophobin ELISA developed is based on polyclonal antibodies, which presents a challenge to develop an assay for wider use. Polyclonal antibodies rely on cross-reactivities that are difficult to control and explain, and therefore long-term reproducibility of the assay is unsure. As such the current test is mainly applicable as a research tool and not for routine standardized analysis carried out in malting houses or breweries. Monoclonal antibodies are preferred in diagnostics. In the future, monoclonal antibodies against *Fusarium* hydrophobins should be produced in order to be able to develop a commercial test for gushing prediction. Monoclonal antibodies can be used as such but also cloned to recombinant antibody fragments and produced in bacteria in a cost efficient way, which is important when large numbers of samples are to be analysed. In addition, the multiplexed miniaturised assay formats based on monoclonal antibodies can further reduce the costs and speed up the analysis. However, the high specificity of monoclonal antibodies may require production of antibodies against several gushing-relevant hydrophobins. This study focused on *Fusarium* hydrophobins. The significance of other fungal hydrophobins in gushing induction needs to be clarified. In addition, the existence of other gushing factors in addition to hydrophobins should be investigated.

Sequence data on hydrophobin-encoding genes are essential for the use of modern molecular biological techniques. With these techniques it will be possible to study the expression of hydrophobin genes. For example VTT has developed a novel transcriptional profiling technique called TRAC for the quantitative analysis of messenger RNAs which precede the formation of proteins in cells (Rautio *et al.* 2006, Satokari *et al.* 2005, Söderlund *et al.* 2001). In future, by studying the hydrophobin gene expression in the field and during malting, new information on induction and regulation of hydrophobin production in fungi could be gained, thus improving the prediction and prevention of hydrophobin formation.

This study revealed that the amount of hydrophobins migrating in the barley-to-beer chain can increase during malting but decrease during brewing. Industrial malting and brewing processes vary between different malt houses and breweries, which may significantly influence the production and the fate of hydrophobins in this chain. The sensitivity to beer gushing is indeed known to vary between breweries. Further large scale studies are required to investigate the exact effects

of industrial malting and brewing processes on the hydrophobin content migrating in the beer production chain.

As reported by Laible and Geiger (2003), fungi can also produce gushing-inhibiting substances, such as polar lipids, in barley and malt. In addition, beer may contain compounds such as hop oils which can contribute to gushing inhibition (Amaha and Kitabatake 1981, Hanke *et al.* 2009, Lutterschmid *et al.* 2010). Furthermore, other gushing factors in addition to hydrophobins may also exist. Full knowledge of both gushing-inducing and -inhibiting substances would enable maltsters and brewers to make a correct prediction of the gushing risk in beer.

The gushing phenomenon is not only restricted to beer, because other carbonated beverages such as sparkling wines, ciders, fruit spritzers etc. may also gush (Christian *et al.* 2010). Lutterschmid *et al.* (2010) demonstrated that addition of the hydrophobin FpHyd5p to several carbonated beverages resulted in gushing. More research is needed to reveal the role of hydrophobins in overfoaming of these other carbonated beverages.

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# Effects of three *Fusarium* species on the quality of barley and malt

# Effects of Three *Fusarium* Species on the Quality of Barley and Malt

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## ABSTRACT

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In this study, field-grown barley was artificially infected during the heading stage with three *Fusarium* species, *F. culmorum*, *F. graminearum*, and *F. poae*. The objective was to investigate possible differences among *Fusarium* species in terms of how severely they affect the quality of barley under Finnish field conditions. Moreover, we studied the effects of heavy infection on corresponding malt quality. Field trials were carried out with two different barley cultivars at two experimental farms. Spike samples collected during the growing period were analyzed for *Fusarium* infection and moisture content. Total precipitation and temperature data were collected daily. In addition, the harvested barley samples were analyzed for overall microbial flora and for mycotoxins. The infection rate and the amount of mycotoxins produced in the barley samples differed among species. *F. graminearum* was found to have the most negative effects on barley quality in terms of the studied parameters. Samples were malted in laboratory scale. All three *Fusarium* species increased the gushing potential of malt. Heavy fungal infection increased the enzyme activities in malt resulting in darker wort color and increased soluble nitrogen and free amino nitrogen content. However, high *Fusarium* contamination reduced lautering performance.

Keywords: Artificial inoculation, Field trials, Gushing, Malting, Mycotoxins

## RESUMEN

### Efectos de Tres Especies de *Fusarium* en la Calidad de Cebada y Malta

En este estudio, cebada cultivada en campos fue artificialmente infectada durante la etapa de espiga con tres especies de *Fusarium, F. culmorum, F. graminearum, y F. poae*. El objetivo era investigar diferencias posibles entre las especies de *Fusarium* en términos de que severamente afectan la calidad de cebada bajo condiciones de campo Finlandesas. Mas a fondo, estudiamos los efectos de infección pesada en la calidad correspondiente de malta. Pruebas de campo fueron realizadas con dos diversas variedades de cebada en dos granjas experimentales. Muestras de espiguillas recogidas durante el período de desarrollo fueron analizadas de infección de *Fusarium* y contenido de humedad. Datos de precipitación total y temperatura fueron tomados diario. Adicionalmente, las muestras cosechadas de cebada fueron analizadas de flora microbiana y micotoxinas totales. El índice de infección y la cantidad de micotoxinas producidas en las muestras de cebada diferenciaron entre especies. *F. graminearum* se descubrió tener los efectos más negativos en la calidad de cebada en términos de los parámetros estudiados. Las muestras fueron malteadas en escala laboratorio. Las tres especies de *Fusarium* aumentaron el potencial de gushing de la malta. La infección pesad de hongos aumentó las actividades de enzimas en la malta dando por resultado un color más oscuro de mosto y aumentó el contenido de nitrógeno soluble y nitrógeno amino. Sin embargo, la alta contaminación de *Fusarium* redujo el funcionamiento del proceso de lauter.

Palabras claves: Inoculación artificial, Gushing, Malteado, Micotoxinas, Pruebas de campo

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The level and diversity of barley mycoflora vary depending on a number of factors that includes climate, cultivation conditions in the field, agricultural practices, resistance of the barley cultivars, lodging, fertilization, soil, and storage conditions after harvesting (19). Weather conditions, particularly the amount of rainfall between emergence of the spikes and harvest, and geographic location determine the abundance of *Fusarium* contamination and the proportions of different *Fusarium* species present on barley (24). *Fusarium graminearum* (teleomorph *Gibberella zeae*) is the most common Fusarium head blight (FHB) pathogen of cereals worldwide as well as a well documented mycotoxin and gushing producer (19,23,27). *F. culmorum*, another common FHB pathogen, is the most harmful species in temperate zones such as northern Europe (8,24). Many studies have indicated that fungi and their metabolites greatly influence barley, malt, and beer quality (6,8,26,29). Production of mycotoxins (i.e., trichothecenes and zearalenone) and gushing factors are the most well known negative effects of *Fusarium* fungi (7,6,18,25,27,29).

It is known that *Fusarium* species differ in terms of how severely they affect barley and malt quality. Haikara (8) observed differences between the ability of *F. avenaceum* and *F. culmorum* isolates to affect germination, mycotoxin contents, and gushing potential of malt. Schwarz (29) reported that *F. graminearum* caused greater losses in barley and malt quality compared with *F. poae* in greenhouse experiments. They showed that, in particular, *F. graminearum* increased mycotoxin contents in barley and decreased germination and kernel plumpness. In Finnish cereals, the amount of mycotoxins produced by fusaria is naturally low, apparently because of the low prevalence of the toxigenic *Fusarium* species (3,11). Moreover, low quality barley that may induce gushing is very rare in Finland and is generally associated with exceptional weather conditions.

In this study, results are presented on the effects of three different *Fusarium* species on the quality of barley and corresponding malts after artificial inoculation of barley in the field. Particular interest was focused on the differences between the infection severity of the *Fusarium* isolates originating from different countries and subjected to Finnish climatic conditions.

## EXPERIMENTAL

### Field Trials

During the summer of 1998, field trials were carried out at two experimental farms, the Experimental Farm of K-group in Hauho (HA) and the Jokioinen Experimental Farm of MTT Agrifood Research Finland in Jokioinen (JO). In mid-May, two different two-row malting barley cultivars (A and B) were seeded in plots of 10 m². Barley was artificially inoculated with *F. culmorum* VTT D-80148, *F. graminearum* (teleomorph *Gibberella zeae*) VTT D-95470, and *F. poae* VTT D-82182 during the heading stage in mid-July. The *F. culmorum* isolate was from barley grown in Finland and was known to produce gushing factors and mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEN) (8,12). The *F. graminearum* isolate was from corn from the United States where this species has been reported to produce DON and cause FHB and gushing of beer (16,27). The *F. poae*

isolate was from oat from Germany. The fungi were cultivated in carboxymethylcellulose broth (2) until a spore concentration of $10^6$–$10^7$/mL was attained. Three sets of three randomized replicate plots were sprayed with 2,000 mL of each *Fusarium* culture using a $CO_2$ sprayer, whereas the control plots were not treated. Ear samples were manually collected four times between spike emergence and harvest for determination of the level of *Fusarium* contamination and moisture content. Weather condition data were also collected at both experimental sites. The barley plots were mechanically harvested on 9 September and 22 September at the HA and JO experimental farms, respectively.

The field trial was repeated with one *Fusarium* species (*F. culmorum*) and one barley cultivar in Hauho during the summer of 2000. In that trial, three replicate plots of cv. A were sprayed with 2,000 mL of *F. culmorum* culture ($3 \times 10^6$ spores/mL). The control plots were again not treated. The barley plots were mechanically harvested on 30 August.

### Barley Analyses

The moisture content of spike samples was determined by drying 5 g of tissue in an oven at 105°C for 1–2 days and measuring the loss of weight. Crop yields were measured by weighing the amount of grain harvested in each replicate. Counts of bacteria and fungi and trichothecene and ZEN contents of harvested barley were analyzed as described below. In addition, kernel size, germinative capacity and energy, and protein content of barley were determined from barley samples cultivated in 2000 only (5).

### Malting Trials

Barley samples (1 kg) were steeped separately for two days at 12°C to a moisture content of 46%. The germination time was six days at 14°C. The steeping and germination steps were performed in a Seeger malting unit (Seeger GmbH, Stuttgart, Germany). Kilning was carried out for 21 hr with a stepwise temperature increase up to 85°C in a forced-air Seeger laboratory kiln (Seeger GmbH). Samples for microbiological, mycotoxin, and gushing analyses were taken after steeping, germination, and kilning, and the analyses were performed as described below.

### Microbiological Analyses

The number of *Fusarium*-infected kernels was assessed by plating 100 non-surface-disinfected kernels on a selective Czapek Dox iprodione dichloran (CZID)-agar and on a wet filter paper (1,5). The results were reported as the percentage of kernels infected with fusaria. *Fusarium* counts (colony-forming units [CFU]) in barley kernels were enumerated by plating homogenized sample slurries on yeast malt (YM) (in 1998) or potato dextrose (in 2000) agar plates (Difco Laboratories, Detroit, MI)

supplemented with 0.01% chloramphenicol, 0.01% chlorotetracycline and 0.02% Triton X. Colonies were counted after the plates were incubated for seven days at 25°C.

In addition to fusaria, other field fungi such as *Alternaria* spp., *Cephalosporium* spp., *Cladosporium* spp., *Drechslera* spp., and *Epicoccum* spp. were also determined on wet filter paper from non-surface-disinfected kernels using direct plating of 100 seeds (5). The results were expressed as a percentage of kernels contaminated with fungi. Yeasts were determined on YM agar (Difco Laboratories), lactic acid bacteria determined on DeMan-Rogosa-Sharpe (MRS) agar (Oxoid Ltd., Basingstoke, Hampshire, England), *Pseudomonas* determined on C-F-C agar (Oxoid Ltd.), and aerobic heterotrophic bacteria determined on plate count agar (Difco Laboratories). Bacteria were incubated for four to five days and yeasts were incubated for seven days at 25°C.

### Mycotoxin Analyses

Mycotoxins were analyzed in the Department of Chemistry at the National Veterinary and Food Research Institute, Finland. Trichothecenes DON, 3-acetyl deoxynivalenol (3-AcDON), nivalenol (NIV), diacetoxyskirpenol (DAS), fusarenon-X (FX), and T-2 and HT-2 toxins were analyzed by gas chromatography mass spectrometry (GC-MS) with electron impact ionization and ZEN by HPLC using a fluorescence detector as described by Eskola et al (3). The detection limit for DON, 3-AcDON, FX, and DAS was 8 µg/kg of cereal, for NIV, T-2, and HT-2 it was 20 µg/kg, and for ZEN it was 7 µg/kg.

### Malt Analyses

Malt modification (calcofluor method), extract content (fine grind, congress mash), color (visual method), free amino nitrogen (FAN), soluble nitrogen (Kjeldahl method), wort β-glucan content (fluorimetric method), and viscosity were analyzed (4). The filtration rate of the congress worts made of coarsely ground malts was assessed by measuring the volumes of the filtrates collected after 15, 30, and 60 min (30). The β-glucanase activity levels of malt extracts were analyzed at 30 and 60°C using dyed barley β-glucan (Megazyme Ltd., Wicklow, Ireland) as substrate (15). Xylanase activity levels of the malt extract were measured at 45°C using Xylazyme AX tablets (Megazyme Ltd.) as substrate (13). α-Amylase activities were analyzed according to the method of McCleary and Sheehan (15).

### Gushing Test

The gushing propensities of the malt samples were measured according to the method of Vaag et al (34). The malt extracts were added to a Finnish commercial beer and agitated with a horizontally rotating shaker (50 rpm).
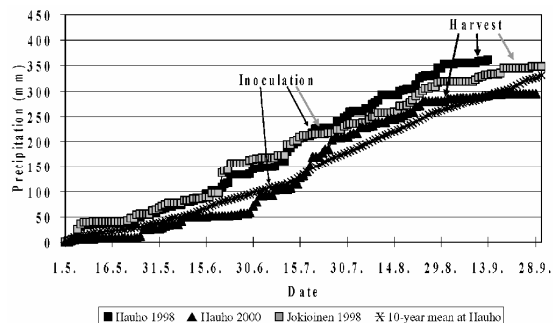


**Fig. 1.** Total precipitation (mm) during the cultivation period of barley at the Hauho and Jokioinen experimental farms, Finland.
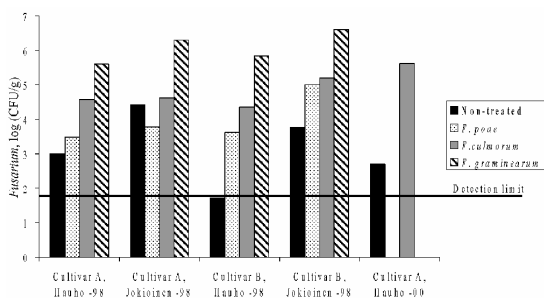


**Fig. 2.** *Fusarium* counts (CFU/g) in homogenized barley samples (cvs. A and B) inoculated with different *Fusarium* species at two experimental farms (Hauho and Jokioinen), Finland in 1998 and 2000.

## RESULTS AND DISCUSSION

### Climatic Conditions

The summer of 1998 was exceptionally rainy in Finland and weather conditions at the experimental farms differed only slightly from each other. Total precipitation was high at both experimental sites (Fig. 1). The mean temperature during August, when the proliferation of fusaria mostly occurred, was 13°C at both sites. The barley was heavily lodged at HA but not at all at JO. However, the moisture content of the barley spike samples collected from JO was higher than that of the samples collected from HA (data not shown). In addition, the crop was harvested two weeks later at JO, where the weather continued to be rainy at that time. The beginning of the summer in 2000 was dry at HA, but heavy rainfall starting in July resulted in total precipitation higher than the 10-year average, as was the case in 1998 (Fig. 1). The mean August temperature in 2000 was 14°C.

### Microflora of Barley

Rainy weather favored the growth of fungi, resulting in a high proliferation of *Fusarium* species during both summers. We reported earlier that the infection rates of *F. culmorum* and *F. graminearum* in spike samples were higher than that of *F. poae* in 1998 (20). However, the infection rates of all the inoculated plots, when measured using a selective CZID agar, were 86% or higher at harvest. The infection rates of the noninoculated plots varied between 74 and 100%. The general *Fusarium* infection rate of barley samples collected at HA and JO experimental fields was 85%, which was much higher than the average *Fusarium* contamination level in Finnish commercial malting barley samples in 1998 (11). The infection rates were also estimated by plating the homogenized barley samples on YM agar. The highest counts were detected in samples inoculated with *F. graminearum* (Fig. 2; 20). The *F. graminearum* counts were 10 to 100 times higher than those of *F. culmorum* and the difference was even greater between *F. graminearum*- and *F. poae*-inoculated barleys. This tendency was observed in both barley cultivars at both experimental farms, although the *Fusarium* counts were generally higher in barley cultivated at the JO experimental farm than it was at HA in 1998 (Fig. 2; 20). Moreover, we measured the activity levels of *Fusarium* serine proteinases of the extracts prepared from the barley samples collected during the growing period or after harvest (20). We observed that the subtilisin-like activities of the *F. culmorum*- and *F. graminearum*-inoculated samples were generally 10–20 times higher than those of the corresponding controls, whereas the subtilisin-like activities of *F. poae*-inoculated samples did not differ from the controls. Our findings imply that the infection had proceeded further in the *F. culmorum*- and *F. graminearum*-inoculated samples than in the *F. poae*-inoculated or the nontreated ones. This is consistent with the

results of previous studies which have indicated that *F. graminearum* is more virulent in terms of causing FHB than is *F. poae* (23,27).

In 2000, the *Fusarium* infection rates of the nontreated and the *F. culmorum*-inoculated spike samples were 93 and 100% (respective *Fusarium* counts at <50 CFU/g and $2 \times 10^5$ CFU/g) 33 days after inoculation, respectively (20). After mechanical harvesting (55 days postinoculation), the *Fusarium* count of the *F. culmorum*-inoculated sample was 1,000 times higher than that of the nontreated barley (Fig. 2; 20). These infection rates and *Fusarium* counts were comparable with those observed in 1998. The general *Fusarium* infection rate of barley samples collected at the HA experimental fields was even slightly higher (90%) in 2000 than it was in 1998. In addition, more pronounced subtilisin-like activity was detected in the *F. culmorum*-inoculated samples collected during the cultivation period in 2000 than in the corresponding samples collected in 1998 (20).

The artificial inoculation of barley with *Fusarium* had only a slight effect on the original microflora, except for fusaria in the infected barley. Only the counts of lactic acid bacteria decreased slightly in *Fusarium*-inoculated samples, whereas the other field fungi, yeasts, *Pseudomonas*, and aerobic heterotrophic bacteria remained at the same relative level as in the nontreated samples (data not shown). Hence, it can be assumed that differences in barley and malt quality between the nontreated and inoculated samples resulted primarily from *Fusarium* infection. In the current study, it was not possible to detect differences in the relative susceptibility of the cultivars to *Fusarium* infection.

### Effects of *Fusarium* Contamination on the Quality of Barley

*Fusarium* inoculation reduced the crop yield of barley. During 1998, the *F. graminearum* isolate decreased yield as much as 20%, whereas the plots that were inoculated with the other two species, had yields similar to the control plot (Table I). According to the Finnish official malting barley studies, the typical crop yield of cvs. A and B is approximately 5,500 kg/ha (reports obtained from the Experimental farm of K-group and from Boreal Plant Breeding Ltd). The crop yields of the nontreated samples, especially in 1998, were much lower (up to 33%) than the expected yield, probably because of exceptional weather conditions and relatively high *Fusarium* infection rates in these samples. The crop yields obtained from the field trials were in general higher at HA than at JO. The crop from the *F. culmorum*-inoculated plots in 2000 was also over 20% lower than the corresponding control (Table I). Furthermore, the kernel plumpness and germination capacity of the inoculated barley were reduced. The percentage of kernels greater than 2.5 mm in diameter decreased from 90% in the control samples to 85% in the inoculated ones (data not shown). Similarly, the germinative capacity decreased from 98 to 88%. Moreover, the infection slightly increased the protein and

**TABLE I**
**Effects of *Fusarium* Species on Crop Yields of Barley Cvs. A and B**
**at the Hauho and Jokioinen Experimental Farms in 1998 and 2000**

| Year | Inoculation | Hauho | | Jokioinen | |
| | | Cv. A | Cv. B | Cv. A | Cv. B |
|---|---|---|---|---|---|
| 1998 | Nontreated | 4,100[a] | 4,320 | 3,931 | 3,678 |
| | *F. culmorum* | 4,020 | 4,660 | 3,977 | 4,006 |
| | *F. graminearum* | 3,250 (–21%)[b] | 3,660 (–15%) | 3,899 (–1%) | 3,318 (–10%) |
| | *F. poae* | 4,250 | 4,410 | 4,413 | 3,990 |
| 2000 | Nontreated | 5,085 | …[c] | … | … |
| | *F. culmorum* | 3,868 (–24%) | … | … | … |

[a] Crop yield (kg/ha) measured by combining the yield from three replicate plots.
[b] Relative loss of yield compared with yield of the corresponding nontreated plots.
[c] … = Not determined.

total nitrogen contents of barley (data not shown). These results are consistent with the observations of Schwarz et al (29).

Trichothecenes and ZEN were analyzed from all mechanically harvested barley samples. All three *Fusarium* isolates studied were able to produce mycotoxins under field conditions (Table II). Most of the samples contained DON and 3-AcDON (Table II), but no other trichothecenes, except traces of NIV, were detected (data not shown). ZEN was also produced by all *Fusarium* species studied. In 1998, the amounts of DON and ZEN were higher in the barley samples inoculated with *F. culmorum,* and particularly with *F. graminearum,* than in those of the samples inoculated with *F. poae* (Table II). This pattern was similar with both barley cvs. A and B. The finding is consistent with the results of Salas et al (23) and Schwarz et al (29), who also detected higher DON levels on barley contaminated with *F. graminearum* than on barley contaminated with *F. poae*. Higher mycotoxin contents were detected in barley cultivated at the JO experimental farm than at HA (Table II). This was likely because of the combination of late harvest and rainy weather at JO, resulting in heavier contamination by fusaria. In 2000, the *F. culmorum*-inoculated sample contained even higher concentrations of DON and ZEN than it did in 1998. The higher proteinase activity level in this sample (20) also supports the conclusion that the *F. culmorum* isolate was more active in 2000 than it was in 1998. The DON contents in the *F. culmorum*- and *F. graminearum*-inoculated barley samples were of the same order of magnitude as in naturally contaminated barleys grown in the Upper Midwestern region of the United States, where severe FHB epidemics occurred in the 1990s (23,26,32). It was estimated that 67–82% of the malting barley crops produced in this region had been contaminated with DON (range of 600 to 60,000 µg/kg) during 1993–1996. *F. graminearum* was the predominant species (26). In 1993, the DON content in 147 barley samples averaged 4,200 µg/kg, ranging from less than 500 to 26,000 µg/kg (33). The DON content of 24 Finnish commercial malting barley samples in 1998 were less than 30 µg/kg and ZEN was undetectable (11). Although *Fusarium* infection was relatively high in Finnish cereals in 1998, Eskola et al (3) also reported low mycotoxin concentrations in rye, barley, oats, and wheat. In 1998, the most frequently isolated *Fusarium* species in Finnish cereals was *F. avenaceum*. The observations suggest that the toxigenic *Fusarium* species have not been predominant in Finnish cereals or that the growing conditions were not favorable for the production of trichothecenes and ZEN by the natural *Fusarium* flora in 1998.

### Effects of *Fusarium* Contamination on the Quality of Malt

*Fusarium Level*. The effect of the malting process on *Fusarium* levels in barley was determined using direct plating of non-surface-disinfected kernels and dilution plating of homogenized samples. Differences in *Fusarium* infection rates between kernel samples plated on CZID agar were very small because of excessive contamination in all samples (data not shown). The dilution plating technique was better suited for estimating the amount of fusaria in artificially inoculated barley during the malting process. As shown in Figure 3, the *F. graminearum* counts remained high throughout the whole malting process, whereas *F. poae* counts were consistently lower. The highest *Fusarium* counts were detected in green malts. Kilning reduced the counts below the initial contamination levels.

*Gushing*. All three *Fusarium* species induced gushing of beer. *F. culmorum* and *F. graminearum* were the most active species (Fig. 3). The gushing propensities of the malt samples inoculated with *F. poae* were variable. According to earlier experiments, the gushing potential of malt increases if the percentage of naturally *Fusarium* contaminated malt kernels exceeds 50% on wet filter papers (7,9). On the basis of the *Fusarium* contamination levels of 36–42% in *F. poae*-inoculated samples, no gushing was expected. In this study, the CFU correlated positively with the gushing results of malts. Thus, if the *Fusarium* counts in malt were $10^3$ CFU/g or higher, the malt frequently induced gushing (data not shown). However, because of the small number of samples, statistical analysis was not feasible. We have previously reported that hydrophobins were able to induce gushing when added to bottled beer and that the amount of these small fungal proteins in malt positively correlated with the gushing potential of malt (10). In the work cited, we measured higher hydrophobin contents in the
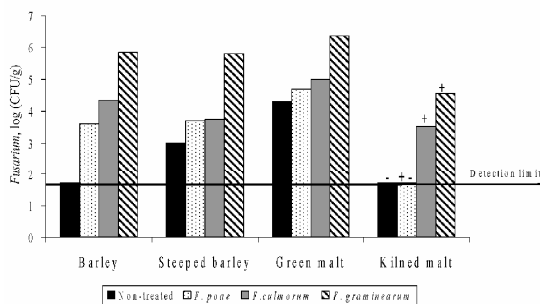


**Fig. 3.** Effect of malting on *Fusarium* counts (CFU/g) of barley (cv. B) inoculated with different *Fusarium* species at Hauho, Finland in 1998 and general gushing propensity of malt. – = No gushing, +– = variable gushing, and + = gushing.

**TABLE II**
**Production of Deoxynivalenol (DON), 3-Acetyl Deoxynivalenol (3-AcDON), and Zearalenone (ZEN)**
**in Barley Cvs. A and B Inoculated with *Fusarium* Species at Hauho (HA) and Jokioinen (JO), Finland in 1998 and 2000**

| Cultivar/Year | Inoculation | DON (µg/kg) | | 3-AcDON (µg/kg) | | ZEN (µg/kg) | |
|---|---|---|---|---|---|---|---|
| | | HA | JO | HA | JO | HA | JO |
| A/1998 | Nontreated | <8 | 235 | <8 | 110 | <7 | <7 |
| | *Fusarium poae* | 75 | 118 | <8 | 20 | 15 | <7 |
| | *F. culmorum* | 760 | 480 | 90 | <8 | 585 | 1,305 |
| | *F. graminearum* | 18,920 | 13,410 | 150 | 285 | 2,010 | 850 |
| B/1998 | Nontreated | <8 | 220 | <8 | 40 | <7 | 110 |
| | *F. poae* | 35 | 2,000 | <8 | 90 | <7 | 210 |
| | *F. culmorum* | 220 | 3,260 | 75 | <8 | 470 | 7,300 |
| | *F. graminearum* | 12,820 | 46,550 | 55 | 2,650 | 4,580 | 9,470 |
| A/2000 | Nontreated | 295 | ...[a] | 30 | ... | 15 | ... |
| | *F. culmorum* | 16,470 | ... | 1,230 | ... | 11,405 | ... |

[a] ... = Not studied.

malt samples produced from *F. culmorum*- and *F. graminearum*-inoculated barleys compared with those produced from the nontreated or *F. poae*-inoculated samples. Gushing potential was followed throughout the malting process by analyzing the samples of barley, steeped barley, green malt, and kilned malt. The gushing activity levels of the malting samples varied during the process depending on the initial *Fusarium* contamination level in barley. The gushing potential of barley, particularly in the case of nontreated samples, decreased in steeping. However, it could again increase during germination or kilning, whereas the gushing propensity of *Fusarium*-inoculated barley remained high throughout the entire malting process (Table III). The more intense *Fusarium* contamination in the samples originating from the JO experimental farm obviously resulted in a more pronounced increase in gushing propensity during germination and kilning

compared with the samples from HA. Commercial malting barley lots with low *Fusarium* contamination did not induce gushing at any stage of malting (data not shown). A similar impact of malting on gushing activity levels of *Fusarium*-contaminated barleys was reported by Munar and Sebree (16).

*Mycotoxins*. Production of DON and ZEN was followed during the malting process. Steeping reduced the level of DON in nontreated samples, whereas the levels increased in artificially inoculated barleys (Table IV). DON is a water soluble compound. Despite of some loss of DON with steeping water, the steeping schedule with long air-rest periods probably activated the DON production of inoculated fusaria. The ZEN contents of all the barley samples decreased during steeping. The highest DON and ZEN contents were detected in green malt, implying that the fungi had produced more mycotoxins during grain germination. Kilned

### TABLE III
**Changes of the Gushing Potential During Malting of Barley Cvs. A and B, Nontreated or Inoculated with *Fusarium culmorum* at the Hauho and Jokioinen Experimental Farm in Finland in 1998**

| Location | Variety | Inoculation | Gushing Tendency[a] | | | |
|---|---|---|---|---|---|---|
| | | | **Barley** | **Steeped Barley** | **Green Malt** | **Kilned Malt** |
| Hauho | A | Nontreated | +++ | + | + | + |
| | B | Nontreated | +++ | − | − | − |
| | B | *F. culmorum* | +++ | +++ | +++ | +++ |
| Jokioinen | A | Nontreated | +++ | + | ++ | ++ |
| | B | Nontreated | +++ | + | + | ++ |
| | A | *F. culmorum* | +++ | +++ | +++ | +++ |

[a] Gushing of beer (g) was determined by weight loss of beer bottle. − = No gushing, + = gushing 1–30 g, ++ = gushing 31–60 g, and +++ = gushing >60 g.

### TABLE IV
**Effect of Malting on the Deoxynivalenol (DON) and Zearalenone (ZEN) Contents of Barley (Cv. B) Inoculated with *Fusarium culmorum* and *F. graminearum* at the Jokioinen Experimental Farm, Finland in 1998**

| Mycotoxin | Inoculation | DON or ZEN (µg/kg) | | | |
|---|---|---|---|---|---|
| | | **Barley** | **Steeped Barley** | **Green Malt** | **Kilned Malt** |
| DON | Nontreated | 220 | 60 | 1,520 | 430 |
| | *F. culmorum* | 3,260 | 15,640 | 10,240 | 12,040 |
| | *F. graminearum* | 46,550 | 55,660 | 100,720 | 47,650 |
| ZEN | Nontreated | 110 | <7 | 1,690 | 30 |
| | *F. culmorum* | 7,300 | 6,000 | 38,500 | 17,950 |
| | *F. graminearum* | 9,470 | 4,400 | 37,640 | 25,770 |

### TABLE V
**The Effect of *Fusarium* Contamination on the Quality of Malt Produced from Artificially Inoculated Barley Cv. A at the Hauho (HA) and Jokioinen (JO) Experimental Farms, Finland in 1998 and 2000**

| | 1998 Crop | | | | | | | | 2000 Crop | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Nontreated | | *F. poae* | | *F. culmorum* | | *F. graminearum* | | Nontreated | *F. culmorum* |
| **Malt Analyses** | HA $n = 2$ | JO $n = 2$ | HA $n = 1$ | JO $n = 1$ | HA $n = 2$ | JO $n = 2$ | HA $n = 1$ | JO $n = 1$ | HA $n = 2$ | HA $n = 1$ |
| Modification, % | 92 | 93 | 95 | 91 | 91 | 90 | 89 | 88 | 97 | 97 |
| Extract content, % dry matter (fine grind) | 81.0 | 81.8 | 80.4 | 80.0 | 80.7 | 81.4 | 78.8 | 79.4 | 80.7 | 78.2 |
| Color, EBC | 5.7 | 4.5 | 6.6 | 7.2 | 6.5 | 5.7 | 9.4 | 8.1 | 6.6 | 9.4 |
| FAN, mg/L | 238 | 194 | 237 | 245 | 228 | 204 | 255 | 228 | 258 | 270 |
| Soluble nitrogen, mg/L | 1,071 | 892 | 1,062 | 1,100 | 1,045 | 935 | 1,146 | 963 | 985 | 1,210 |
| β-Glucans, mg/L | 69 | 190 | 40 | <40 | 72 | 145 | 48 | 98 | 47 | <40 |
| Wort viscosity, cP | 1.50 | 1.52 | 1.46 | 1.46 | 1.47 | 1.49 | 1.47 | 1.48 | 1.50 | 1.56 |
| Filtration rate, mL/30 min (coarse grind) | 170 | 60 | 180 | 150 | 170 | 80 | 110 | 55 | 130 | 45 |
| α-Amylase, U/kg | 330 | 230 | 325 | 355 | 315 | 250 | 270 | 215 | 345 | 415 |
| β-Glucanase 30°C, U/kg | 625 | 590 | 505 | 515 | 490 | 575 | 410 | 445 | 835 | 690 |
| β-Glucanase 60°C, U/kg | 50 | 65 | 60 | 60 | 90 | 120 | 90 | 90 | 65 | 120 |
| Xylanase, U/kg | 305 | 210 | 270 | 270 | 410 | 520 | 465 | 365 | 300 | 935 |

[a] $n$ = Number of maltings.

samples contained lower amounts of mycotoxins than did the green malt. This reduction might be partly because of the removal of rootlets from kilned malts before mycotoxin analyses. The fate of naturally occurring *Fusarium* toxins during malting was also studied by Schwarz et al (27), and Munar and Sebree (16). Reductions of DON and ZEN contents during steeping were observed in both studies. Schwarz et al (27) observed production of DON and ZEN during germination. They also reported a minor decrease in the DON content during kilning, whereas the ZEN content was found to be even higher in malt than it was in green malt. In the study of Munar and Sebree (16), the DON content sharply increased during the early stages of kilning (18 h, 52°C), resulting in a higher DON level in malt than in green malt, although a decrease in the DON content was observed during the last three hours at 85°C. Later, Schwarz et al (29) reported that in the case of limited *Fusarium* growth, the levels of DON, ZEN, 15-AcDON, and NIV declined during malting. The findings reported above imply that the effects of malting on *Fusarium* toxins depend on the initial contamination of barley as well as on malting conditions.

**Malt Analyses**

The effects of *Fusarium* contamination on the quality parameters of malt were studied in detail. The results of malt analyses of cv. A cultivated during the field trials in 1998 and 2000 are shown in Table V. Trends in malt quality observed in cv. B, with respect to *Fusarium* infection, were similar to those in cv. A, and these results are therefore excluded.

All of the *F. culmorum*- and *F. graminearum*-infected malt samples from 1998 and 2000 contained higher xylanase activity levels than did the nontreated samples. The increased activity levels were probably because of the presence of fungal enzymes in these malts. Interestingly, the amount of endogenous β-glucanase was lower in the *Fusarium*-inoculated samples than in the nontreated samples when the enzyme activity levels were measured at 30°C. This indicated that the endogenous enzyme production of barley was decreased because of very heavy *Fusarium* infection. However, the malt β-glucan content was reduced in all *Fusarium*-inoculated samples. This was also suggesting the presence of fungal enzymes. Higher activity levels of β-glucanase at 60°C were found in the *Fusarium*-inoculated samples, indicating the contribution of thermostable microbial β-glucanase. The effects of *F. poae* infection on malt enzyme activity levels were low. Notably higher β-glucanase (60°C) and xylanase activity levels were detected in *F. culmorum*- and *F. graminearum*-infected malts compared with *F. poae*-infected malts. This current study clearly shows that the intensity of the effects of *Fusarium* infection was species dependent; the effects of *F. culmorum* and *F. graminearum* on the malt quality were more pronounced than those of *F. poae*. The small effect of *F. poae* could be partly explained by its lower count during malting compared with the other two species.

The low wort β-glucan content, which was expected to increase the filtration rate, did not consistently correlate with wort lautering performance. For example, the sample inoculated with *F. culmorum* in 2000 had a very low β-glucan level, but a slow filtration rate (Table V). It is well known that in addition to β-glucans, the filtration rate is affected by many parameters such as complexes formed between gel proteins and pentosans, residual starch, and lipids (17). Sadosky et al (22), reported that filterability of a beer model solution was decreased by β-glucan and especially, by arabinoxylan. In this current study, the increased xylanase activity levels of *F. culmorum*- and *F. graminearum*-inoculated samples could have increased soluble arabinoxylan contents in worts, resulting in poor filtration performance.

Furthermore, previously it has been shown that *F. culmorum* and *F. graminearum* can produce proteinases that hydrolyze ce-

real storage proteins (20). These proteinases were present in low concentrations in the mechanically harvested *F. culmorum*- and *F. graminearum*-inoculated barleys, but since fusaria can grow during malting, it is likely that more of the fungal proteinases were synthesized during the grain germination. Increased wort soluble nitrogen content and color are considered to be a consequence of enhanced proteolysis. This current study showed that *Fusarium* infection tended to darken wort color and to increase the amount of soluble nitrogen and FAN, suggesting that *Fusarium* proteinases may have degraded barley proteins already in the field or during malting and mashing (Table V). Although the differences in wort soluble nitrogen and FAN levels were negligible among the malt samples of cv. A in 1998 (Table V), the results for cv. B malts supports this conclusion (data not shown). The highest increases were observed in the *F. graminearum*-inoculated samples of cv. B in 1998, in which the amount of soluble nitrogen and FAN were 30 and 60% higher than in nontreated samples, respectively (data not shown). Our findings are consistent with the results of Schwarz et al (28), who detected higher proteinase, β-glucanase, and xylanase activity levels in barley samples artificially infected with *F. graminearum* and *F. poae* compared with the control sample. The authors concluded that enzyme activity levels in the barley samples were so high that they might affect the quality of corresponding malts. Furthermore, several authors have reported that heavy *Fusarium* infection decreases β-glucan content and simultaneously increases wort color, soluble nitrogen, and FAN (8,21,28,29,31).

## CONCLUSIONS

All the *Fusarium* species studied were able to proliferate in the field in southern Finland and they affected barley and malt quality. The extent of the effects was species dependent. The growth of *F. graminearum* and *F. culmorum* in barley was faster and more intense than that of *F. poae*. Moreover, *F. graminearum* produced higher amounts of mycotoxins in the field and during malting than did *F. culmorum* and particularly, *F. poae,* in the trials of 1998. *F. graminearum* and *F. culmorum* also reduced the crop yield. In addition, *F. culmorum* infection reduced kernel size and germination of barley. The *F. culmorum* isolate had greater effect on barley and malt quality in 2000 than it did in 1998. All the species studied induced gushing of beer, but *F. poae* induced the weakest gushing propensity. The gushing potential of barley decreased during steeping but increased during germination or kilning. Reduction in gushing potential during steeping was more evident in nontreated barley than in samples heavily contaminated with *Fusarium*. *Fusarium* infection was not observed to affect the other microflora in barley.

*Fusarium* infection increased wort color and slightly increased the amount of soluble nitrogen and FAN. Activity levels of β-glucanase at 60°C and of xylanase were elevated, especially, in *F. graminearum*- and *F. culmorum*-infected malt samples. These higher levels of cell-wall-degrading enzyme activities, most likely originating from *Fusarium*, contributed to the decreased content of β-glucans in wort. However, high *Fusarium* contamination in some cases decreased the wort filtration rate, indicating the presence of other components or complexes affecting the lautering performance.

*Fusarium* contamination, mycotoxins in barley, and gushing propensity of malt were higher in barley cultivated at the JO experimental farm than it was at HA, although barley was heavily lodged at the latter site in 1998. The high moisture content of spikes and delayed harvesting at JO obviously decreased the quality of barley and of the corresponding malt. Differences between the cultivars in terms of susceptibility to *Fusarium* infection were not detected.

Mycotoxin contents in Finnish cereals have been shown to be naturally low. In this study, all three *Fusarium* species were able to produce DON, 3-AcDON, and ZEN. This result supports the conclusion that the prevalence of naturally occurring toxigenic *Fusarium* species in Finland is rather low. The diversity of toxigenic *Fusarium* species and the effects of climatic conditions on the aggressiveness of fusaria in Finland should be further studied. Emergence of new, potentially toxigenic *Fusarium* species should be monitored constantly. In addition, the ability of different *Fusarium* species to produce hydrophobins contributing to gushing should also be investigated in more detail.

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# Fungal hydrophobins as predictors of the gushing activity of malt

# Fungal Hydrophobins as Predictors
# of the Gushing Activity of Malt

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**ABSTRACT**

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Fungal infection of barley and malt, particularly by strains of the genus *Fusarium,* is known to be a direct cause of beer gushing. We have shown previously that small fungal proteins, hydrophobins, isolated from strains of the genera *Fusarium, Nigrospora* and *Trichoderma* act as gushing factors in beer. A hydrophobin concentration as low as 0.003 ppm was sufficient to induce gushing. The gushing-inducing abilities of the isolated hydrophobins varied probably due to their structural differences. The hydrophobins did not affect beer foam stability. A correlation was observed between the hydrophobin level analyzed by the hydrophobin ELISA developed and the gushing potential of malt. The risk of gushing was found to increase with hydrophobin concentrations above 250 µg/g malt. The levels of hydrophobin and the *Fusarium* mycotoxin deoxynivalenol (DON) in malts were not correlated which indicated that the formation of those two fungal metabolites may not be linked. Furthermore, we did not observe a correlation between the DON content and the gushing potential of the malt studied. Our observations suggest that the accuracy of predicting gushing could be improved by measuring the amount of the actual gushing factors, hydrophobins, in barley or malt.

**Key words:** Beer gushing, ELISA, hydrophobin, prediction of gushing risk in malt.

## INTRODUCTION

Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously over foams out from the package immediately on opening[10]. Two types of gushing exist in beer[3,6]. Primary gushing is induced by fungal metabolites, so-called gushing factors, which are present in malt or in other cereal raw materials of beer. Non-malt related gushing, i.e. secondary gushing, may occur if beer contains e.g. haze, impurities from bottles, metal ions, calcium oxalate crystals, cleaning agent residues or excess of gas. Primary gushing is commonly caused by *Fusarium* fungi but other genera such as *Aspergillus, Nigrospora, Penicillium* and *Stemphylium* are also reported to induce gushing[4]. Gushing factors produced by fungi have been studied for decades. As shown in Table I, they have been reported to be polypeptides or peptide-containing substances. Very small amounts of these substances, quantities

in the ppm range or lower, have been reported to induce gushing of beer. Aastrup *et al.*[2] observed that addition of proteolytic enzymes to gushing-inducing malt extract significantly reduced gushing tendency, suggesting that the gushing-inducing factors present in malt were proteins or polypeptides. Gushing factors are assumed to be surface active molecules which stabilize carbon dioxide bubbles in beer by forming a layer around the microbubbles[7,26]. This layer may prevent breakdown of the bubbles, leading to overfoaming.

Gushing negatively affects the image of beer, incurring economic losses for breweries and maltsters. An increasing percentage of European malt samples analysed during the past five years have shown gushing tendency[1]. In the USA severe epidemics of *Fusarium* Head Blight in barley have occurred during the last decade, which have increased the risk of gushing[28,31]. Currently the gushing potential of barley and malt can be predicted by quantifying the presence of *Fusarium* fungi or their antigens[8,22,32]. The principle weakness inherent to these methods lies in the fact that they do not directly detect the actual gushing-inducing factors. Some studies have also shown that the actual *Fusarium* level of barley or malt is a poor predictor of gushing propensity[23,28]. The gushing test described by Vaag *et al.*[33] and modified for barley by Aastrup[1], relies on an aqueous extract of barley or malt being added to bottled beer so that the gushing tendency of beer can be measured after three days of shaking. However, the test is labor-intensive and time-consuming, and is impractical for screening large numbers of samples.

Our recent studies indicated that small fungal proteins called hydrophobins act as the gushing factors of beer[13,18]. Hydrophobins are highly surface active, moderately hydrophobic proteins produced by filamentous fungi[34]. A characteristic feature of these proteins is their eight conserved cysteine residues forming four disulphide bridges in the molecule made up of $100 \pm 25$ amino acids. Under some conditions hydrophobins form aggregates. Based on sequence comparison, hydrophobins are divided into two different classes, I and II[34]. Hydrophobins self-assemble at their hydrophilic-hydrophobic interfaces to form amphipathic membranes[35]. This property allows hydrophobins to fulfill a broad spectrum of functions in fungal growth and development. Hydrophobins are present in fungal cell walls, where they are involved in the formation of structures of mycelium and spores[34]. Fungi secrete hydrophobins into their surroundings, where the proteins can decrease the surface tension of water or change the nature of a surface from hydrophilic to hydrophobic or *vice versa*[36]. These properties are useful when the fungus penetrates

the air-water interface or attaches to hydrophobic surfaces like cuticular waxes of plant tissues. The aim of this study was to isolate and characterize hydrophobins from gushing active fungi and to demonstrate that these hydrophobins are able to induce gushing in beer. The main goal of our work was to develop a test for detection of gushing potential of barley and malt by analysing the hydrophobin levels in samples.

## MATERIALS AND METHODS

### Fungal strains, media and cultivation

*Fusarium poae* VTT D-82182 (D182), *Nigrospora* sp. VTT D-79122 (D122) and *Trichoderma reesei* VTT D-74075 (D75) obtained from the VTT Culture Collection (Helsinki, Finland) were used. The *Fusarium* strain was cultivated in Czapek-Dox Broth (Difco Laboratories, Detroit, USA) or Potato Dextrose Broth (Difco Laboratories), the *Nigrospora* strain in Potato Dextrose Broth (Difco Laboratories) and the *Trichoderma* strain in *Trichoderma* minimal medium[27] buffered to pH 6. All strains were cultivated in shake flasks for at least 7 days at room temperature. The mycelium was separated from culture medium by filtration through GF/B glass fiber filter (Whatman International Ltd., Maidstone, UK), washed with water and frozen at –20°C.

### Purification of hydrophobins

Hydrophobins were isolated as described by Nakari-Setälä *et al.*[25] using the sequential extraction of mycelium or by bubbling air through culture medium in which the fungal strains had been grown and then collecting the foam produced. The protein samples were further purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 column using the Äkta Explorer system (Pharmacia Biotech, Sweden). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions eluted with 40–50% of acetonitrile were collected.

### Protein analyses

Hydrophobins were detected using SDS-PAGE performed with 17.5% or 20% gels[21] using the 2050 Midget Electrophoresis System (Pharmacia LKB Biotechnology, Sweden) or PhastSystem (Pharmacia LKB Biotechnology), followed by visualization of the proteins by silver staining (Silver Stain Kit, Bio-Rad Laboratories, Hercules, USA) or immunoblotting. Polyclonal antibodies against the hydrophobins of *T. reesei* D75 and *F. poae* D182 were raised in rabbits. Immunisation took place four times within three months using the Freunds adjuvant. The hydrophobin antibodies together with goat anti-rabbit IgG (H+L)–alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) were used for the immunoblot analysis. The blot was developed using BCIP/NPT Colour Development Substrate (Promega, USA). Protein concentrations of the purified hydrophobin samples were determined using BC Assay Protein Determination Kit (Uptima, France) or BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). In addition, a concentration of *T. reesei* hydrophobin samples was determined from the HPLC-chromatogram by using known amounts of the purified hydrophobins HFBI[25] or HFBII[24] as a standard. Partial N-terminal amino acid sequences of the purified proteins were determined by degradative Edman chemistry in the Protein Chemistry Laboratory of the Institute of Biotechnology, Finland[14].

### ESI-MS analysis

The hydrophobins molecular weights were determined using the ESI-MS (electrospray–mass spectrometry) technique. RP-HPLC purified hydrophobin samples were diluted 1:5 in 0.1% formic acid in 50% acetonitrile and introduced into a Micromass Quattro Micro triple quadrupole mass spectrometer (Manchester, UK) and analyzed by continuous-flow injection using a syringe pump. The sample flow rate was 5 μL/min. The electrospray ion source was operated at a capillary voltage of 3.00 kV and cone voltage of 20 V. Source and desolvation temperatures were 80°C and 130°C, respectively. Desolvation gas flow was 500 L/h and cone gas flow 20 L/h. The scan range was 400 to 2000 $m/z$. Data was acquired and processed with MassLynx 3.5 software (Waters, Milford, USA).

### Beer foam stability

The effect of hydrophobins on beer foam stability was studied by adding 0.1, 1 and 10 μg of the RP-HPLC purified HFBI[25] and HFBII[24] hydrophobins from *T. reesei* D75 into 0.33 L of bottled beer. The beer bottles were inverted once and the foam stability was measured using the NIBEM foam stability apparatus Model B (Haffmans BV Venlo, Holland) according to the manufacturer's instructions. The NIBEM apparatus recorded the collapse times in seconds required by foam to fall down 10, 20 and 30 mm.

### Hydrophobin ELISA

A competitive ELISA (Enzyme Linked ImmunoSorbent Assay) was developed for detection of hydrophobins in barley and malt. Kernels were ground with a Universal Laboratory Disc Mill DLFU (Bühler-Miag GmbH, Braunschweig, Germany) and 5 g of the fine flour was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. After centrifugation the supernatant was transferred to a clean

**Table I.** Properties of the gushing factors produced by different fungi: historical background 1963–1980.

| Isolated from | Chemical structure | Molecular weight | Concentration needed for gushing | Reference |
|---|---|---|---|---|
| *Nigrospora* sp. | Polypeptide | 16.5 kDa | 0.05 ppm | 4, 16 |
| *Stemphylium* sp. | Peptidoglycan | nd | 4 ppm | 4 |
| *Fusarium graminearum* | Hexapeptide | nd | 0.4 ppm | 4, personal communication |
| *Penicillium crysogenum* | Cyclic tetrapeptide | nd | 0.3 ppm | 17 |
| Northern European Malt | Peptide-like | 10 kDa | 0.5 ppm | 15 |

nd: not determined.

tube and antibodies against the hydrophobin of *F. poae* D182 were added. After incubation the sample-antibody mixture was transferred into triplicate wells of immunoplates (Nunc-Immuno Modules, MaxiSorp polystyrene strips, Nunc, Rochester, USA) coated with a hydrophobin extract of *F. poae* D182. Goat anti-rabbit IgG (H+L)–alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma, St. Louis, USA) in diethanolamine-MgCl₂ buffer (Oy Reagena Ltd, Toivala, Finland) were used for AP detection. After incubation for 30 min at room temperature, the absorbance was read at 405 nm using a Multiskan Ex microtitre plate reader (Labsystems, Helsinki, Finland). Because of the nature of the competitive ELISA, a lower absorbance value corresponded to a higher amount of hydrophobins in the sample.

### Gushing potential

The gushing test was carried out according to the method of Vaag *et al.*[33] using a horizontal rotating shaker (50 rpm)[11] in order to analyze the gushing potential of malt. In this method, an aqueous extract of ground malt was added to bottled beer and the pasteurized bottles were shaken for three days. After shaking the bottles were kept still for 10 min, inverted three times and opened after 30 sec. The amount of gushing was determined from the change in weight of the bottle. The gushing-inducing ability of hydrophobins was studied by adding the purified hydrophobins into beer bottles and shaking the bottles as described above. The test was performed in duplicate except with the hydrophobin of *F. poae.*

### Deoxynivalenol analysis

The deoxynivalenol content of barley and malt was analysed using the EZ-Quant High Sensitivity Deoxynivalenol (DON) Test Kit (Diagnostix, Mississauga, Canada) or the EZ-Quant DON Test Kit (Diagnostix) according to the manufacturer's instructions.

## RESULTS AND DISCUSSION

### Isolation and characterization of hydrophobins

Hydrophobins were isolated from *F. poae* D182, *Nigrospora* sp. D122 and *T. reesei* D75 by bubbling the culture broths with air and collecting hydrophobin enriched foam or by sequential extraction of mycelium. The hydrophobins were subsequently purified by separation of these extracts by preparative RP-HPLC. The purified hydrophobin samples were analysed by SDS-PAGE which indicated that molecular weights of the protein bands were below 14 kDa (Fig. 1). The protein bands of *F. poae* (isolated by bubbling from Czapek-Dox Broth) and *Nigrospora* sp. (isolated by sequential extraction of mycelium cultivated in Potato Dextrose Broth) were slightly larger than that of *T. reesei*. Nakari-Setälä *et al.*[25] reported a predicted molecular weight of 7.5 kDa for the hydrophobin HFBI of *T. reesei*. When the RP-HPLC purified foam samples of *F. poae* and *Nigrospora* sp. were analysed by ESI-MS, both proteins had a molecular weight of approximately 8.5 kDa, which was consistent with their SDS-PAGE migration.

Partial N-terminal amino acid sequences of the RP-HPLC purified proteins of *F. poae* and *Nigrospora* sp. are presented in Fig. 2. When sequence data were compared to the consensus sequence of hydrophobins[34], the typical conserved cysteine pattern of hydrophobins was found in the sequences of the *F. poae* and *Nigrospora* sp. proteins. Kitabatake and Amaha[16] reported that the gushing-inducing factor of *Nigrospora* (NFG) had a molecular weight of 16.5 kDa, consisted of 166 amino acid residues and had approximately 16 cysteine residues. If NGF is assumed to be in a dimeric form, then the number of amino acid and cysteine residues of the monomeric protein corresponds to those of hydrophobins.

In immunoblot analysis the polyclonal antibodies raised against the hydrophobin from *F. poae* D182 reacted only with its own antigen and not with the hydrophobins from *T. reesei* D75 and *Nigrospora* sp. D122 (data not shown). Correspondingly, the polyclonal antibodies raised against the hydrophobins from *T. reesei* D75 did not react with the hydrophobins of *F. poae* D182 or *Nigrospora* sp. D122 (data not shown). These results suggested that the three hydrophobins differed from each other to such an extent that they could not be detected with the same polyclonal antibodies. However, gushing of beer is considered to be most commonly caused by *Fusarium*[11,28], which justifies the use of the *F. poae* hydrophobin antibodies in the ELISA test for prediction of the gushing propensity of barley and malt.

### Gushing and foam stability effects of hydrophobins

RP-HPLC purified hydrophobin fractions from *F. poae, Nigrospora* sp. and *T. reesei* were added to bottled beer and the bottles were rocked according to the gushing test protocol. Addition of an amount as low as 1 µg of *T. reesei* hydrophobins into bottled beer (0.33 L), corre-
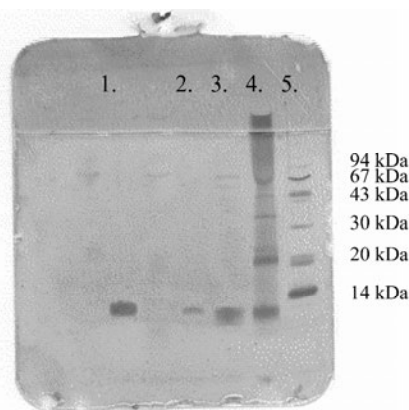


**Fig. 1.** 20% SDS-PAGE of the hydrophobin samples isolated from mycelium or culture medium of the following fungi: *F. poae* D182 (1), *Nigrospora* sp. D122 (2) and *T. reesei* D75 (purified 3 and unpurified 4). The molecular weight markers are shown on the right (5). Total protein detected by silver staining.
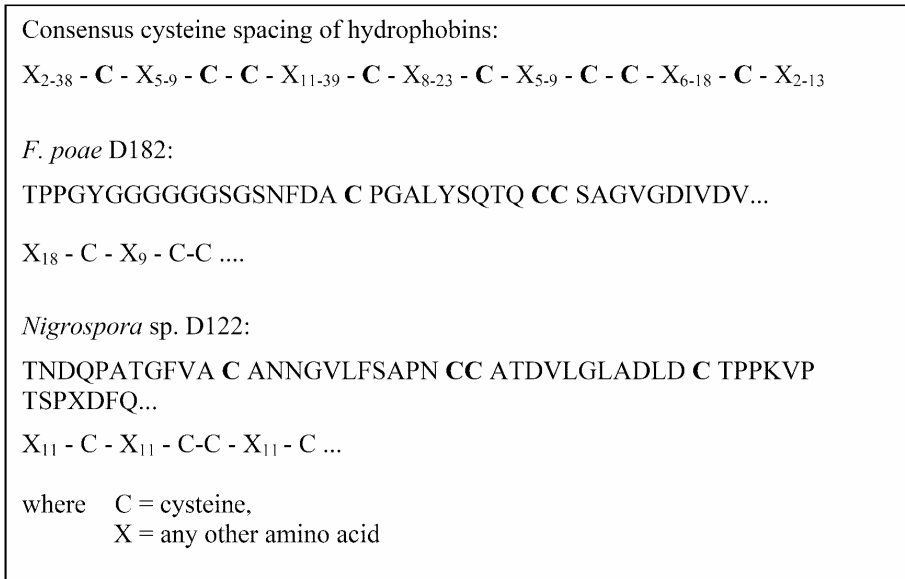
Consensus cysteine spacing of hydrophobins:

$X_{2-38}$ - C - $X_{5-9}$ - C - C - $X_{11-39}$ - C - $X_{8-23}$ - C - $X_{5-9}$ - C - C - $X_{6-18}$ - C - $X_{2-13}$

*F. poae* D182:

TPPGYGGGGGGGSGSNFDA **C** PGALYSQTQ **CC** SAGVGDIVDV...

$X_{18}$ - C - $X_9$ - C-C ....

*Nigrospora* sp. D122:

TNDQPATGFVA **C** ANNGVLFSAPN **CC** ATDVLGLADLD **C** TPPKVP TSPXDFQ...

$X_{11}$ - C - $X_{11}$ - C-C - $X_{11}$ - C ...

where    C = cysteine,
              X = any other amino acid

**Fig. 2.** Partial N-terminal amino acid sequences of hydrophobins of *F. poae* D182 and *Nigrospora* sp. D122. Cysteine spacing in the hydrophobins of *F. poae* D182 and *Nigrospora* sp. D122 are compared to the consensus cysteine spacing found for fungal hydrophobins according to Wessels[34].

sponding to a concentration of 0.003 ppm, was sufficient to cause gushing (Table II). A tenfold higher amount of the *Nigrospora* hydrophobin (0.03 ppm) was needed to induce gushing. Gushing activity of the hydrophobin from *F. poae* was the weakest at inducing gushing as 0.1 ppm. The concentrations of the *T. reesei, F. poae* and *Nigrospora* sp. hydrophobins needed to induce gushing were of the same order of magnitude as those of the gushing factors reported by other investigators and presented in Table I. Moreover, in a previous investigation we reported that the isolated hydrophobins could also induce gushing in mineral water, although the gushing was more pronounced in beer than in mineral water[18].

The *T. reesei* hydrophobins, HFBI and HFBII, did not affect beer foam stability as determined by the NIBEM apparatus (data not shown), although 10 μg of the hydrophobin samples in beer was sufficient to induce gushing even when the bottles were not shaken but only gently in-

verted once. These results indicate that the properties of hydrophobins enable them to induce gushing rather than to stabilize foam in beer. According to current knowledge hydrophobic proteins in beer, such as Lipid Transfer Protein, increase beer foamability, but other components, such as bitter substances and polysaccharides, increase foam stability by cross-linking with the proteins[5]. It can be assumed that the hydrophobins from *T. reesei* did not favour the formation of cross-linkages.

**Validation of the hydrophobin ELISA test**

Standards with known hydrophobin concentrations ranging from 5 to 200 μg/mL were prepared by diluting the RP-HPLC purified hydrophobin sample of *F. poae* with an aqueous extract of a gushing negative malt. Two sets of standards were analysed with the hydrophobin ELISA developed. A linear correlation ($r^2 \approx 0.95$) was found between the logarithm of hydrophobin concentrations below 100 μg/mL and the results of the hydrophobin ELISA (A 405 nm) (Fig. 3). The differences between the absorbance values of the two standard sets are probably due to inaccuracy of the protein concentration analysis of the RP-HPLC purified hydrophobin samples. In addition, because of their hydrophobic nature, some of the hydrophobins may have adhered to the walls of tubes and pipette tips, which could have led to errors in hydrophobin concentrations particularly in the standards with low hydrophobin content. The hydrophobin ELISA could not distinguish between hydrophobin concentrations higher than 100 μg/mL, which means that the assay can be used to estimate hydrophobin levels below 1000 μg/g barley or malt without sample dilution.

**Table II.** Gushing of beer induced by RP-HPLC purified hydrophobin fractions of *T. reesei* D75, *F. poae* D182 and *Nigrospora* sp. D122. Two different hydrophobins of *T. reesei,* HFBI and HFBII, were tested.

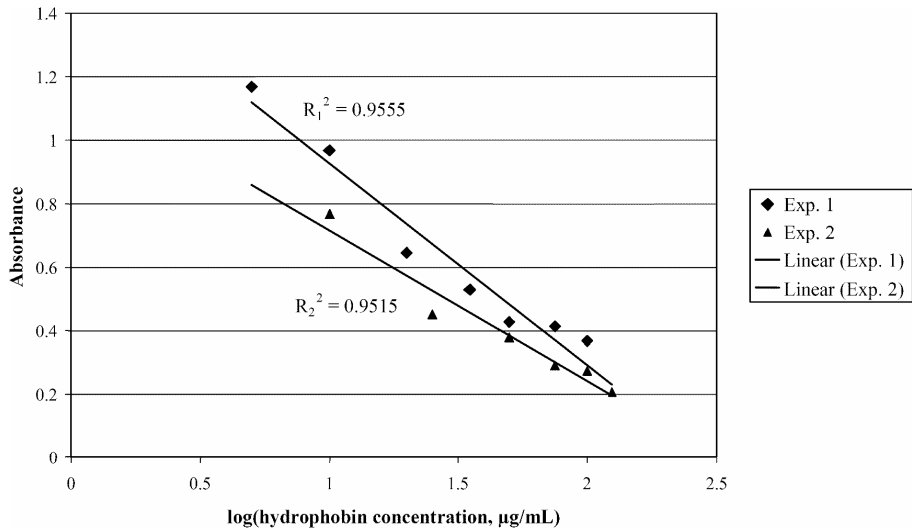| Amount of hydrophobin μg/0.33 L beer | Amount of beer gushed (g) | | | |
|---|---|---|---|---|
| | *T. reesei* HFBI | *T. reesei* HFBII | *F. poae* | *Nigrospora* sp. |
| 0.01 | 0 | 0 | nd | nd |
| 0.1 | 0 | 0 | nd | nd |
| 1 | 10 | 12 | 0 | 0 |
| 10 | 189 | 192 | 0 | 66 |
| 45 | nd | nd | 27 | 183 |
| 60 | nd | nd | 80 | nd |

nd = not determined.

**Fig. 3.** Logarithmic concentrations of the hydrophobin standards of *F. poae* (µg/mL) diluted in a malt extract versus the results of the hydrophobin ELISA (A 405 nm). Two different sets of standards (Exp. 1: 5, 10, 20, 35, 50, 75, 100, 150 and 175 µg/mL, and Exp. 2: 10, 25, 50, 75, 100, 125, 150 and 200 µg/mL) were tested in duplicate. The results of the two highest standard concentrations are excluded from the standard curve because they were outside the linear range. The standard protein concentration experiments were the result of separated determinations of different hydrophobin preparations of *F. poae.*
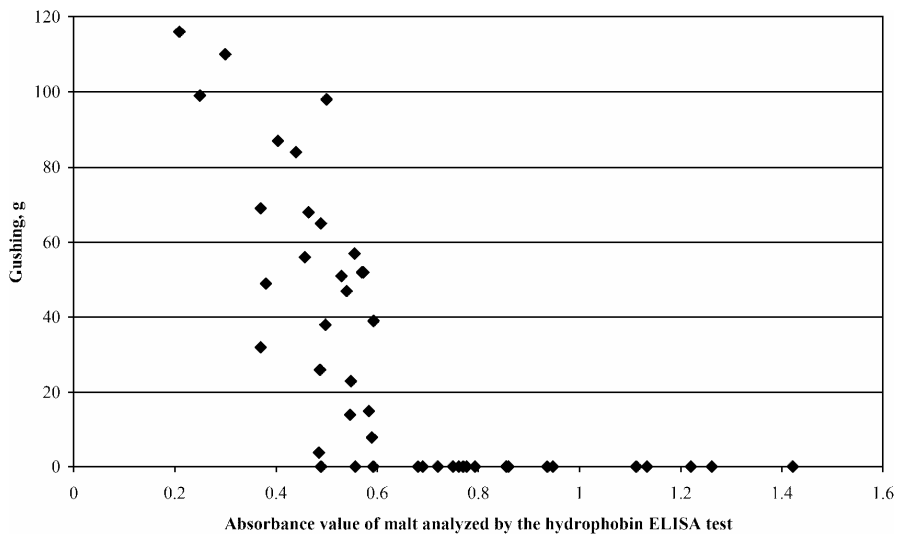


**Fig. 4.** Absorbance values of the malt samples analyzed by the hydrophobin ELISA versus gushing potentials of the malts (n = 44). In the competitive ELISA, low levels of absorbance reflect high levels of hydrophobin in the sample.

### Hydrophobin levels in barley and malt samples compared to gushing potential and deoxynivalenol content of malt

The hydrophobin ELISA developed was used to assess the levels of hydrophobin in barley and malt samples infected both naturally and artificially with *Fusarium* fungi.

The results of the hydrophobin ELISA were compared to the results of the gushing test. A correlation was found between the hydrophobin level and the gushing potential of malt; the risk of gushing was observed to be increased if the absorbance value of the malt in the hydrophobin ELISA was below 0.6, corresponding to a hydrophobin concentration of ca. 250 µg/g malt (Fig. 4). All the malt
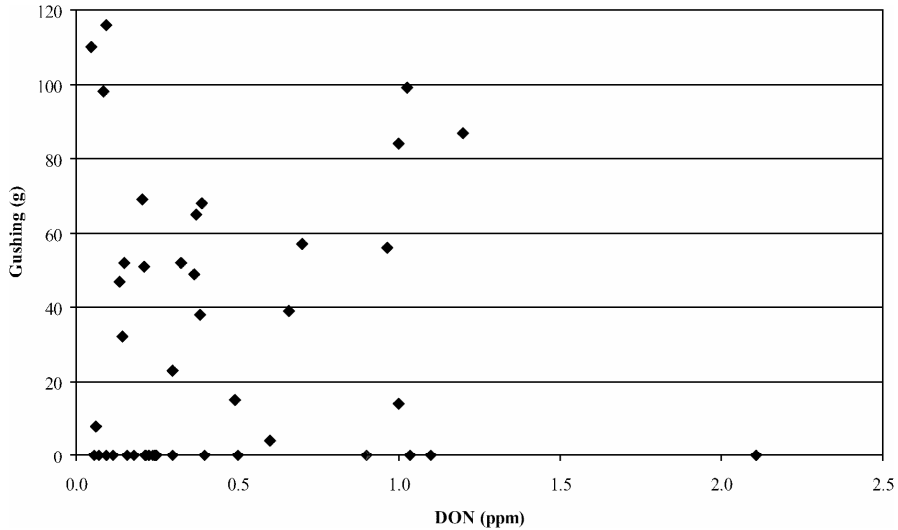
**Fig. 5.** DON content of malt versus gushing potential of malt (n = 44).

samples with absorbance values of 0.4 or lower, corresponding to hydrophobin concentrations of ca. 500 µg/g malt, repeatedly induced gushing in the gushing test.

When the results of the hydrophobin ELISA of the barley samples were compared to the gushing potential of the corresponding malts, no clear correlation was found except in the cases where artificially or otherwise heavily *Fusarium*-infected barleys were studied (data not shown). This is probably due to the production of hydrophobins during the malting process, which could be concluded from the lower hydrophobin levels detected in the barleys compared to those of the corresponding malts (data not shown). Previous studies also support this conclusion because fungi, especially fusaria, have been shown to proliferate and to produce mycotoxins and gushing factors in the malting conditions[12,23,28,30].

Furthermore, no connection was found between the results of the hydrophobin ELISA and the deoxynivalenol (DON) contents of the malts (data not shown). This observation implies that the accumulation of hydrophobin and DON are not necessarily metabolically linked. Munar and Sebree also stated that the formation of DON and the gushing factors might be independent of each other[23]. We also compared the DON content of malt with its gushing potential. Once again, we did not observe any correlation between these two parameters (Fig. 5). This finding is inconsistent with the results of Schwarz *et al.*[28] who found strong correlation between the DON level and the gushing potential of malt produced from North American barley samples. Most of the samples analyzed in this study were grown in Finland. The DON contents of Finnish barleys have been reported to be low[9,20]. For example in the seasons of 1995–1999, all Finnish commercial malting barley samples studied contained less than 96 µg DON/kg barley[20]. The most common *Fusarium* species in Finnish grain has been reported to be *F. avenaceum* which is incapable to produce DON[9,37]. On the contrary, a strong

DON producer, *Fusarium graminearum,* has been reported to be the predominating *Fusarium* species in North American barley[28,31]. It was estimated that 67–82% of the malting barley crops produced in the upper Midwestern region of the USA had been contaminated with DON (range 600–60 000 µg/kg) during 1993–1996[29].

## CONCLUSIONS

Fungal hydrophobins are able to induce gushing of beer even at low concentrations. They can be assumed to be one of the gushing factors produced by fungi, but not necessarily the only ones. The ability of hydrophobins to induce gushing varied, and this was assumed to be due to differences in their structure resulting from variation in their amino acid sequences (Table II). A correlation was found between the hydrophobin level in malt (below absorbance 0.6) and the gushing potential of malt, suggesting that a hydrophobin analysis could be used for predicting the risk of gushing in malt. The possible formation of hydrophobins during malting complicates the use of the hydrophobin ELISA to predict the gushing potential directly from barley. Detection of hydrophobin coding genes from barley could enable more accurate gushing prediction. All mycelial fungi are assumed to produce hydrophobins, but only some fungal species are able to produce mycotoxins, which makes hydrophobins more reliable indicators for gushing than for example DON. In general, determination of the actual gushing-inducing factors improves the accuracy of gushing prediction. Hydrophobin analysis of malt offers a means for improved prediction. As reported by Laible and Geiger[19], fungi could also produce gushing-inhibiting substances, such as polar lipids, in barley and malt. Knowledge of both gushing-inducing and -inhibiting substances would enable maltsters and brewers to make a correct prediction of gushing risk in beer.

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# Identification and characterization of gushing-active hydrophobins from *Fusarium graminearum* and related species

## Research Paper

# Identification and characterization of gushing-active hydrophobins from *Fusarium graminearum* and related species

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Fungal infection of barley and malt, particularly by the *Fusarium* species, is a direct cause of spontaneous overfoaming of beer, referred to as gushing. We have shown previously that small fungal proteins, hydrophobins, act as gushing-inducing factors in beer. The aim of our present study was to isolate and characterize hydrophobins from a gushing-active fungus, *Fusarium graminearum* (teleomorph *Gibberella zeae*) and related species. We generated profile hidden Markov models (profile HMMs) for the hydrophobin classes Ia, Ib and II from the multiple sequence alignments of their known members available in public domain databases. We searched the published *Fusarium graminearum* genome with the Markov models. The best matching sequences and the corresponding genes were isolated from *F. graminearum* and the related species *F. culmorum* and *F. poae* by PCR and characterized. One each of the putative *F. graminearum* and *F. poae* hydrophobin genes were expressed in the heterologous host *Trichoderma reesei*. The proteins corresponding to the genes were purified and identified as hydrophobins and named GzHYD5 and FpHYD5, respectively. Concentrations of 0.003 ppm of these hydrophobins were observed to induce vigorous beer gushing.

## Introduction

Gushing is a phenomenon in which beer spontaneously foams out from the container immediately on opening (Fig. 1). Two types of gushing can be distinguished based on the origin of gushing inducing substances [1, 2]. Primary gushing is due to abnormalities in the raw materials of beer and is known to be caused by fungal infection of barley and malt, particularly by the species of *Fusarium* [3–6]. Non-malt related gushing, i.e. secondary gushing, is due to faults in the beer produc-

tion process or to the incorrect treatments of packaged beer. *Fusarium graminearum* (teleomorph *Gibberella zeae*), is the most common *Fusarium* head blight (FHB) pathogen of cereals world wide as well as a well-documented gushing inducer [6–8]. In addition to *F. graminearum*, *F. culmorum* and *F. poae* have also been shown to produce gushing factors in the field during the growing period of barley as well as during the malting process [9, 10].

We have shown previously that small fungal proteins called hydrophobins act as the gushing factors of beer [11, 12]. Hydrophobins are highly surface active, moderately hydrophobic proteins produced by filamentous fungi [13–15]. A characteristic feature of these proteins is their eight conserved cysteine residues forming four disulphide bridges in the molecule. Hydrophobins have

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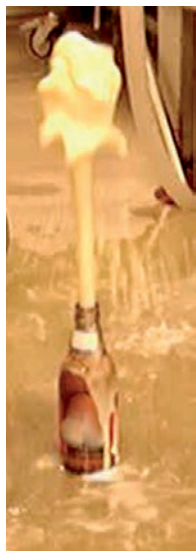**Fax:** +358 20 722 7071

**Figure 1.** Gushing of beer induced by the hydrophobin GzHYD5. 50 µg of purified GzHYD5 was added directly in bottled beer and the bottle was agitated according to the laboratory gushing test [29, 30] prior opening.

diverse roles in fungal growth and development [15, 16]. They act as structural components and in interactions between fungi and their environments. Hydrophobins can be found in the structural parts of fungi or they can be secreted to the culture medium. Based on sequence comparison, hydrophobins are divided into two different classes, I and II [17]. Class II hydrophobins have been observed thus far only in Ascomycetes, whereas class I hydrophobins have been observed in both Ascomycetes and Basidiomycetes [14]. Thus, the class I hydrophobins can further be divided into two sub-groups Ia and Ib, which represent the class I hydrophobins of Ascomycetes and Basidiomycetes, respectively.

In the GenBank sequence database (www.ncbi.nlm. nih.gov) about 140 hydrophobins can be found, all from filamentous fungi. Fuchs *et al.* [18] identified five hydrophobin genes encoding both class I and II hydrophobins in *F. verticillioides*. In addition, two hydrophobin genes of *F. culmorum* have been identified and expressed in *Saccharomyces cerevisiae* [19] and in *Pichia pastoris* [20].

The aim of our work was to isolate and characterize hydrophobins from a gushing-active fungus *Fusarium graminearum* and related species. Detailed physico-chemical study of relevant hydrophobins has previously been hindered by the difficulties of producing and purifying well-characterized hydrophobins in sufficient

amounts. To overcome this problem, we have used recombinant production of two relevant hydrophobins. Unlike most other attempts to produce hydrophobins we have used a filamentous fungus as the production host. The advantage of this approach is that the secretion pathway of this host is very similar to that of the original organism. Therefore, we could expect that production levels would be higher and that the mature proteins would be similar in structure (folding – disulfide formation, post-translational modifications) to the wild-type. The use of highly purified protein also allows a quantitative analysis.

## Materials and methods

### Microbial strains, media and culture conditions

Fungal strains used in this study were obtained from the VTT Culture Collection and are listed in Table 1. The *Fusarium graminearum* (teleomorph *Gibberella zeae*) strain VTT D-051036 (NRRL 31084) used for isolation of the putative hydrophobin genes in this study was the same strain whose genome has been sequenced and published by the Broad Institute (http://www.broad. mit.edu). Potato Dextrose Agar (Difco Laboratories, Detroit, USA) was used for maintenance of the strains. For mycelium production, the strains were cultivated in a shake flask containing Potato Dextrose Broth (Difco Laboratories) for four days at room temperature (approx.

**Table 1.** Fungal strains used for screening the presence of the putative hydrophobin genes in their genomes.

| Species | Strain | Origin |
|---|---|---|
| *Fusarium culmorum* | VTT D-80148 | Barley, Finland |
| *F. graminearum* | VTT D-82082 | Barley |
| *F. graminearum* | VTT D-82086 | Barley |
| *F. graminearum* | VTT D-82169 | Barley |
| *F. graminearum* | VTT D-95472 | Corn, USA |
| *F. graminearum* | VTT D-051036 | Wheat or barley, USA |
| *F. poae* | VTT D-76038 | Barley |
| *F. poae* | VTT D-82182 | Oat, Germany |
| *F. sporotrichioides* | VTT D-72014 | Grain |
| *F. avenaceum* | VTT D-80141 | Barley, Finland |
| *F. oxysporum* | VTT D-80134 | Grain |
| *F. oxysporum* | VTT D-98690 | Bulb of Tulipa sp., Germany |
| *F. sambucinum* | VTT D-77056 | Grain |
| *F. solani* | VTT D-77057 | Grain |
| *F. equiseti* | VTT D-82087 | Rotting fruit of Cucumis melo, Turkey |
| *F. tricinctum* | VTT D-96600 | Barley, Finland |
| *Cochliobolus sativus* | VTT D-76039 | Barley, Finland |
| *Alternaria alternata* | VTT D-76024 | Barley |
| *Aspergilluis ochraceus* | VTT D-00808 | Barley, Finland |
| *Penicillium chrysogenum* | VTT D-96661 | Moldy house, Finland |

25 °C). The mycelium was separated from culture medium by filtration through a GF/B glass fiber filter (Whatman International Ltd., Maidstone, USA), washed with sterilized water and freeze-dried.

Electrocompetent *Escherichia coli* DH5α cells were used to generate entry and expression clones. LB agar plates containing 50 µg kanamycin ml$^{-1}$ or 125 µg hygromycin B ml$^{-1}$ were used for selective growth of entry and expression clones, respectively.

The (HFBII) hydrophobin deletion strain of *Trichoderma reesei* VTT D-99676 [21] was used for the expression of the hydrophobin gene isolated from *F. graminearum* and *F. poae*. Positive *T. reesei* transformants were selected on agar plates containing per liter 182.2 g sorbitol, 20 g glucose, 15 g KH$_2$PO$_4$, 18.6 g Agar Noble, 1 ml of trace element concentrate, pH 5.5, 2.4 ml of 1 M MgSO$_4$, 4 ml of 1 M CaCl$_2$, 10 ml of 1 M acetamide and 12.5 ml of 1 M CsCl. Positive transformants were grown on *Trichoderma* minimal medium [22] supplemented with 3% lactose at 28 °C for four to seven days in order to promote the expression of the inserted *Fusarium* hydrophobin gene.

### Extraction of genomic DNA

Genomic DNA was extracted from the freeze-dried mycelia using FastDNA® Spin Kit for Soil (Qbiogene, Carlsbad, USA) according to the manufacturer's instructions. The lysing step was performed using a FastPrep® Cell Disrupter, model FP120 (Qbiogene) with four cycles of 30 s at a setting of 6.0 m s$^{-1}$. Between the disruption cycles the samples were cooled on ice. In addition, genomic DNA of the strain *F. graminearum* VTT D-051036 was extracted from freeze-dried mycelium as described by Raeder and Broda [23].

### Generation of profile HMMs

Profile Hidden Markov Models (profile HMMs) are statistical models of multiple sequence alignments [24]. They capture position-specific information about how conserved each column of the alignment is, and which residues are likely in that column. The basic assumption of the models is that the identity of a particular position is independent of the identity of all other positions. In this study profile HMMs were generated using the version 2.3.2 of the HHMER software (http://hmmer.org) for the hydrophobin classes Ia, Ib and II from the multiple sequence alignments of their known members gathered from the Ref. [14]. The classes Ia and Ib represent the class I hydrophobins of *Ascomycetes* and *Basidiomycetes*, respectively. The *Fusarium graminearum* genome database of predicted proteins published by the Broad Institute (http://www.broadinstitute.org) was searched with

the models. The lower the E-value calculated by the software, the better the sequence matches with the model. The best matching sequences and the corresponding genes were chosen for further studies.

### Isolation of putative hydrophobin genes from *Fusarium*

PCR primers with *att*B sites were designed for the gene of the best matching hydrophobin sequence found from the *F. graminearum* genome database under the locus tag FG01831.1. The attB-PCR primers were as follows: 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TCA TGA AGT TCT CAC TCG CCG C-3′ (sense) and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA GTC CTG GAC ACC AGT AG-3′ (antisense). PCR reactions were set up in a total volume of 50 µl by mixing 300 ng of target DNA per reaction with 1 µl of DyNAzyme EXT enzyme (Finnzymes, Espoo, Finland), 5 µl of DyNAzyme F514 buffer (Finnzymes), 4 µl of dNTPs mix containing 2.5 mM of each dNTP (Sigma-Aldrich, Suffolk, UK), 5 µl of both oligonucleotide primer solutions (1 µM, Sigma-Aldrich) and PCR grade water to the final volume. PCR grade water instead of DNA was used as a negative control. The PCR program consisted of initial denaturing at 94 °C for 4 min, followed by 25 cycles of denaturing at 94 °C for 30 s, primer annealing at 45 °C, 50 °C, 55 °C and 60 °C for 30 s, and elongation at 72 °C for 20 s, and a final extension at 72 °C for 10 min. *att*B-PCR products were separated in 1% (w/v) agarose gels and visualized using UV light. Bands containing the expected size PCR-fragment were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA concentration was measured using the Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany). Instead of using all four annealing temperatures mentioned above, the annealing temperature of 50 °C was used for screening the presence of the putative hydrophobin genes from all fungal strains listed in Table 1.

### Cloning and expression of the putative *Fusarium* hydrophobin genes in *Trichoderma reesei*

The putative hydrophobin genes were cloned using the Gateway® Technology kit (Invitrogen, Carlsbad, CA, USA). The purified *att*B-PCR fragments generated with the *att*B-PCR primers as described above were ligated into the pDONR221 vector (Invitrogen) with a BP recombination reaction and transformed into the electrocompetent *E. coli* DH5α cells according to the manufacturer's instructions in order to generate entry clones. Positive transformants were selected using LB agar plates containing kanamycin. Entry clones were puri-

fied with the QIAprep Spin Miniprep Kit using a micro-centrifuge (Qiagen) according to the manufacturer's instructions. Restriction of entry clones with suitable restriction enzymes was performed to check the size of the inserts. Both strands of the entry clones containing inserts of the right size were sequenced independently to verify correct insertion.

An entry clone with the correct insert was used to create an expression clone by ligating the insert into the *T. reesei* expression vector pMS186 [25] with a LR recombination reaction. The pMS186 contains the Gateway reading frame cassette C inserted between the *cbh1* (cellobiohydrolase 1) promoter and terminator of *T. reesei*, and a hygromycin resistance cassette. The created expression vector was transformed into the electrocompetent *E. coli* DH5α cells, and positive transformants were selected using the LB agar plates containing hygromycin B. Generated expression clones were purified and verified by sequencing the both strands. The expression cassette was released from the vector with NotI restriction enzyme (New England Biolabs, Ipswich, MA, USA) and purified from a 1% (w/v) agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

The expression cassette was transformed together with the plasmid pToC202 containing an acetamide selection marker into the protoplasts of the hydrophobin deletion strain of *T. reesei* VTT D-99676 [21], essentially as described by Penttilä *et al.* [22]. Transformants were selected for acetamide resistance on plates containing 10 mM acetamide. The transformants were streaked on the selective medium for two successive rounds and isolated by single-spore cultures. The presence of the expression cassette in the genome was checked by PCR using the *att*B-PCR primers, as described above. Positive transformants were grown in shake flasks containing *Trichoderma* minimal medium [22] supplemented with 3% lactose at 28 °C for four to seven days in order to promote the expression of the insert.

**Purification of expressed hydrophobins**
Putative hydrophobins were isolated from the culture filtrates of the positive transformants grown in 50 ml of *Trichoderma* minimal medium [22] supplemented with 3% lactose by bubbling air through culture filtrates and then collecting the foam produced [26]. Aqueous two-phase extraction using Berol 532 surfactant (Akzo Nobel Surface Chemistry AB, Stenungsund, Sweden) was used for hydrophobin isolation from culture filtrates (500−1000 ml) at large scale, as described by Linder *et al.* [27]. Purification of hydrophobins from

the fungal mycelium was also attempted. For this, mycelium was mixed with 4 M guanidine hydrochloride in 0.2 M Tris/HCl at pH 7.5 for 2 h, and then centrifuged. The supernatant was diluted with an equal volume of water before extraction with surfactant (Berol 532). The surfactant extraction was performed as for the culture filtrates. The samples from the foam and from the surfactant extraction were analyzed by SDS-PAGE and by reversed phase high performance liquid chromatography (RP-HPLC). RP-HPLC was performed using a Vydac C4 column (Vydac, Hesperia, CA, USA) with an ÄKTAexplorer chromatographic system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Elution was performed with a 0−100% gradient of acetonitrile in 0.1% trifluoroacetic acid. Detection was by UV at 215, 280 and 375 nm. Fractions eluted with 35−50% acetonitrile were collected.

**Protein analytical methods**
Samples were analyzed by SDS-PAGE under reducing conditions on 17.5% gels [28] using the 2050 Midget Electrophoresis System (Pharmacia LKB Biotechnology, Sweden), followed by visualization of the proteins by silver staining (Silver Stain Kit, Bio-Rad Laboratories, Hercules, USA). Protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) or by HPLC using a standard which had a concentration known by amino acid analysis. Putative hydrophobins were characterized by N-terminal protein sequencing and mass spectrometry (performed at the Protein Chemistry Laboratory, Institute of Biotechnology, University of Helsinki, Finland) and by amino acid analysis (performed at the Department of Biochemistry and Organic Chemistry of Uppsala University, Sweden). N-terminal protein and peptide sequencing was performed by degradative Edman chemistry using a Procise 494A Sequencer (Perkin Elmer, Applied Biosystem Division, Foster City, CA, USA). Protein and peptide molecular masses were determined with Autoflex™ or Ultraflex™ MALDI-TOF/TOF mass spectrometers (Bruker-Daltonics, Bremen, Germany) in the positive ion reflector mode. The samples were dried on the target plate together with an equal volume of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/acetonitrile (1:1, v/v). The MALDI-TOF spectra were externally calibrated with standard protein or peptide mixtures from Bruker-Daltonics (Bremen, Germany). For structural characterisation, proteins were alkylated with 4-vinylpyridine, desalted by reversed-phase chromatography and subjected to enzymatic digestion with trypsin (1% w/w, Sequencing Grade Modified Trypsin, V5111; Promega, USA) over-

night at 37 °C, as described by Selinheimo *et al.* [25]. Separation of tryptic peptides was performed by reversed-phase chromatography. For amino acid analysis, protein samples were hydrolysed in 2 ml of 6 M HCl/0.1% phenol at 110 °C for 24 h prior to analysis with the Biochrom model 20 analyser. Cys, Pro and Trp residues could not be accurately quantitated by this method.

## Determination of gushing-inducing ability of hydrophobins

The gushing-inducing ability of hydrophobins was studied by adding 1, 10 and 50 µg of the RP-HPLC purified hydrophobins directly into bottled beer or carbonated mineral water (0.33 l, $CO_2$ content of mineral water 0.6% (w/w)). The bottles were agitated gently in a horizontally rotating shaker (50 rpm) for three days as described in the procedure of a gushing test commonly used for prediction of gushing potential of malt [29, 30]. After agitation, the bottles were kept still for 10 min, inverted three times and opened after 30 s. The amount of gushing was determined from the change in weight of the bottle.

## Results

### Putative hydrophobin genes of *Fusarium graminearum*

Statistical profile hidden Markov models (profile HMMs) were generated for the hydrophobin classes Ia, Ib and II from the multiple sequence alignments of their known members [14]. The *Fusarium graminearum* genome database of predicted proteins (http://www.broadinstitute.org) was searched using the generated profile HMMs of the hydrophobin classes. This revealed five uncharacterized genes showing similarity with known hydrophobin sequences. Four of them, the locus tags FG01763.1, FG01764.1, FG03960.1 and FG09066.1, were classified as genes encoding class I hydrophobins and one, the locus tag FG01831.1, as a gene encoding a class II hydrophobin. The most significant hits were FG01831.1 with the model of class II (E-value 4.5e-41), FG03960.1 with the model of class Ib (E-value 1.7e-07) and FG09066.1 with the model of class Ia (E-value 0.0079). The gene FG03960.1 was also found with the model of class Ia (E-value 0.043). Analysis of the deduced protein sequences encoded by the putative hydrophobin genes with the program SignalP [31] indicated that all the proteins have a signal sequence, and thus are predicted to be secreted.

The gene FG01831 was chosen for further studies. Amplification of the gene FG01831 from genomic *F. graminearum* D-051036 DNA by PCR with the attB primers as described above revealed an amplicon of about 500 bp. The size of the amplicon was consistent with an approximate gene size of 392 bp plus 62 bp added by the FG01831 *att*B-PCR primers. The purified amplicon was ligated and transformed into *E. coli* DH5α cells, as described above. The sequencing of the both strands revealed a hydrophobin gene with an expected DNA sequence, referred to as *Gibberella zeae hyd5* gene with an EMBL accession number FN668637. The deduced amino acid sequence of the corresponding protein, GzHYD5, is given in Fig. 2.

```
Seq. 1    MKFSLAAVALLGAVVSALPANEKRQAYIPCSGLYGTSQCCATDVLGVADL 50

Seq. 2    MKFSLAAVALLGAVVSALPANEKRQAYIPCSGLYGTSQCCATDVLGVADL 50

Seq. 3    MKFSLAAVTLLGAVVSALPANEKRQAYVPCTGLYGSSQCCATDVLGVANL 50

          ********:******************:**:****:*************:*


Seq. 1    DCGNPPSSPTDADNFSAVCAEIGQRARCCVLPILDQGILCNTPTGVQD 98

Seq. 2    DCGNPPSSPTDADNFSAVCAEIGQRARCCVLPILDQGILCNTPTGVQD 98

Seq. 3    DCGTPPSVPANATDFSAVCAEIGQRARCCVLPILDQGILCNTPTGVQD 98

          ***.*** *::* :**********************************
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**Figure 2.** Comparison of predicted protein sequences of *F. graminearum* (GzHYD5, seq. 1), *F. culmorum* (seq. 2) and *F. poae* (FpHYD5, seq. 3) corresponding to the hypothetical protein coded by the gene with the locus tag FG01831.1 in the *Fusarium graminearum* genome database of predicted proteins. Signal peptides predicted using the program SignalP are marked in bold. The signal peptide cleavage site determined in GzHYD5 and FpHYD5 is indicated by the arrow.

PCR using the FG01831 *att*B-PCR primers was used to screen the *Fusarium* strains listed in Table 1 for the presence of a *G. zeae hyd5* homolog in their genomes. The results obtained suggested that all five isolates of *F. graminearum* had the gene (data not shown). Moreover, an amplicon was also produced with the strains of *F. culmorum*, *F. equiseti*, *F. poae* and *F. sporotrichioides* (data not shown). The amplicons produced from *F. culmorum* (VTT D-80148) and *F. poae* (VTT D-82182) DNA were purified, ligated into the pDONR221 vector, cloned in *E. coli* DH5α cells and sequenced, as described above. The DNA sequences obtained revealed that the *G. zeae hyd5* gene shared 96% and 88% identity with the corresponding genes of *F. culmorum* and *F. poae*, respectively (introns excluded). Comparison of translated amino acid sequences without predicted signal peptides indicated 100% and 88% identity between GzHYD5 and the corresponding proteins of *F. culmorum* and *F. poae*, respectively (Fig. 2). The *G. zeae hyd5* homolog in *F. poae* is referred to as *Fusarium poae hyd5* gene with an EMBL accession number FN669508. The *G. zeae hyd5* and the *F. poae hyd5* were chosen for the expression studies in *T. reesei*.

## Expression of G. zeae hyd5 and F. poae hyd5 genes in Trichoderma reesei

The *G. zeae hyd5* insert as well as the *F. poae hyd5* insert from the pDONR221 vectors were transferred to the *T. reesei* expression vector pMS186, as described above, in order to create an expression construct in which the protein-coding region of the genomic *G. zeae hyd5* or the genomic *F. poae hyd5* was between the *T. reesei cbh1* promoter and terminator. The *cbh1* promoter is a strong inducible promoter and active throughout the cultivation when the fungus is grown in inducing conditions, e.g. with cellulose and lactose. The expression constructs were transformed into a strain of *T. reesei* from which the cellulose- and lactose-inducible HFBII hydrophobin had been deleted, and the transformants were selected for acetamide resistance on selective acetamide-containing plates and tested with PCR, as described above. A number of transformants with a correct expression cassette were found (data not shown). The positive transformants were cultivated in shake flasks containing 50 ml of *Trichoderma* minimal medium supplemented with 3% lactose in order to promote the expression of the heterologous hydrophobins. Proteins were isolated as described above. The SDS-PAGE analysis of the foam samples revealed that the culture filtrates of some transformants contained proteins with a molecular mass about 10 kDa as expected for hydrophobins (data not shown). These trans-

formants were cultivated in shake flasks (500–1000 ml) and the culture filtrates were subjected to aqueous two-phase extraction. The extracts were further purified by RP-HPLC, as described above. To illustrate the use of two phase extraction, an example of chromatograms from the different stages of the procedure for one *G. zeae hyd5* transformant is shown in Fig. 3. For *F. poae hyd5* transformants the results were similar although hydrophobin concentrations were higher. The chromatogram peaks that were most efficiently enriched in the two phase extraction were collected and subjected to more detailed characterization. The *G. zeae hyd5* culture supernatant showed a peak at 41.8% acetonitrile and
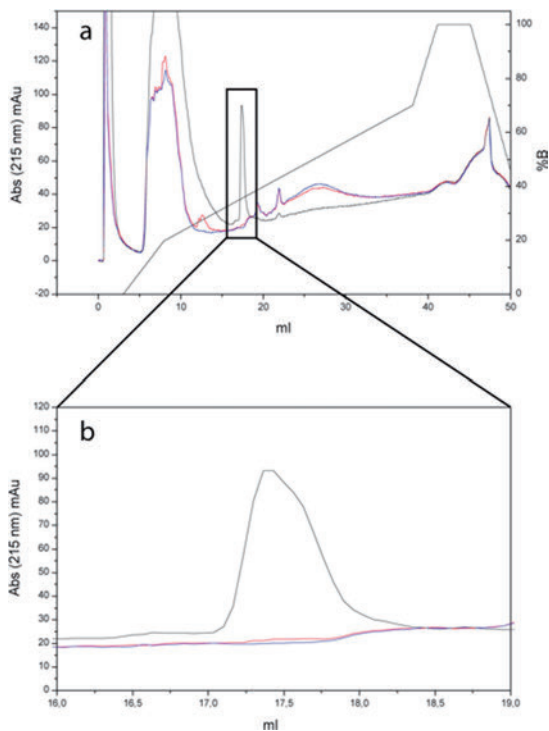


**Figure 3.** Chromatograms from the aqueous two-phase system (ATPS) purification of the GzHYD5 protein. The entire chromatogram is shown in (a) and in (b) the hydrophobin peak is zoomed, showing only the part from 16 to 19 ml (as indicated by the lines). The red curve is the growth medium containing secreted GzHYD5, the blue curve is the medium after the hydrophobin has been extracted, and the black curve is the extracted hydrophobin. The GzHYD5 is seen as an almost undetectably small peak in the red curve and it becomes noticeable only when compared to the blue curve where it is removed. The 50× concentrating effect of the ATPS results in a clearly detectable peak in the black curve. The content of other peaks in the chromatograms than the GzHYD5 at 17.5 ml was not determined. In all three chromatograms the same volume was injected.

the *F. poae hyd5* culture supernatant showed one peak that eluted at 40.5% acetonitrile. As described below they were identified to be the expected hydrophobins and named GzHYD5 and FpHYD5 for the *G. zeae hyd5* and *F. poae hyd5* transformants, respectively.

The yields of the hydrophobins were as follows. We purified 1 mg of GzHYD5 from one liter of culture supernatant. No GzHYD5 protein was found associated with the mycelium (data not shown). The yield of FpHYD5 was 18 mg from one liter of supernatant. About 1.5 mg of FpHYD5 was also extracted from 20 grams of mycelium, which corresponds to the amount produced in one liter cultivation.

## Detailed characterization of GzHYD5

MALDI-TOF MS of the RP-HPLC purified putative GzHYD5 hydrophobin protein fraction gave a signal corresponding to an average molecular mass of 7 571 Da. The theoretical average molecular mass of GzHYD5 calculated from the encoded amino acid sequence (including the signal sequence) is 10033.5 Da. N-terminal sequence analysis of the purified protein (100 pmol) did not result in any PTH-amino acid signals from the 10 cycles of Edman degradation performed, indicating that the protein has a blocked N-terminus. Cleavage of the signal sequence is predicted to occur between Arg-24 and Gln-25 residues. The sequencing result obtained thus suggests that the newly exposed N-terminal Gln is cyclized to a pyroglutamic acid. Cyclization of N-terminal Gln residues after signal sequence cleavage is frequently observed in fungal proteins [25]. The calculated average mass of the mature protein with the signal sequence cleaved between residues Arg-24 and Gln-25 and with a pyroglutamic acid at the N-terminus is 7577.5 Da. If the mature protein contains 4 disulfide bridges between its 8 Cys residues the calculated average mass will be 7569.5 Da, which is in good agreement with the obtained mass of 7571 Da.

For further characterization of the putative GzHYD5 protein, the sample was reduced and alkylated with 4-vinylpyridine. During these reactions the possible disulfide bonds of the protein are reduced and 4-vinyl-pyridine molecules react with the –SH groups of the Cys residues generating an additional mass of 105.1 Da per Cys residue. The molecular mass of the alkylated RP-HPLC purified protein analyzed by MALDI-TOF MS was 8422 Da ($\Delta m = 851$ Da) indicating the presence of eight Cys residues in the protein as expected for a hydrophobin.

For further characterization, the alkylated GzHYD5 was fragmented by trypsin which was expected to result in three tryptic peptides with monoisotopic masses

of 5687.550 Da, 245.129 Da and 2516.226 Da based on the primary sequence. MALDI-TOF MS analysis showed two peptides of mass 5683 Da and 2517 Da. For sequence analysis the peptides were separated by reversed phase chromatography. For the 5683 Da peptide no sequence was obtained. This peptide corresponds to the N-terminus of the protein and the result was in line with the previous result that the N-terminal amino acid of the protein is a pyroglutamic acid. The mass of the 2517 Da peptide corresponds to the C-terminal tryptic peptide of GzHYD5. The identity of this peptide was further confirmed by N-terminal sequencing which gave the sequence CCVLPILDQGILC. The 245 Da peptide (AR) was too small to be recovered by reversed phase chromatography for sequencing.

Finally the putative GzHYD5 protein was subjected to amino acid analysis. The results are presented in Table 2 and corresponded to the expected composition. In summary, the molecular mass, tryptic digestion, number of Cys-residues, internal sequencing, and amino acid composition confirm that the purified protein was the correct GzHYD5 protein.

## Detailed characterization of FpHYD5

The calculated average molecular mass for FpHYD5 based on the amino acid sequence is 10 003.6 Da including the signal peptide. The signal sequence is predicted to be cleaved between residues Arg-24 and Gln-25 analogously as was seen for GzHYD5. The calculated

**Table 2.** The calculated and the measured amino acid content of GzHYD5 and FpHYD5.

| Amino acid | GzHYD5 | | FpHYD5 | |
|---|---|---|---|---|
| | Mole of residues per mole of protein | | Mole of residues per mole of protein | |
| | calculated | measured | calculated | measured |
| N + D | 10 | 9.8 | 8 | 7.9 |
| T | 5 | 5.0 | 6 | 6.0 |
| S | 5 | 5.1 | 4 | 4.2 |
| Q + E | 6 | 6.3 | 6 | 6.3 |
| P | 6 | 7.4 | 6 | 7.2 |
| G | 7 | 7.2 | 7 | 7.2 |
| A | 7 | 7.1 | 8 | 8.1 |
| C | 8 | NA | 8 | NA |
| V | 5 | 4.8 | 7 | 6.7 |
| M | 0 | 0.0 | 0 | 0.0 |
| I | 4 | 3.6 | 3 | 2.7 |
| L | 6 | 5.9 | 6 | 5.9 |
| Y | 2 | 2.0 | 2 | 1.9 |
| F | 1 | 1.0 | 1 | 1.0 |
| H | 0 | 0.0 | 0 | 0.0 |
| K | 0 | 0.1 | 0 | 0.1 |
| R | 2 | 2.0 | 2 | 2.0 |

NA: not analysed

mass of the mature protein, with the N-terminal Gln cyclized to a pyroglutamic acid, would then be 7517.6 Da. In MALDI-TOF MS the RP-HPLC purified putative FpHYD5 showed a major peak with a mass of 9213 Da (data not shown). In addition to the major mass peak heavier molecules with a repeating mass difference of about 80 Da were detected (data not shown). The molecular mass of the alkylated protein analyzed by MALDI-TOF MS was 10 062 Da ($\Delta m = 849$ Da) indicating the presence of eight Cys residues in the FpHYD5 protein. To verify the identity of the isolated protein, amino acid analysis was performed (Table 2). This showed a very good fit with the expected composition. Because the amino acid composition was correct but the mass too large, we conclude that the FpHYD5 was glycosylated. Consistent with this, there is a typical N-glycosylation site, NATD, in the sequence starting at position 37. Additionally the amino acid analysis also revealed that the sample contained glucosamine (unlike the GzHYD5). Looking at the structures of the homologous proteins HFBI and HFBII from *T. reesei* [32, 33] we note that the predicted glycosylation site is positioned in a surface-exposed part of the protein on its hydrophilic side. Thus, we conclude that all data support the conclusion that the isolated protein is FpHYD5 and that it is post-translationally modified by glycosylation corresponding to a mass of 1695 Da.

### Gushing inducing ability of GzHYD5 and FpHYD5

Our previous studies have shown that pronounced gushing can be induced by adding hydrophobins directly into bottled beer and shaking the bottles gently for three days before opening [12]. For example, addition of an amount as low as 1 µg of *T. reesei* hydrophobins HFB I and HFB II into bottled beer (0.33 l), corresponding to a concentration of 0.003 ppm, was sufficient to cause gushing. In this study, RP-HPLC purified

GzHYD5 and FpHYD5 were also observed to induce beer gushing (Table 3). Similar to *T. reesei* hydrophobins amounts as low as 1 µg of GzHYD5 and FpHYD5 were able to cause vigorous overfoaming in beer. In addition, GzHYD5 and FpHYD5 were observed to induce gushing in carbonated mineral water (Table 3).

## Discussion

Hydrophobins are produced ubiquitously by filamentous fungi. Hydrophobin genes have been identified in many Ascomycetes and Basidiomycetes including some *Fusarium* species [14, 18, 19]. In many cases, more than just one hydrophobin is present in one species. We generated profile hidden Markov models (profile HMMs) for the different hydrophobin classes and searched the *F. graminearum* genome database of predicted proteins with these models. The search revealed five putative hydrophobin genes belonging to both the hydrophobin classes I and II. The hypothetical proteins encoded by these genes had a specific Cys pattern of hydrophobins in their predicted amino acid sequences. The best matching sequences were found from the hypothetical proteins under the locus tags FG01831.1, FG03960.1 and FG09066.1. The finding was in accordance with the findings of Fuchs *et al.* [18] and Zapf *et al.* [19]. The translated protein sequence of the locus FG09066.1 was the most closely related to the deduced protein sequences of class I hydrophobins Hyd3p from *F. verticillioides* [18] and FcHyd3p from *F. culmorum* [19]. The hypothetical protein of FG03960.1 was found with both the class I profile HMMs, indicating that the protein has sequence similarities with hydrophobins of both Ascomycetes and Basidiomycetes. Only low sequence homology was observed between the predicted protein sequence of FG0390.1 and other hydrophobins. The translated protein sequence of the locus FG01831.1 was closely related to the deduced protein sequence of class II hydrophobin Hyd5p from *F. verticillioides* (identity 89%) [18] and was identical with the deduced protein sequence of FcHyd5p from *F. culmorum* [19] (predicted signal peptides excluded). Our study confirmed the results of Zapf *et al.* [19] in respect of the identification of FcHyd5 hydrophobin gene from *F. culmorum*. In addition, we identified the corresponding gene also from *F. poae* encoding a mature protein that was 88% identical to the corresponding predicted mature proteins of *F. garminearum*, *F. culmorum* and *F. verticillioides*. Moreover, our study indicated that the corresponding gene is most likely present in *F. equiseti* and *F. sporotrichioides* as well.

**Table 3.** Gushing in beer and in carbonated mineral water induced by GzHYD5 and FpHYD5. Purified hydrophobins were added in bottled beer and mineral water in duplicate, and the bottles were agitated according to the laboratory gushing test [29, 30]. After agitation, the bottles were opened and the amounts of overfoaming beer were determined from the change in weight of the bottle. The results are the means of two replicates.

| Amount of hydrophobin, microgram in 0.33 l | Gushing, g | | | |
| --- | --- | --- | --- | --- |
| | in beer (*n* = 2) | | in mineral water (*n* = 2) | |
| | GzHYD5 | FpHYD5 | GzHYD5 | FpHYD5 |
| 0 | 0 | 0 | 0 | 0 |
| 1 | 106 | 34 | 68 | 101 |
| 10 | 178 | 167 | 133 | 143 |
| 50 | 207 | 207 | 150 | 159 |

The gene FG01831.1, referred to as *Gibberella zeae hyd5* gene (the EMBL accession number FN668637), was isolated from genomic DNA of *F. graminearum* by PCR and transformed into the HFBII hydrophobin deletion strain of *T. reesei*. The protein GzHYD5 was successfully expressed in *T. reesei* and purified from the culture filtrate of one over-producing transformant. The molecular mass of the mature protein was 7571 Da, which agrees with the molecular masses of other hydrophobins [26, 36]. Although the deduced protein sequences of GzHYD5 and *F. culmorum* hydrophobin FcHyd5p showed 100% identity, Stübner *et al.* [20] reported that the transgenic FcHyd5p produced by *P. pastoris* clones had an approximate size of 12 kDa according to SDS-PAGE. They concluded that post-translational modifications might occur in FcHyd5p.

Characterization of GzHYD5 revealed that the protein contains eight Cys residues. The pattern of eight Cys residues is one main unifying feature of hydrophobins [16]. The molecular masses of the tryptic peptides were observed to be as predicted supporting the protein sequence deduced for GzHYD5. In addition, the determined N-terminal sequence of the C-terminal tryptic fragment was identical to the predicted one. Moreover, the results of the amino acid analysis showed a very good fit with the expected composition of GzHYD5. These findings confirmed that the purified protein was the correct GzHYD5 protein.

The *G. zeae hyd5* homolog of *F. poae* referred to as *Fusarium poae hyd5* gene (the EMBL accession number FN669508) was also successfully expressed in *T. reesei* and the corresponding protein FpHYD5 was purified from the culture filtrate of one over-producing transformant. The presence of eight Cys residues was also observed in FpHYD5. Although the results of the amino acid analysis corresponded to the expected composition of FpHYD5, the measured molecular mass (9213 Da) was greater than the calculated one (7518 Da) indicating post-translational modifications. As mentioned above, post-translational modifications were also suggested to occur in the transgenic FcHyd5p produced by *P. pastoris* clones [20]. The SC3 hydrophobin of *Schizophyllum commune* has been observed to be glycosylated containing 17−22 mannose residues [39]. Glycosylation of proteins has also been shown to occur in *T. reesei*. In the case of *Trichoderma reesei* tyrosinase TrTyr2 the protein with a sodium adduct was found to be glycosylated with a glycan consisting of two N-acetylglycosamines and five hexoses [25]. Mature FpHYD5 contains one putative N-glycosylation site at the asparagine residue N(37). Molecular modeling suggests that this residue is on the opposite side than the hydrophobic binding site

in the protein (Fig. 4). This positioning would not interfere with the surface binding function of the hydrophobin and seems very likely. The observed mass difference (1695 Da) could correspond to the sodium adduct (23 Da) of FpHYD5 with a glycan consisting of two N-acetylglycosamines ($2 \times 203.20$ Da) and seven hexoses ($7 \times 162.14$ Da). The phosphorylation of this construct could explain the ladder of 80 Da detected by MALDI-TOF MS.

The hydrophobin yields purified from the culture supernatants of *G. zeae hyd5* and *F. poae hyd5* transformants were 1 mg $l^{-1}$ of GzHYD5 and 18 mg $l^{-1}$ of FpHYD5. In the laboratory fermenter cultivations of the wild-type *Trichoderma reesei* strains, the maximum production of 500 mg $l^{-1}$ and 30 mg $l^{-1}$ of *T. reesei* HFB I and HFB II have been observed, respectively [40]. *Schizophyllum commune* has been reported to secrete the SC3 and SC4 hydrophobins in quantities up to 60 mg $l^{-1}$ and 10 mg $l^{-1}$, respectively [41, 42].

Previous studies have demonstrated that hydrophobins are able to induce beer gushing [12, 18, 20]. Hydrophobins can be produced in the field or during malting of fungal infected barley, and they can be transmitted through the brewing process ending up in the final beer [10]. Hydrophobins are assumed to stabilize carbon dioxide bubbles in beer by forming a layer around the microbubbles [43, 44]. This layer may prevent breakdown of the microbubbles in stored beer by hindering the gas diffusion out of the bubbles. Upon bottle opening the external pressure in the bottle drops and the stabilized microbubbles may expand leading to overfoaming. This theory was supported by the studies of Stübner *et al.* [20] which showed that a very stable foam was formed by introducing air into the cell-free
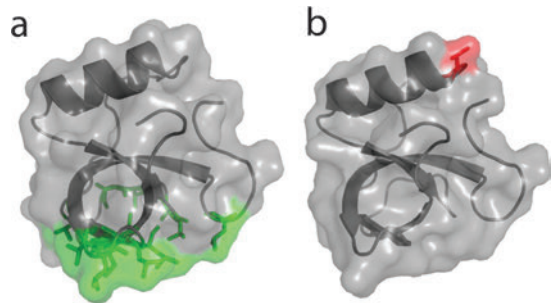


**Figure 4.** Homology model of FpHYD5 showing the position of the N-glycosylation site. In (a) the previously determined structure of *T. reesei* HFBII [32] is shown with the surface binding hydrophobic face coloured green. In (b) the model of FpHYD5 is shown with the glycosylated Asn-37 shown in red. The FpHYD5 model was made based on the HFBII structure using the SWISS-MODEL automated homology modelling service (swissmodel.expasy.org).

culture supernatants containing the *F. culmorum* hydrophobin FcHyd5p. In this study, we demonstrated that addition of an amount of 0.003 ppm of GzHYD5 and FpHYD5 directly in beer was sufficient to induce gushing. The amount of hydrophobins needed to induce beer gushing varies. In our previous studies, an amount of 0.003 ppm of *T. reesei* hydrophobins HFBI and HFBII in beer was able to induce gushing whereas 10 and 30 times higher amounts of hydrophobins produced by *Nigrospora* sp. and *F. poae* were needed for gushing induction, respectively [12]. The gushing inducing capability of GzHYD5 and FpHYD5 corresponds to that of *T. reesei* hydrophobins HFBI and HFBII. An amount of approx. 0.2 ppm of FcHyd5p with 6× His-tag was needed to induce beer gushing in the study of Stübner *et al.* [20]. The significantly higher activity of the tag-free hydrophobins characterized in this study suggests that it is important to retain a native structure of hydrophobins in order to understand their function in a quantitative way. Gushing phenomenon is not only restricted to beer, because also other carbonated beverages like sparkling wine, ciders, fruit spritzers etc. may gush [45]. Hydrophobins might be a cause of overfoaming of other carbonated beverages in addition to beer as demonstrated with mineral water in this study.

A connection has been observed between the hydrophobin content determined by the hydrophobin ELISA and the gushing potential of malt [12]. In addition, previous studies have indicated that despite the substantial loss of hydrophobins during brewing a portion survived the brewing process, ending up in the final beer [10]. However, there is still limited knowledge of the effects of brewing process on hydrophobins. Detailed studies are needed to investigate the extent of the effects of different process steps on the gushing-inducing ability and on the concentration of hydrophobins migrating in the brewing process.

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## Conflict of interest statement

No financial/commercial conflicts of interest exist.

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# Fungal hydrophobins in the barley-to-beer chain

# Fungal Hydrophobins
# in the Barley-to-Beer Chain

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### ABSTRACT

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Fungal hydrophobins have been shown to induce gushing of beer. In order to study the occurrence and fate of hydrophobins at different stages of the production chain of beer, barley samples artificially infected in the field with *Fusarium culmorum, F. graminearum* and *F. poae* were collected during the growing period as well as during various stages of the malting process. In addition, naturally infected malt was brewed in pilot scale and samples were collected throughout the process. The samples were assayed for hydrophobin content using an ELISA method. The results showed that fungi produced hydrophobins that accumulated during barley grain development in the field, but that production was more pronounced during malting. Prolonged storage of barley tended to reduce the ability of fungi to produce hydrophobins in malting. Studies on the fate of hydrophobins during the brewing process revealed that mashing released hydrophobins from the malt into the wort. Some loss of hydrophobins occurred throughout the brewing process with spent grains, cold break (wort boiling) and surplus yeast. In addition, the beer filtration step reduced hydrophobin levels. Despite the substantial loss of hydrophobins during brewing, the level was high enough to induce the gushing detected in the final beer.

**Key words:** Brewing, *Fusarium,* hydrophobins, malting.

## INTRODUCTION

Hydrophobins are small surface-active proteins produced by filamentous fungi[17,18]. A characteristic feature of these proteins is their eight cysteine residues, whose sequence position is conserved. Hydrophobins self-assemble at their hydrophilic-hydrophobic interfaces to form amphipathic membranes[19]. This property allows hydrophobins to fulfil a broad spectrum of functions in fungal growth and development[2,20]. By self-assembly, hydrophobins are able to stabilise air bubbles and oil droplets in water, decrease the surface tension of water, and change the nature of a surface from hydrophilic to hydrophobic and *vice versa*[18].

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Our recent studies revealed that fungal hydrophobins induced undesirable beer gushing[6,7]. Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously overfoams out from the container immediately on opening. Fungal infection of barley and malt, in particular by species of *Fusarium,* is known to cause beer gushing[3,5,8,13]. However, the distribution and fate of fungal gushing factors, hydrophobins, in the beer production chain is still largely unknown. We have developed an enzyme linked immunosorbent assay (ELISA) for hydrophobins and showed that the hydrophobin ELISA can be used for prediction of the gushing risk of malt[12]. In addition, the genus *Fusarium* contains toxigenic species which produce mycotoxins, such as deoxynivalenol (DON), during barley grain development in the field and during malting[8,11,14]. A high portion of DON present in malt has been shown to survive the brewing process and end up in the beer[14]. Although the presence of hydrophobins is not completely predictive of the presence of mycotoxins[8,12], in some ways their presence and gushing is a consumer warning of the more sinister quality concerns with gushing beer.

The aim of this investigation was to study the occurrence and fate of hydrophobins at different stages of the beer production chain by analyzing the hydrophobin levels of samples taken throughout the barley-to-beer chain with the hydrophobin ELISA.

## MATERIALS AND METHODS

### Barley and malt samples

Barley samples non-inoculated and artificially inoculated in the field with *Fusarium culmorum* VTT D-80148, *F. graminearum* (teleom. *Gibberella zeae*) VTT D-95470 and *F. poae* VTT D-82182 as previously described by Pekkarinen *et al.*[9] were studied. The gushing positive and negative malt samples for lab-scale mashing studies were purchased from Carlsberg Research Laboratory, Denmark. For pilot scale brewing studies, a heavily *Fusarium* infected malt (six-rowed malting variety Robust, crop year 2002) was kindly provided by Dr. Paul Schwarz, North Dakota State University, USA.

### Laboratory scale malting

Barley samples (1 kg) were steeped separately for two days at 12°C to a moisture content of 46% and germinated for 6 days at 14°C in a Seeger laboratory malting unit (Seeger GmbH, Germany). The moisture contents of the samples were determined daily and if necessary, distilled

water was added to adjust the moisture content to 45%. Kilning was carried out in 21 h with a stepwise temperature increase up to 85°C in a forced-air Seeger laboratory kiln (Seeger GmbH, Germany). Samples for the hydrophobin analysis were taken after steeping, germination and kilning. Moist samples were freeze-dried prior to the analysis.

## Laboratory scale mashing

Laboratory scale high gravity mashing[15] was performed using the gushing positive and negative malt samples purchased from Carlsberg Research Laboratory, Denmark. The former sample had repeatedly induced vigorous beer gushing in the gushing test. Ground malt, 50 g, was mixed with 200 ml of preheated water (50–52°C) containing 100 ppm $CaCl_2$ and 0.75 mM $H_2SO_4$. The sample mixture was then transferred to the mashing bath (Bender & Hobein, Munich, Germany) and the following mashing procedure was performed with a temperature increase rate of 2°C/min: 48°C/30 min – 63°C/30 min – 72°C/30 min – 80°C/10 min. After mashing the hot mash was filtered through a GF/A glass fiber filter (Whatman International Ltd., Maidstone, UK) and the wort was boiled for 45 min. The boiled wort was left to stand in the cold overnight and the formed cold break was removed by centrifugation. The amounts of hydrophobins in malt grist, spent grains, cold break and wort were analyzed using the hydrophobin ELISA[12]. Spent grains and cold break were freeze-dried prior to the hydrophobin analysis.

## Pilot scale brewing

Both gushing positive and negative malt samples were brewed on a 10 litre scale. Heavily *Fusarium* infected six-rowed Robust malt, which induced beer gushing in the gushing test, was used as a gushing positive sample. The gushing negative two-rowed Scarlett-malt (crop year 2001), with a low *Fusarium* contamination rate, was produced in the pilot malting house at VTT Biotechnology. About 2 kg of malt grist was mashed with 8 litres of water according to the following mashing program: 48°C/30 min, 64°C/30 min, 72°C/30 min and 80°C/10 min (temperature increase rate 1°C/60 s). Wort was lautered at 75°C and boiled with hops for 60 min. After clarification wort was pitched with the lager yeast strain VTT A-63015 and fermented at 10°C for 8 days. Secondary fermentation was performed at 15°C for 15 days and stabilisation at –1°C for 5 days. Beer was filtered through Seitz EK sheets (SEITZ-FILTER-WERKE GmbH, Bad Kreuznach, Germany) and bottled. Process samples (malt grist, spent grains, wort before and after boiling, cold break, yeast after the main fermentation, and beer before and after filtration) were collected and frozen at –20°C. Spent grains, cold break and yeast were freeze-dried prior to the hydrophobin analysis.

## Wort boiling and beer filtration studies

In order to study the effects of wort boiling on the gushing-inducing ability of hydrophobins, approximately 0, 10, 50 and 100 μg of *Trichoderma reesei* HFBII hydrophobins were added to 1 ml of unboiled wort or to water. The hydrophobin + wort and hydrophobin + water samples were kept in the boiling water bath for 1 h. Precipi-

tates formed during boiling were centrifuged and the clear supernatants were added to commercially brewed and bottled beer (0.33 L). The bottles were shaken according to the gushing test (see below). Unboiled hydrophobin + wort samples were used as controls. The test was performed in duplicate.

The effects of the beer filtration step on hydrophobin levels were studied by adding purified hydrophobins of *F. poae* to distilled water. Distilled water was used instead of beer because beer naturally contains high amounts of many proteins which could interfere with the determination of the low hydrophobin contents of the samples. Two different hydrophobin concentrations, approximately 5 μg/mL and 15 μg/mL, were examined. Water-hydrophobin samples, 30–50 mL, were filtered through Seitz K150 filter sheets (SEITZ-FILTER-WERKE GmbH, Bad Kreuznach, Germany) in the laboratory scale beer filtration unit (Sartorius, Goettingen, Germany) with a pressure of 1–1.4 bar. The hydrophobin concentrations in water before and after filtration were determined in duplicate with the BC Assay Protein Determination Kit (Uptima, France) according to the manufacturer's instructions.

## Hydrophobin analysis

Competitive ELISA (Enzyme Linked ImmunoSorbent Assay) developed for detection of hydrophobins in barley and malt was used to determine the hydrophobin levels in the samples collected from the field or during malting and brewing[12]. Briefly, 5 g of the ground sample was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. Liquid samples, such as wort and beer, were tested directly without the extraction. The extract was centrifuged and the supernatant was transferred to a clean tube, and antibodies against the hydrophobin of *F. poae* were added. After incubation the sample-antibody mixture was transferred to at least three replicate wells of an immunoplate (Nunc-Immuno Modules, MaxiSorp polystyrene strips, Nunc, Rochester, USA) coated with a hydrophobin extract of *F. poae*. Goat anti-rabbit IgG (H + L)–alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma, St. Louis, USA) in diethanolamine-$MgCl_2$ buffer (Oy Reagena Ltd, Toivala, Finland) were used as substrate for AP detection. The absorbance was read at 405 nm using a Multiskan Ex microtitre plate reader (Labsystems, Helsinki, Finland) and the mean of the absorbance values of the replicate wells was calculated. Because of the nature of the competitive ELISA, a lower absorbance value corresponds to a higher amount of hydrophobins in the sample. The hydrophobin level is expressed as the inverse of the mean absorbance value (1/Abs). A standard deviation was calculated using the Microsoft Office Excel 2003 program. A standard curve generated from purified hydrophobins of *F. poae* was used to approximate the hydrophobin content in the samples collected and analysed by the hydrophobin ELISA[12].

## Gushing potential

Gushing potential of malt was determined according to the gushing test described by Vaag *et al.*[16] using a horizontally rotating shaker (50 rpm)[4]. In this test, an aqueous
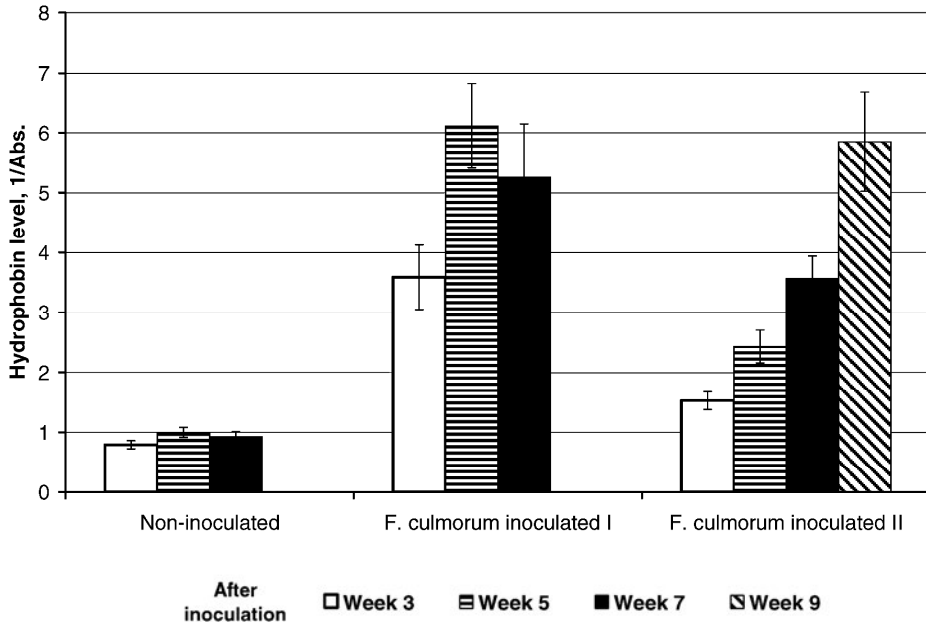
**Fig. 1.** Hydrophobin levels in the non-inoculated and *F. culmorum* inoculated barley head samples collected during the field trials at approximately 3, 5, 7 weeks and from the field II trials also 9 weeks after inoculation (n = 8). Standard deviation bars are included.

extract of ground malt was added to bottled beers and the pasteurized bottles were shaken for three days. After shaking, the bottles were kept still for 10 min, inverted three times and opened after 30 sec. The amount of gushing was determined from the change in weight of the bottle.

## RESULTS AND DISCUSSION

### Formation of hydrophobins during the growing period of barley

Fungi, especially *Fusarium* species, are known to be able to produce gushing factors during the growing period of barley[1,8]. In order to study the formation of hydrophobins during the growing period, head samples of the non-inoculated and two *F. culmorum* inoculated barleys were collected from two fields approximately 3, 5, 7 and 9 weeks after inoculation. The weather conditions during the field trials favored the growth of *Fusarium* spp., resulting in high infection rates in the head samples, and in the harvested barleys 78–100% of the kernels were contaminated with *Fusarium* spp[9]. Hydrophobin levels in the freeze-dried head samples were determined in duplicate using the hydrophobin ELISA. The results revealed that hydrophobins were present in the *F. culmorum* infected heads three weeks after inoculation and the formation of hydrophobins continued throughout the growing period (Fig. 1). Differences between the infection rates of the individual heads randomly collected from the 10 m² test blocks could cause fluctuations in the results of the sensitive hydrophobin ELISA. This could explain the reduc-

tion in hydrophobin level of the *F. culmorum* inoculated sample I taken 7 weeks after inoculation, compared to the hydrophobin level of the sample taken 2 weeks earlier.

### Hydrophobins in the malting process

*Fusarium* fungi are able to proliferate and produce gushing factors during the malting process[8,11,13]. The results of the hydrophobin ELISA of the two non-inoculated and the three *Fusarium*-inoculated barley samples collected during the lab-scale malting process revealed that hydrophobins were also produced during malting, especially during the steeping and germination steps (Fig. 2). Lower hydrophobin levels were detected in the final malt after removal of the rootlets. A comparison between the estimated amounts of hydrophobins detected in barley and in malt revealed that over tenfold higher amounts of hydrophobins were found in malt, compared to those in the corresponding barley (data not shown). The highest hydrophobin levels were detected in the malts produced from barleys inoculated with *F. culmorum* and *F. graminearum* (Fig. 2). These malts also induced the most vigorous gushing in the gushing test (data not shown).

Relatively high initial hydrophobin levels were detected in barleys inoculated with *F. culmorum* and *F. graminearum* (Fig. 2), which made them easily distinguishable from the non-gushing barley samples based on the results of the hydrophobin ELISA. However, the formation of hydrophobins during malting complicates the use of the hydrophobin analysis to predict the gushing potential directly from barley with a low initial hydrophobin level, as in the case of the *F. poae* inoculated samples
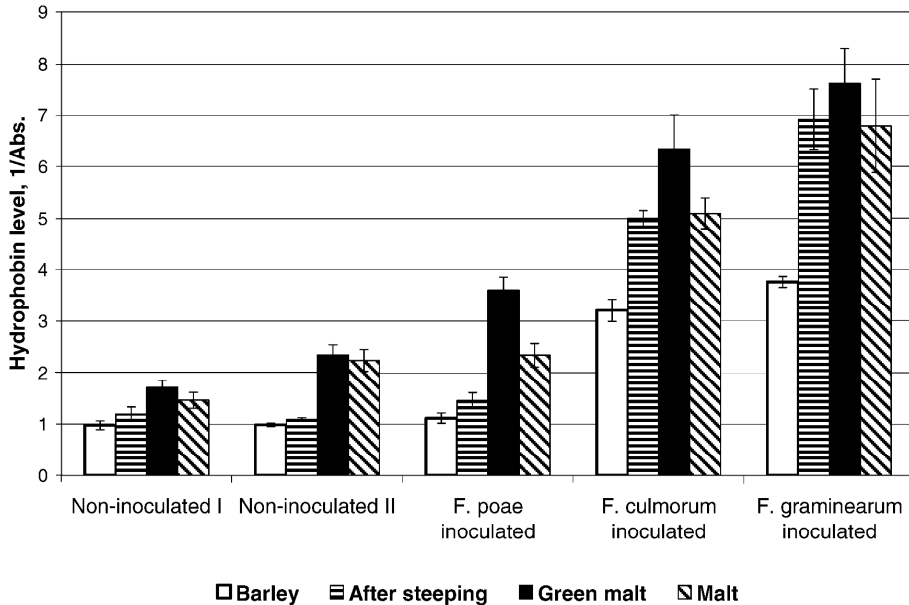
**Fig. 2.** Changes in hydrophobin levels of the non-inoculated and *Fusarium*-inoculated barley samples taken at different stages of the laboratory scale malting process. Hydrophobin levels were analyzed using the hydrophobin ELISA (n = 4). Standard deviation bars are included.

(Fig. 2). A short incubation of moist barley in order to activate the hydrophobin production prior to the hydrophobin analysis could help to overcome this problem.

Samples of the two non-inoculated barleys, I and II, from the field trials were malted two or three times during the storage period of 17 months at 15°C. The hydrophobin level and the gushing potential of the malts were determined after each malting. The results showed reduction in hydrophobin formation as well as in the gushing potential as a result of prolonged storage (Table I). These findings are consistent with the results reported by Munar and Sebree[8], who also observed a substantial loss of gushing-factor formation after 180 days of storage. Differences in hydrophobin levels of the non-malted barleys I and II were small, but after malting of barley stored for 5–6 months, the hydrophobin level of the malt IIa produced from barley II was three times higher than that of the malt Ia produced from barley I (Table I). This observation revealed the significant effect of malting on the hydropho-

**Table I.** The effect of the storage time of barley on the hydrophobin level and on the gushing potential of the corresponding malt.

| Sample | Storage of barley (months) | Hydrophobin level (1/Abs.) | Gushing (g) |
|---|---|---|---|
| Non-inoculated barley I | | 0.71 | nd |
| Malt Ia | 5 | 1.52 | 0 |
| Malt Ib | 9 | 1.11 | 0 |
| Non-inoculated barley II | | 0.90 | nd |
| Malt IIa | 6 | 4.41 | 49 |
| Malt IIb | 12 | 2.22 | 3 |
| Malt IIc | 17 | 1.96 | 0 |

nd: not determined.

bin formation if the barley lot is contaminated with viable gushing-active *Fusarium* species, as in the case of the non-inoculated barley II.

### Hydrophobins in mashing

Hydrophobins must be released from malt in the mashing process, survive the process conditions and end up in the wort in order to be able to induce gushing in beer. In order to study the behavior of hydrophobins during mashing, we mashed both gushing positive and negative malt samples on a laboratory scale and determined the hydrophobins in malt grist, freeze-dried spent grains, freeze-dried cold break and wort. The results of the hydrophobin ELISA indicated that hydrophobins were partly extracted into the wort during mashing, although hydrophobins were also detected in spent grains and cold break (Fig. 3). When the estimated extractable hydrophobin content of malt grist was calculated and compared with the hydrophobin content of the corresponding wort (data not shown), a substantial loss of hydrophobins was observed to occur during mashing; at most 20% of the hydrophobins present in malt grist was found in wort. In this study we mashed the malt samples according to one standard mashing procedure. Changes in mashing procedure, such as in a proteolytic stand or in agitation speed, could affect the hydrophobin levels that end up in the wort.

### Purified hydrophobins in wort boiling and beer filtration

The results of the gushing test of the boiled and unboiled hydrophobin + wort and hydrophobin + water samples are shown in Table II. The gushing inducing potential
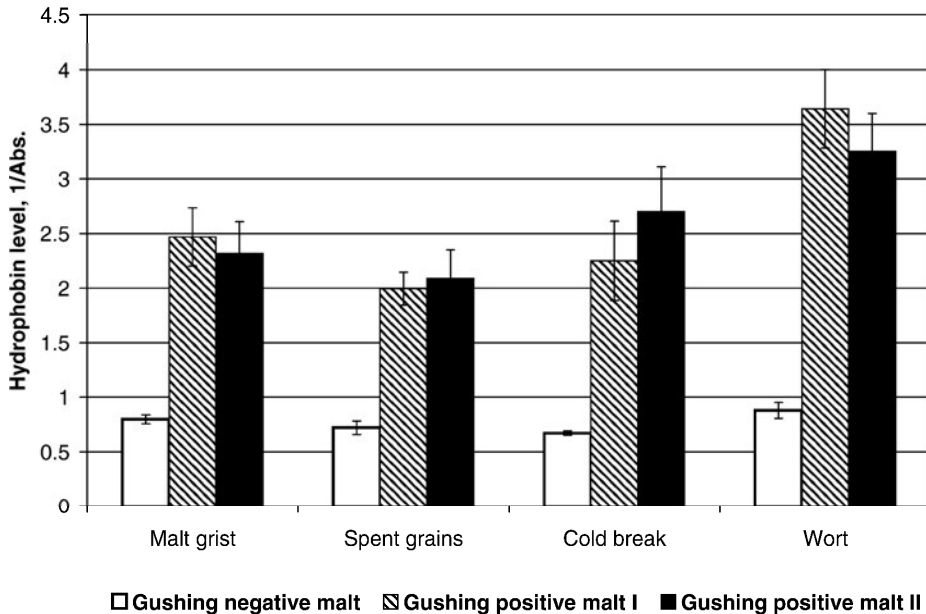
**Fig. 3.** Hydrophobin levels in aqueous extracts of malt grist, spent grains and cold break, and in wort of the gushing negative and positive malt samples mashed on a laboratory scale. Hydrophobin levels were analyzed using the hydrophobin ELISA (n = 3). Standard deviation bars are included.

of the boiled hydrophobin + wort samples was observed to be lower than that of the corresponding unboiled samples, suggesting that some of the hydrophobins were precipitated or inactivated during wort boiling. Boiling alone could not destroy or inactivate hydrophobins, because the gushing inducing potential of the boiled hydrophobin (50 µg) + water samples was comparable to that of the unboiled hydrophobin (50 µg) + wort samples (Table II). The results indicate that wort contains substances which interact with hydrophobins in wort boiling, causing a reduction in the gushing activity of hydrophobins.

The effect of beer filtration on hydrophobin levels was also studied by filtering the hydrophobin-water mixtures through the beer filtration unit. The results of the filtration studies suggested that part of the hydrophobins can be removed by filtration (data not shown). Based on the properties of hydrophobins, it can be assumed that hydrophobins adhere to the filter sheets or that they form aggregates large enough to be retained by the filters. However,

**Table II.** The effect of boiling on the gushing inducing ability of *T. reesei* HFBII hydrophobin boiled in wort or in water. The test was performed in duplicate.

| HFBII addition | Beer gushing (g) | |
| --- | --- | --- |
| | Boiled | Unboiled |
| 0 µg in wort | — | 0 |
| 10 µg in wort | 0 | 102 |
| 50 µg in wort | 38 | 188 |
| 100 µg in wort | 149 | 199 |
| 50 µg in water | 173 | — |

—: not determined.

the level of reduction was dependent on the initial hydrophobin concentration of the sample; with a hydrophobin concentration of 15 µg/mL, 60% of the hydrophobins were lost during the filtration, compared to a loss of only 15% with a hydrophobin concentration of 5 µg/mL. Robinson *et al.*[10] observed that the filter sheets removed proteins until saturated, after which the protein contents of the filtrated beers were at the same level as that of the beers before filtration. The saturation of filter sheets could also occur with hydrophobins decreasing the removing effect of filtration.

**Hydrophobins in the pilot-scale brewing process**

Hydrophobin levels were studied throughout the brewing process by brewing the gushing negative and positive malts on a 10-liter scale. As can be seen from Fig. 4, the beer brewed from the gushing positive malt contained a higher amount of hydrophobins than the beer brewed from the gushing negative malt. The former beer overfoamed when shaken according to the gushing test, indicating that a hydrophobin level high enough to induce gushing of beer was first extracted from the malt and then survived the brewing process. As in the mashing studies (Fig. 3), some of the hydrophobins were removed with spent grains and cold break (wort boiling), but also with surplus yeast (data not shown). In addition, the beer filtration step reduced the hydrophobin levels (Fig. 4).

The changes in the relative levels of hydrophobins during brewing were calculated based on the approximation of the total hydrophobin content of the gushing positive malt and the corresponding brewing liquors (Fig. 5). The
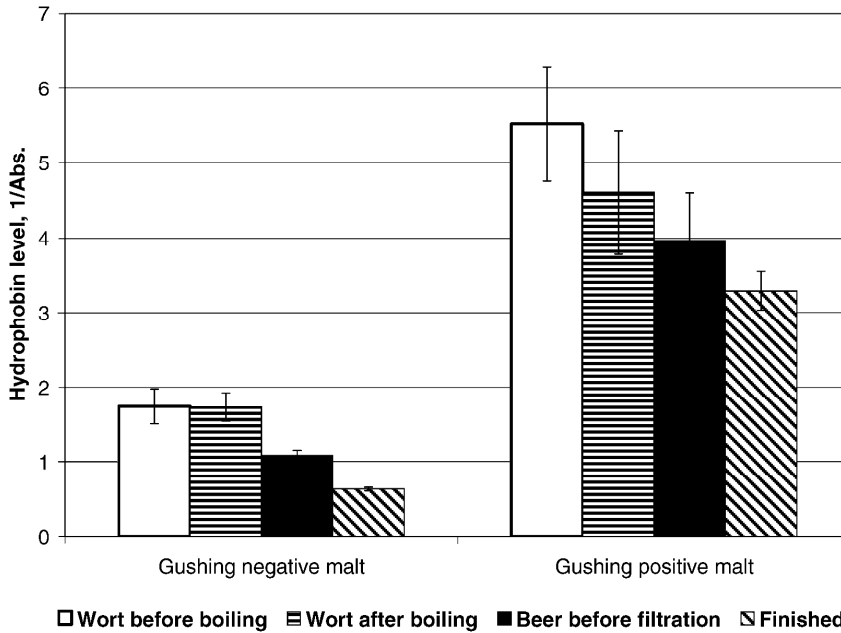
**Fig. 4.** The effects of wort boiling and beer filtration on the hydrophobin levels of brewing liquors produced from the gushing negative and positive malts. Hydrophobin levels were analyzed using the hydrophobin ELISA (n = 4). Standard deviation bars are included
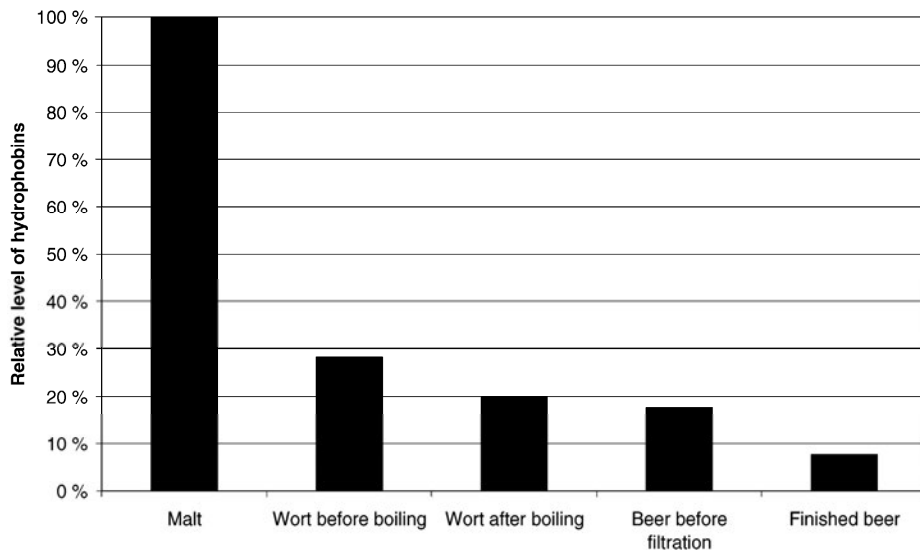


**Fig. 5.** Relative levels of hydrophobins in the pilot-scale brewing process. The calculated amounts of hydrophobins in brewing liquors were compared to the calculated amount of hydrophobins in gushing positive malt used for brewing.

calculation revealed that most of the hydrophobins originating from the gushing positive malt were removed with the spent grains (up to 70%). In addition, wort boiling, fermentation and beer filtration steps reduced the hydrophobin content to such an extent that only approximately 10% of the original hydrophobin content of the gushing positive malt was present in the finished beer. These results are in accordance with the results obtained in the

laboratory scale mashing studies, as well as with the results obtained in the boiling and filtration studies of the purified hydrophobins.

## CONCLUSIONS

*Fusarium* fungi were able to produce hydrophobins in the field during the growing period of barley, as well as during the malting process, especially during the steeping and germination steps. Due to the decreased viability of *Fusarium* spp, the capability of fungi to produce hydrophobins was reduced during prolonged storage of barley. Hydrophobins were extracted during mashing and a portion survived the brewing process, ending up in the final beer where they induced gushing when present in sufficiently high levels. Hydrophobins were removed from the brewing process mainly with spent grains, but also with cold break and surplus yeast. In addition, the beer filtration step reduced the hydrophobin level of the final beer. The estimation of hydrophobin content, based on the hydrophobin ELISA results of the samples collected during the pilot-scale brewing study, showed that only about 10% of the hydrophobin present in the gushing positive malt was found in the final beer.

Our studies revealed that the amount of hydrophobins can increase during malting, but decrease during brewing. Malting and brewing processes vary between different malt houses and breweries, which may significantly influence the production and the fate of hydrophobins during the beer production chain. Further large scale studies are required to investigate the extent of the effects of commercial malting and brewing processes on the hydrophobin content migrating in the beer production chain.

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# Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors

ORIGINAL PAPER

# Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors

**Arja Laitila · Tuija Sarlin · Erja Kotaviita ·
Timo Huttunen · Silja Home · Annika Wilhelmson**

**Abstract** *Fusarium* infection of barley and malt can cause severe problems in the malting and brewing industry. In addition to being potential mycotoxin producers, *Fusarium* fungi are known to cause beer gushing (spontaneous overfoaming of beer). Cereal-derived bacteria and yeasts are potential biocontrol agents. In this study, the antifungal potential of selected yeasts (12 strains) derived from the industrial malting ecosystem was studied in vitro with a plate-screening assay. Several ascomycetous yeast strains showed antagonistic activity against field and storage moulds, *Pichia anomala* being the most effective strain. The effects of *P. anomala* VTT C-04565 (C565) were examined in laboratory scale malting with naturally contaminated barley exhibiting gushing potential. *P. anomala* C565 restricted *Fusarium* growth and hydrophobin production during malting and prevented beer gushing. Grain germination was not disturbed by the presence of yeast. Addition of *P. anomala* C565 into the steeping seemed to retard wort filtration, but the filtration performance was recovered when yeast culture was combined with *Lactobacillus plantarum* VTT E-78076. Well-characterized microbial cultures could be used as food-grade biocontrol agents and they offer a natural tool for tailoring of malt properties.

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## Introduction

The fungal community characteristic to malting barley develops before harvest, during storage and during the malting process. More than 150 species of filamentous fungi and yeasts may be found on cereal grains as surface contaminants or as internal invaders [53]. It is well known that barley-derived fungi and their metabolites greatly influence malt and beer quality [17, 42, 65]. *Fusarium* moulds are important members of the indigenous fungal community of barley. The abundance of *Fusarium* contamination and the diversity of the species are dictated particularly by crop susceptibility, agricultural practices, climate and geographic location [7, 61]. Contamination of the barley crop by fusaria is of concern particularly in years when poor weather conditions favor the growth of toxigenic and gushing-active *Fusarium* species. Gushing is a term used to describe spontaneous overfoaming of beer on opening of the packaged product, and it is often associated with heavy *Fusarium* infection of barley or malt [2, 57]. Gushing is a complex phenomenon, which can at least partially be explained by the secretion of specific factors by fungi in barley in the field, during storage, or during the malting process [2, 41, 52]. Gushing factors are assumed to be surface-active molecules, which stabilize $CO_2$ bubbles in beer by forming a layer around microbubbles [46].

Our recent studies indicated that fungal proteins called hydrophobins act as the gushing factors of beer [26, 30, 52]. Hydrophobins are small, secreted, cysteine-rich proteins ($100 \pm 25$ amino acids) that are produced by filamentous fungi [68]. Hydrophobins are among the most

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abundantly produced proteins of fungi and they have various biological roles and unique properties [36]. They have the property of self-assembly at hydrophilic–hydrophobic interfaces forming very stable insoluble amphipathic films, and are involved in fungal adherence to surfaces [68]. These protein films are commonly found on surfaces of aerial structures such as hyphae, conidia, and fruiting bodies [62]. A hydrophobic coating has also been proposed to have a protecting role against both desiccation and wetting, and to assist spore dispersal. Hydrophobins play key roles in development and in the interactions of fungi with the environment and other organisms, particularly plants [67].

Strict control of incoming barley lots is vitally important in order to reject contaminated material prior to purchasing. However, malting conditions favor the growth of *Fusarium* fungi, including species which might produce mycotoxins and gushing factors during the process [22, 41, 51, 56, 57]. Therefore, there is a need for efficient and safe ways to control growth and metabolic activity of fungi in raw materials, as well as during the processing. Due to current environmental and health concerns, research has been directed toward developing natural means of prevention of fungal grain diseases and spoilage. Biological control with well-characterized, antagonistic microbes or with natural plant-derived and microbial compounds has been introduced into many fields of food and feed processing. The plant-derived microbes, mainly bacteria and yeasts, have shown strong antagonistic activity against various fungal contaminants [37, 44]. Biocontrol candidates will most likely persist in the habitat from which they were isolated [16]. Starter technology, in which barley is inoculated with well-characterized microbes, has also been introduced to the malting industry [5, 8, 25].

Our previous study revealed that a numerous and diverse yeast community consisting of both ascomycetous and basidiomycetous species was a significant part of the malting ecosystem [35]. Several yeasts produced plant cell wall-degrading enzymes with potentially positive contribution to malt processability. It has been reported that many species of ascomycetous and basidiomycetous yeasts of the saprophytic phyllosphere community have strong antagonistic activity against various fungal pathogens [3]. Several strains have successfully been applied to prevent pre- and post-harvest fungal diseases of fruits and vegetables [4, 28] and to control spoilage moulds during the storage of high moisture feed grains [12, 47]. However,it is rather little known about the antifungal potential of the diverse yeast community occurring in the industrial malting ecosystem. Boivin and Malanda [6] demonstrated that the addition of specific, malt-derived *Geotrichum candidum* (teleomorph *Galactomyces geotrichum*) into the malting process restricted fungal growth and prevented mycotoxin forma-

tion. This application has been developed into commercial scale.

Biocontrol strains are often introduced to various applications as single cultures. Recently, research has also been directed to combining several biocontrol agents or linking microbial cultures with other preservation methods [66]. Yeast and lactic acid bacteria often occurring together in plant-based bioprocesses and synergistic interactions between these two groups are utilized in many cereal fermentations [4]. We have previously shown that the addition of lactic acid bacteria (LAB) into malting activated the indigenous yeast community and enhanced production of microbial $\beta$-glucanase and xylanase in the malting process [34]. Combining antagonistic yeast with lactic acid bacteria might further enhance the usefulness of starter technology in complex bioprocesses such as malting.

The present study was designed to elucidate the antifungal potential of yeasts isolated from industrial maltings. Furthermore, the effects of a selected strain, *Pichia anomala* VTT C-04565 (C565) were investigated in a true malting environment with naturally infested barley showing gushing potential. The ultimate goal was to suppress *Fusarium* growth and to prevent the production of gushing-inducing hydrophobins during malting. We also studied the effects of *P. anomala* C565 in combination with *Lactobacillus plantarum* VTT E-78076 starter culture in order to enhance malt processability.

## Materials and methods

### Fungal cultures

The yeast cultures, including 7 ascomycetous and 5 basidiomycetous strains, and 21 filamentous fungi were provided by the VTT Culture Collection (Table 1). The yeasts were chosen on the grounds that they occur spontaneously in the malting ecosystem [35]. Furthermore, strains belonging to the species *A. pullulans, C. sake, C. saitoana, Cr. albidus, G. geotrichum, P. anomala*, and *P. guilliermondii* had also shown antifungal potential in other plant applications [4, 6, 44, 70]. In addition, 4 basidiomycetous yeasts (*Cr. albidosimilis, Cr. curvatus, Cr. magnus*, and *R. pinicola*) were tested, as they were shown to produce plant cell wall-degrading enzymes [35]. Yeast strains were grown on yeast-malt extract agar, YM-agar (Difco Laboratories, Detroit, MI, USA) at 25°C for 2–3 days. The yeast cultures were stored in 10% glycerol at −70°C for long-term storage, and on YM-slants at 4°C for short-term storage. The filamentous fungi originated from barley and malted barley samples. The mould cultures were grown at 25°C for 7 days on Potato Dextrose Agar (PDA, Difco) and maintained on PDA-slants at 4°C

**Table 1** Antifungal potential of various yeasts and yeast-like fungi isolated from an industrial malting ecosystem against field and storage moulds in a plate-screening assay

| Fungal strain | | Ascomycetous yeasts and yeast-like fungi | | | | | | | Basidiomycetous yeasts | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Aureobasidium pullulans | Candida sake | C. saitoana | Geotrichum sp. | Pichia anomala | P. anomala | P. guilliermondii | Cryptococcus albidus | Cr. albidosimilis | Cr. curvatus | Cr. magnus | Rhodotorula pinicola |
| | VTT-D | D-041014 | C-95520 | C-04524 | D-04559 | C-04564 | C-04565 | C-04568 | C-92012 | C-04508 | C-04536 | C-04540 | C-04571 |
| *Achremonium polychronum* | 96653 | – | – | + | + | + | + | + | – | – | – | – | – |
| *Alternaria alternata* | 76024 | + | – | – | + | + | + | – | – | – | – | – | – |
| *Aspergillus ochraceus* | 00808 | – | – | + | + | + | + | – | – | – | + | – | – |
| *Cochliobolus sativus* | 76039 | + | – | + | + | + | + | + | – | + | + | + | – |
| *Eurotium amstelodami* | 03923 | + | + | + | + | + | + | + | + | + | + | – | – |
| *Fusarium avenaceum* | 80141 | + | + | + | + | + | + | + | + | + | + | + | + |
| *F. cerealis* | 96601 | + | + | + | + | + | + | + | + | + | + | – | – |
| *F. culmorum* | 80148 | – | – | + | + | + | + | + | – | – | – | – | – |
| *F. equiseti* | 82087 | – | + | + | + | – | + | – | – | – | – | – | – |
| *F. graminearum* | 82169 | – | – | + | + | + | + | + | – | – | – | – | – |
| *F. graminearum* | 95470 | + | – | + | + | + | + | + | – | + | – | – | – |
| *F. langsethiae* | 03931 | – | – | + | + | + | + | – | – | – | – | – | – |
| *F. oxysporum* | 80134 | – | – | – | + | + | + | – | – | – | – | – | – |
| *F. poae* | 76038 | – | – | + | – | + | + | + | – | – | – | – | – |
| *F. sambucinum* | 77056 | – | – | + | + | + | + | + | – | – | – | – | – |
| *F. sporotrichioides* | 82175 | – | – | – | + | + | + | + | – | – | – | – | – |
| *F. sporotrichioides* | 72014 | + | – | + | + | + | + | – | + | – | – | – | – |
| *F. tricinctum* | 96607 | – | – | – | + | + | + | – | – | – | – | – | – |
| *Penicillium verrucosum* | 01847 | + | – | + | + | + | + | + | – | + | – | – | – |
| *P. verrucosum* | 00831 | + | – | + | + | + | + | + | – | – | – | – | – |
| *Pyrenophora teres* | 89395 | + | + | + | + | + | + | + | – | – | – | – | – |

– no inhibition, + suppression of mould growth

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## Antifungal screening in vitro with plate-assay

The yeast strains were screened for antifungal potential using a dual-culture overlay assay adapted from Magnusson et al. [38]. Yeasts were inoculated along a 2 cm line on replicate YM plates and allowed to grow at 25°C for 2–3 days. The plates were overlaid with 10 ml of temperated malt extract soft agar (0.05% malt extract, Difco) containing $10^4$ fungal spores per ml. Spore suspension was prepared by removing the spores from the PDA plates of a 7 day-culture. Sterile saline (10 ml) was added to the plates and spores were harvested with a bacteriological spreader. The suspension was filtered through sterile glass wool to remove mycelial debris. The number of spores was counted microscopically using a counting chamber (Thoma, Knittle Gläser, Germany) and adjusted by adding sterile distilled water. The growth inhibition was measured after 5 and 7 days of incubation at 25°C. The results were considered as positive (+) if the mould could not overgrow the yeast or if a clear inhibition area was observed around the colony. In the negative (−) samples, the whole plate was covered with mycelia.

## Malting experiments with naturally infested barley

Barley (*Hordeum vulgare* L, Poaceae, two-row variety Scarlett cultivated in Finland 2005) samples (1 kg) were malted in a specially designed, computer-controlled micro-malting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). Due to intensive *Fusarium* contamination and gushing potential, this sample was unacceptable for commercial purposes but suitable for this study. Fungal gushing factors were not found in the native barley, but were produced during the malting process. Before malting, barley samples were sieved to remove grains <2.5 mm. All barley samples were steeped in 3 l of water or in water containing microbial cultures at 18°C for 8 h, followed by a 16 h air rest (20°C) and a second steep (2 h, 18°C). The moisture content of grains was measured daily and kept constant (46–47%) by adding water. The barley was then allowed to germinate for 5 days at 16°C and dried (kilned) in warm air (4 h 50°C, 3 h ramp to 60°C, 2.5 h 60°C, 3 h ramp to 85°C, 1 h 85°C) in a separate kiln. The rootlets were removed before analyses.

Liquid cultures of *P. anomala* C565 strain were grown in Erlenmayer flasks containing YM-broth and incubated on a rotary shaker at 100 rpm at 25°C for 3 days. Cells were harvested by centrifugation at 5,300$g$ for 10 min. Cell counts were determined microscopically using a Thoma counting chamber and adjusted to the desired level with sterile distilled water. Three individual malting experiments were carried out with a pure culture of *P. anomala* C565. In experiment 1 (Exp. 1) yeast cultures were added into the first steeping water at a level of $10^6$ cfu/ml. In experiments 2 (Exp. 2) and 3 (Exp. 3) *P. anomala* C565 was added into both steeping waters ($10^6$ cfu/ml). In malting experiment 4 (Exp. 4), *P. anomala* C565 was combined with *L. plantarum* VTT E-78076 (E76) in duplicate samples. *L. plantarum* E76 strain was grown in MRS-broth (Oxoid, Basingstoke, Hampshire, UK) at 30°C for 3 days. LAB culture, including cells and spent medium, was added into the first steeping water at a level of 4% v/v of the steeping water. The LAB were enumerated on MRS agar plates (Oxoid) incubated in anaerobic conditions at 30°C for 72 h. *P. anomala* C565 cells were added into the second steeping water.

The number of germinated grains was counted daily from a sample of about 150–200 kernels until the germination rate exceeded 90%. The concentration of ethanol in the head space of each malting drum was analyzed continuously using a Fourier Transform Infrared Spectroscopy (FTIR) multicomponent gas analyzer Gasmet® (Temet Instruments Ltd., Helsinki, Finland) with a heated, flow-through, 5 m path length sample cell.

## Microbiological analyses of process samples

Samples for the microbiological analyses were taken from untreated barley, and from barley after steeping, germination and kilning (after rootlet removal). The following microbial groups were analyzed from homogenized barley samples: aerobic heterotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria, and yeasts. Duplicate samples were prepared in each experiment. A sample of 10 g was homogenized for 10 min with 90 ml of sterile saline in a Stomacher Lab Blender 400 (Seward Medical, London, UK). Aerobic heterotrophic bacteria were determined on plate count agar (PCA, Difco Laboratories) and *Pseudomonas* spp. on C–F–C agar (Oxoid Ltd.). Samples were incubated in aerobic conditions at 30°C for 2–3 days. The number of LAB was determined on MRS agar (Oxoid) and samples were incubated in anaerobic conditions at 30°C for 5 days. To prevent fungal overgrowth of bacterial determinations, 0.001% cycloheximide (Sigma Chemical, St. Louis, MO, USA) was added to PCA, C–F–C and MRS media. Yeast counts were determined on YM agar (Difco Laboratories). Samples were incubated in aerobic conditions at 25°C for 3–5 days. Chlortetracycline and chloramphenicol (both at 0.01%) were added to YM medium to prevent bacterial growth. In addition, 0.02% of Triton-X 100 (BDH) was used to limit the spreading of fungal colonies on YM-agar. The bacteria and yeast results are expressed as colony forming units/gram barley (cfu/g).

For *Fusarium* analyses, 100 randomly selected kernels were placed on a selective Czapek–Dox agar containing Iprodion and Dichloral (CZID-agar) [1, 15]. The CZID

plates were incubated at 25°C for 7 days. Other filamentous fungi such as *Alternaria* spp., *Cephalosporium* spp., *Cladosporium* spp., *Drechslera* spp., *Epicoccum* spp., *Mucor* and *Rhizopus* spp. were determined from barley, steeped barley and malt samples on wet filter paper using direct plating of 100 kernels [15]. Filter paper plates were incubated at 25°C for 21 days. Fungi were identified under a stereomicroscope on the basis of typical colony form and color. Identification was confirmed by conidia morphology with a light microscope (magnification 400×). The results are expressed as percent of kernels contaminated with fungi.

Determination of fungal hydrophobins and gushing potential

The hydrophobin levels in the malt samples were determined with competitive ELISA (Enzyme Linked Immuno-Sorben Assay) as described by Sarlin et al. [52]. Ground sample (5 g) was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. After centrifugation, the supernatant was removed to a clean tube and antibodies against *F. poae* VTT D-82182 (D182) were added. After incubation, the sample-antibody mixture was transferred to an immunoplate (Nunc-Immuno Modules, MaxiSorp polystyrene strips, Nunc, Rochester, NY, USA) coated with hydrophobin extract of *F. poae* D182 Goat anti-rabbit IgG (H + L)-alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories, Hercules, CA, USA) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma) in diethanolamine-MgCl$_2$ buffer (Oy Reagena Ltd., Toivala, Finland) were used as substrate for AP detection. The absorbance was read at 405 nm. Due to the nature of the competitive ELISA, a lower absorbance value corresponded to a higher amount of hydrophobin in the samples. In the present study, the results are expressed as the inverse of the mean absorbance value. The results are means of the analyses of four replicates. The significance of *P. anomala* C565 for malt hydrophobin levels in three malting experiment was evaluated using one-way variance analysis (ANOVA) with Tukey's Honestly Significant Difference (HSD) test. Statistical significance was assessed at $P < 0.05$. The software SPSS 14.0 for Windows was used for the statistical analyses.

The gushing potential of malt samples was measured as described by Vaag et al. [64]. The malt extracts were added to commercial, bottled beer (0.33 l) and pasteurized bottles were agitated for 3 days with a horizontally rotating shaker at 50 rpm [21]. The gushing positive and negative malt samples obtained from Carlsberg Research Laboratory, Copenhagen Valby, Denmark were included in the studies. After shaking, the bottles were kept still for 10 min, inverted three times and opened after 30 s. The amount of gushing was determined from the change in weight of the bottle. The test was performed in triplicate.

Malt and wort analyses

High gravity mashing and the Büchner filtration test for evaluation of lautering performance were performed, as described by Sjöholm et al. [59]. The high gravity laboratory mashing conditions resemble those used in commercial brewery practice, so the results give a better prediction of the brewing performance of malt than the standard EBC Congress mash. Samples were analyzed using the following EBC recommended methods: malt friability, wort extract content, wort soluble nitrogen, wort free amino nitrogen, wort viscosity and wort $\beta$-glucan [14]. α-Amylase activity was analyzed with a Ceralpha kit (Megazyme International Ireland Ltd., Wicklow, Ireland) using an extraction time of 30 min and assay conditions as specified by the manufacturer. $\beta$-Glucanase activity was analyzed with the Azo-barley glucan method kit using azo-barley glucan as substrate (Megazyme). The assay was performed at both 30 and 60°C in order to distinguish between $\beta$-glucanase of barley and microbial origin. Xylanase was analysed with an *endo*-1,4-$\beta$-xylanase assay procedure using Xylazyme AX tablets (Megazyme) as substrate. Milled malt (1.00 g) was extracted in 8.0 ml of sodium acetate buffer (25 mM, pH 4.5) for 15 min at room temperature with continuous stirring (200 rpm). The flour was separated by centrifugation (1,000*g*). Xylanase activity was measured at 45°C. A substrate tablet was added to 0.5 ml of extract and incubated for 30 min. The reaction was stopped by adding 5.0 ml of 1% Trizma base. Absorbance was measured at 590 nm. The results are expressed as difference in absorbance between the sample and a reagent blank.

**Results**

Antifungal potential of yeasts and yeast-like fungi against filamentous field and storage fungi

The antifungal potential of seven ascomycetous and five basidiomycetous yeast strains was screened against common field and storage contaminants using a dual-culture plate assay, in which yeast cultures were first grown in YM-agar and then overlayed with mould spore suspension in soft malt agar (Table 1). The main emphasis of this study was on the suppression of *Fusarium* growth, and therefore 13 different *Fusarium* strains were tested. The results indicated that the ascomycetous yeasts had better antifungal potential than the basidiomycetous yeasts. As seen from Table 1, *C. saitoana* C524, *Geotrichum s*p. D559, *P. anomala*

C564 and C565, and *P. guilliermondii* C568 were the most prominent strains with respect to antagonistic activity against filamentous fungi. When grown together on solid media, these yeasts clearly suppressed the growth of several indicator moulds. However, great variation in growth inhibition was observed among different mould species and even between strains. All the yeast strains tested could prevent the overgrowth of *F. avenaceum* D141 in the plate assay, whereas *F. oxysporum* D134 and *F. tricinctum* D607 strains were inhibited only by *Geotrichum* sp. D559, *P. anomala* C564 and C565.

## Antimicrobial effects of *P. anomala* C565 in malting of naturally infested barley

*Pichia anomala* C565 was selected for the malting experiments because it suppressed the growth of all indicator organisms in the in vitro study. Three individual malting experiments were carried out with *P. anomala* C565 single strain culture. In Exp. 1, the yeast culture was added into the first steeping water, and in Exp. 2 and 3 into both steeping waters. The cells were added into the steeping waters at a level of $10^6$ cfu/g of barley. As seen from Fig. 1., the counts of *P. anomala* C565 increased over 1 log unit during the first days of malting and reached their maxima ($3 \times 10^8$ cfu/g) at the end of germination. Kilning had little effect on the viable counts. The yeast counts in the final malt were over $10^6$ cfu/g in Exp. 1 and over $10^7$ cfu/g in Exp. 2 and 3. *P. anomala* C565 suppressed the growth of other yeasts on the YM-plates and only *P. anomala* colonies were detected, whereas a diverse yeast population was detected in the control samples.

Many antifungal studies have been carried out in controlled laboratory environments with pure cultures or with artificially contaminated material. In this study, the antifungal potential of malt-derived yeast was evaluated with naturally contaminated material showing gushing potential. The addition of *P. anomala* C565 into the steeping water clearly suppressed the intensity of *Fusarium* contamination and obviously modified the *Fusarium* population (Fig. 2). Although the CZID-analysis showed that 100% of the kernels were contaminated after steeping, a clear visual difference in *Fusarium* populations was observed between the control (Fig. 2a) and *P. anomala* C565 treated samples (Fig. 2b) after steeping. The *Fusarium* contamination in the final malt samples remained high (99% of the kernels were contaminated with fusaria). Only ∼7% lower counts were measured in the malt samples after *P. anomala* C565 treatment. Direct plating method with CZID-agar had limited quantitative value and indicated only the fraction of kernels contaminated with fungi, not the degree of infection. Therefore, the effects of *P. anomala* C565 on *Fusarium* fungi were also evaluated indirectly by determination of fungal hydrophobins, also known as gushing inducers.

The results of the hydrophobin-ELISA revealed that the addition of *P. anomala* C565 clearly restricted the production of *Fusarium* hydrophobins during malting (Fig. 3). Analysis of variance showed that the malt hydrophobin levels of the control and *Pichia*-treated samples were significantly ($P < 0.05$) different. As also can be seen from Fig. 3, the hydrophobin levels in the control samples of Exp. 1 differed significantly ($P < 0.05$) from those of Exp. 2 and 3. The first malting experiment was carried out with barley after six months of storage and the subsequent experiments with the same barley sample after 7 and 7.5 months of storage. The results indicated that the hydrophobin formation capability of fusaria was reduced during the prolonged barley storage.

The gushing test confirmed that beer gushing was prevented when *P. anomala* C565 was added into the steeping



**Fig. 1** Growth of indigenous yeasts (control) and inoculated *P. anomala* yeasts (C565) during the malting experiments. Duplicate grain samples were determined in each experiment. The control counts are mean values obtained from three individual malting experiments (±SD). *P. anomala* C565 counts are averages of duplicate samples from Exp. 1 (C565 added into the first steeping water) and averages of four samples from Exp. 2 and 3 (C565 added into both steeping waters)
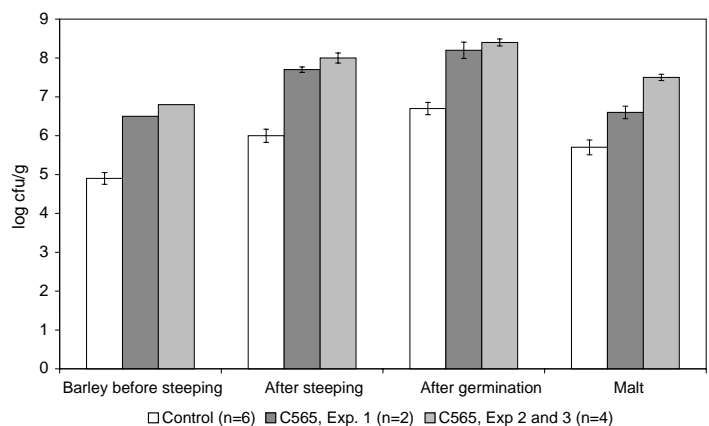
**Fig. 2** *Fusarium* growth restriction by *P. anomala* C565 added to the steeping water. Kernels contaminated with *Fusarium* fungi after 2 days of malting (after steeping) on CZID plates. **a** control sample, **b** *P. anomala* C565 added to both steeping waters
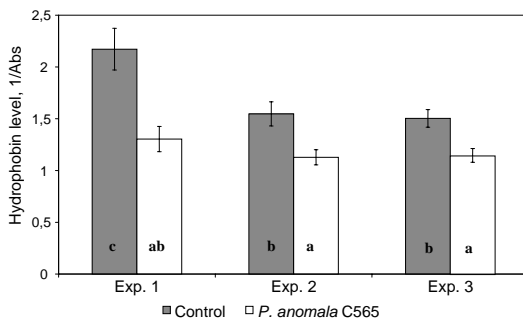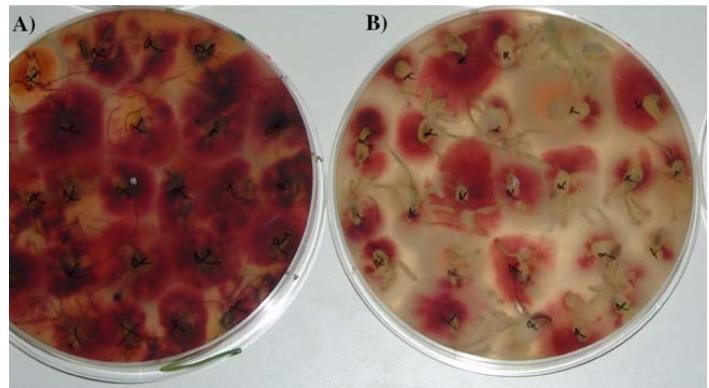




**Fig. 3** Effects of *P. anomala* C565 on malt hydrophobin levels. *P. anomala* C565 was added into the first steeping water in Exp. 1 and to both the first and second steeping water in Exp. 2 and 3. Values are means of four replicates (±SD). Bars labeled with different *letters* are statistically different at the significance level of 0.05

waters of barley. All the control samples induced overfoaming of beer, whereas gushing tendency was not observed in the *P. anomala* treated samples (Table 2).

We also studied the effect of *P. anomala* C565 addition on the growth of other filamentous fungi during malting. As seen from Fig. 4, the common field fungi *Alternaria, Cephalosporium*, *Cladosporium*, and *Drechslera*, were not

**Table 2** Effects of *P. anomala* C565 on malt gushing potential

| Experiment | Gushing tendency[a] | |
| --- | --- | --- |
| | Control | *Pichia anomala* C565 |
| Exp. 1 | $17 \pm 16$ | $0 \pm 0$ |
| Exp. 2 | $32 \pm 5$ | $0 \pm 0$ |
| Exp. 3 | $1 \pm 1$ | $0 \pm 0$ |

*P. anomala* C565 was added into the first steeping water in Exp. 1 and to both first and second steeping water in Exp. 2 and 3

[a] Gushing of beer was determined as the beer overflowing (g) from the bottles. The test was performed in triplicate

restricted by the addition of *P. anomala* C565. On the contrary, slightly higher (10%) *Drechslera* and *Cephalosporium* counts were observed after steeping of *P. anomala* C565 treated samples compared to the control. The *Mucorales* fungi, such as *Mucor* or *Rhizopus*, did not belong to the indigenous fungal community of barley (Fig. 4). They are commonly detected as process contaminants at elevated moisture conditions, especially during the early hours of kilning. The fungal analysis of malt samples revealed that over 80% of the control kernels were contaminated with *Mucor* fungi. A significant reduction of this fungus was measured in *P. anomala* C565 treated samples. Only 26% of the malt kernels contained *Mucor* fungi after *P. anomala* treatment.

Addition of *P. anomala* C565 as single culture into the steeping waters had no effect on the bacterial community consisting of both Gram-negative and -positive bacteria. The aerobic bacterial count reached $10^9$ cfu/g after 5 days of germination in both control and *Pichia*-treated samples. In the final malt samples after rootlet removal, the number of aerobic heterotrophic bacteria was $10^8$ cfu/g. A significant proportion of this aerobic bacterial population was composed of pseudomonads ($10^6$ cfu/g). The indigenous LAB population was low in barley, but it increased considerably during malting in both control and *P. anomala* C565 samples. The final malt contained $10^7$ LAB/g.

### Effects of *P. anomala* C565 on grain germination and malt quality

*Pichia anomala* C565 had no notable effect on grain germination (Table 3). Over 96% of the kernels had germinated in both samples after 3 days of malting. Interestingly, *P. anomala* C565 clearly decreased the ethanol concentration in the head space of a malting drum (Fig. 5). After carbon dioxide, ethanol was the second most abundant volatile detected in the control samples. The present results indicate that the ethanol produced by the grain was rapidly consumed by *P. anomala*. The composition of the gas atmosphere in

**Fig. 4** Effects of *Pichia ano-mala* C565 added to the first and second steeping water on the occurrence of *Alternaria, Cephalosporium, Cladosporium, Drechslera*, and *Mucor* fungi in barley samples after steeping and in final malt. The values are means of the two malting experiments (Exp 2. and 3.)
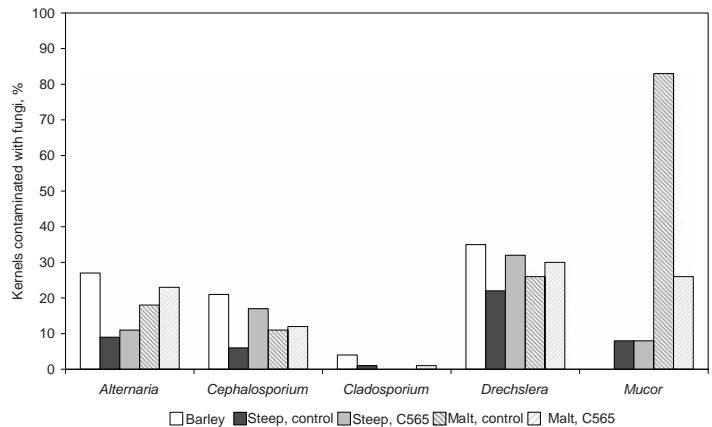


**Table 3** Effects of *P. anomala* C565 addition on grain germination

| Malting time, day | Germinated grains, % | |
|---|---|---|
| | Control | *Pichia anomala* C565 |
| 1 | 16 ± 1 | 11 ± 4 |
| 2 | 79 ± 2 | 71 ± 4 |
| 3 | 97 ± 3 | 96 ± 1 |

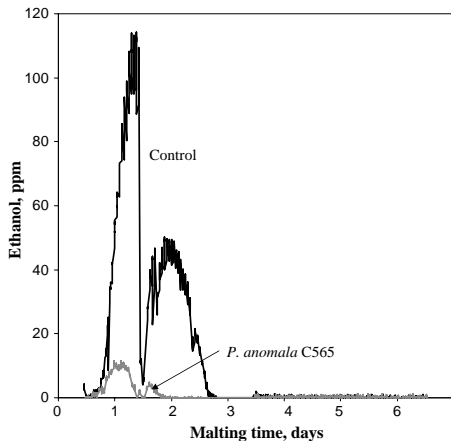The values are mean ± standard deviation of three individual malting experiments (Exp 1–3)



**Fig. 5** Effects of *P. anomala* C565 on ethanol production during the first days of malting. The results represent one of the duplicate malting experiments (Exp. 2)

the malting drum also differed with respect to ethyl acetate, which was only detected in *Pichia*-inoculated samples, at low levels (1.5–7 ppm) during the first 2 days of malting.

Table 4 shows the effects of *P. anomala* C565 on malt quality and on the properties of high gravity wort. The malts were all well modified based on the high friabilities. How-

ever, this study indicated that *P. anomala* C565 addition may retard mash filterability (Fig. 6a). The difference between the filtration curves of the control and *Pichia* treated samples was small but consistent. Approximately 10% less filtrate was obtained within one hour of *Pichia* treated samples (when added into both steeping waters) compared to control samples. As seen from Table 4, *P. anomala* appeared to suppress the microbial β-glucanase (assayed at 60°C) and xylanase activities in malt in the Experiments 2 and 3, which could partly explain the impeded wort filtration.

**Combination of *P. anomala* C565 with *Lactobacillus plantarum* E76 starter culture**

In order to improve the wort filtration performance, *P. anomala* C565 was combined with *L. plantarum* E76 in Exp. 4. *L. plantarum* E76 was added to the first steeping water and *P. anomala* C565 to the second steeping water. As seen from Fig. 6b, the filtration performance was recovered when these two treatments were combined. *L. plantarum* E76 treatment enhanced the production of plant cell wall hydrolysing enzymes of microbial origin: slightly higher microbial β-glucanase activities relative to the control were observed when *L. plantarum* E76 was combined with *P. anomala* C565 (Table 5). In addition, part of the beneficial effects obtained with lactic acid starter treatment can be explained by reduced growth of gram-negative bacteria, particularly pseudomonads (Fig. 7) with a negative influence on mash filterability.

**Discussion**

Malting can be considered as a complex ecosystem consisting of germinating grain and a complex microbial community including a number of aerobic bacteria, lactic acid

**Table 4** Effects of *P. anomala* C565 on malt and wort (High gravity) properties

| | Control[a] | *P. anomala* C565 | |
| --- | --- | --- | --- |
| | Mean (n = 3) | Exp 1. (n = 1) | Exp. 2 and 3 (n = 2) |
| Malt analyses | | | |
| Friability, % | 88 ± 2 | 88 | 86 |
| α-Amylase, U/g | 320 ± 6 | 352 | 321 |
| β-Glucanase, 30°C, U/kg | 722 ± 46 | 720 | 684 |
| β-Glucanase, 60°C, U/kg | 114 ± 15 | 94 | 105 |
| Xylanase, abs × 1000 | 0.209 ± 0.05 | 0.213 | 0.184 |
| High gravity wort analyses | | | |
| Wort extract content, w-% | 16.8 ± 0.1 | 17.1 | 16.7 |
| Color, EBC | 5.7 ± 0.2 | 5.5 | 5.6 |
| Free amino nitrogen, mg/l | 367 ± 9 | 404 | 366 |
| Soluble nitrogen, mg/l | 1,819 ± 14 | 1,891 | 1,807 |
| pH | 5.6 ± 0 | 5.6 | 5.5 |
| β-Glucan, mg/l | 263 ± 15 | 260 | 270 |
| Wort viscosity, cP | 2.17 ± 0.03 | 2.18 | 2.26 |

*P. anomala* C565 was added into the first steeping water in Exp. 1 and to both the first and second steeping water in Exp. 2 and 3

[a] The values for control samples are mean ± standard deviation of three individual malting experiments (Exp 1–3)

bacteria, yeasts and filamentous fungi [35, 42, 49]. It is obvious that microbes greatly influence malt quality, wort filtration and fermentation and therefore, have a significant impact on beer processing and quality. Depending on the nature and extent of microbes, the effects may be either beneficial or deleterious to malt quality [6, 10, 17, 23, 33, 37, 51, 58, 63, 65]. The most negative consequences linked to intensive mould growth, especially fusaria, are the production of mycotoxins and gushing factors [52, 56].

This study indicated that *Fusarium* growth during malting and the production of fungal hydrophobic proteins, also known as gushing factors, could be suppressed with yeasts naturally occurring in the industrial malting ecosystem. In vitro screening with a plate-assay indicated that ascomycetous strains belonging to species of *A. pullulans*, *C. sake, C. saitoana, G. geotrichum, P. anomala*, and *P. guilliermondii* were the most potential yeasts with respect to antifungal activity. These results were in agreement with previous investigations [6, 19, 45, 50, 54, 70]. *P. anomala* VTT C-04565 (C565) was selected for malting experiments in order to verify the antifungal potential of malt-derived yeast in malting with naturally infested barley. To our knowledge, this is the first report showing the effects of *P. anomala* against *Fusarium*-fungi in malting and on overall malt quality.

*Pichia anomala* is a robust organism, which is occurring naturally in plant materials such as in cereals [44]. It is tra-
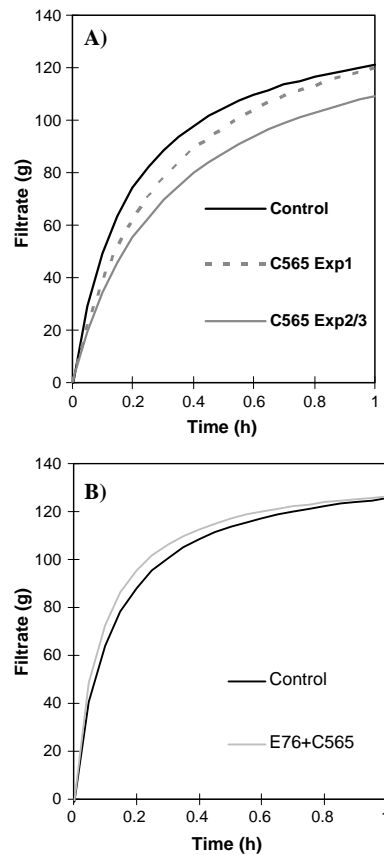


**Fig. 6** Effects of *P. anomala* C565 (**a**) and of a combination of *P. anomala* C565 with *L. plantarum* E76 (**b**) on mash filterability measured as the Büchner filtration test. Values are means of triplicate (Control in **a**), duplicate (C565 Exp2/3 in **a**; E76 + C565 B) or single (C565 Exp1 in **a**; Control in Fig **b**) malting samples. The repeatability of the filtration curve has been evaluated by including standard malt in each analysis during several years. In the standard malt, the standard deviation of the amount of filtrate measured at 0.25 h is 5.1 g

ditionally used in fermented products in Africa and Asia [43]. This species is classified as safe (biosafety level 1), and is potentially a suitable biocontrol agent in a variable environment [12, 44]. *P. anomala* has previously shown antimicrobial activity against a wide range of unrelated microbes such as bacteria, yeasts, and filamentous fungi [44]. *P. anomala* J121 has been extensively studied in the preservation of moist grains (wheat, barley and oats) for animal feed [12, 18, 19, 47, 48].

We demonstrated that *P. anomala* C565 added to the steeping water restricted *Fusarium* growth. Steeping can be regarded as the most important step in malting with respect to microbiological safety because it activates rapid growth of bacteria and fungi [42]. Therefore, *P. anomala* C565 was

**Table 5** Effects of combined treatment with *L. plantarum* E76 (added to first steeping water) and *P. anomala* C565 (added to second steeping water) on malt and wort (High gravity) properties

| | Control (n = 1) | *L. plantarum* E76 + *P. anomala* C565 (n = 2) |
|---|---|---|
| Malt analyses | | |
| Friability, % | 86 | 84 |
| α-Amylase, U/g | 352 | 356 |
| β-Glucanase, 30°C, U/kg | 673 | 638 |
| β-Glucanase, 60°C, U/kg | 94 | 166 |
| Xylanase, abs × 1,000 | 0.203 | 0.278 |
| High gravity wort analyses | | |
| Wort extract content, w-% | 16.6 | 16.6 |
| Color, EBC | 6.0 | 7.0 |
| Free amino nitrogen, mg/l | 356 | 392 |
| Soluble nitrogen, mg/l | 1,809 | 1,882 |
| pH | 5.5 | 5.5 |
| β-Glucan, mg/l | 210 | 205 |
| Wort viscosity, cP | 2.12 | 2.07 |
| Gushing tendency[a] | 23 ± 17 | 0 ± 0 |

[a] Gushing of beer was determined as the beer overflowing (g) from the bottles. The test was performed in triplicate
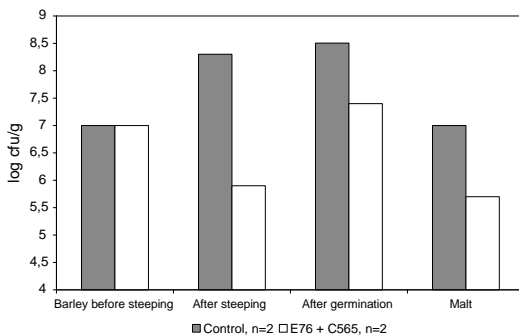


**Fig. 7** Effects of *L. plantarum* E76 and *P. anomala* C565 combination on the growth of *Pseudomonas* spp. during malting. Values are means of duplicate malting samples

inoculated at this stage. Although direct plating had little quantitative value in *Fusarium* biomass evaluation, clear suppression of *Fusarium* growth was observed on grains cultivated on CZID-agar. Apparently, the majority of the *Fusarium* community was located in and on the outermost layers of barley tissues and was therefore restricted by the addition of *P. anomala* C565. In addition to *Fusarium* inhibition, *P. anomala* C565 treatment restricted *Mucor*-contamination. Mucorales fungi, such as *Mucor* and *Rhizopus*, are considered as surface contaminants of grains and they proliferate during germination and the early stages of kilning [11]. This finding also suggested that yeasts may suppress the attachment of fungal surface contaminants.

However, *P. anomala* did not totally inhibit fusaria. Moreover, the growth of other field fungi was not inhibited by the *P. anomala* addition into the malting process, although inhibition of several filamentous fungi was observed in in vitro screening with a plate assay. On the contrary, suppression of *Fusarium* growth most probably provided more nutrients and space for the growth of certain other fungi such as *Cephalosporium* and *Drechslera*. This finding supports the theory that some species were located deeper in the husk layers and were not necessarily influenced by the external addition of biocontrol agent. The field fungi occur in different parts of the husk and pericarp layers in barley [55]. Therefore, this study highlights the importance of verification of the results obtained from in vitro studies with pure cultures by using naturally infested material *in vivo*. Furthermore, the plate-screening assay indicated that differences in sensitivity might occur among *Fusarium* species and even between strains. However, *Fusarium* diversity after *Pichia* treatment was not analyzed in this study, and therefore we cannot conclude which specific species were inhibited during malting. In Finland, the most common *Fusarium* species in barley during the recent years have been *F. avenaceum, F. athrosporioides, F. sporotrichioides*, and *F. culmorum* [72]. Our further studies will be directed toward investigating the effect of biocontrol yeasts on *Fusarium* diversity during processing.

This study also indicated that *P. anomala* C565 suppressed the production of fungal hydrophobic proteins during malting. Hydrophobins are among the most important structural proteins found in the filamentous fungi [13]. They are produced in response to changes in the environment and they react to interfaces between fungal cell walls and the air or between fungal cell walls and solid surfaces [29]. We recently showed that fungal hydrophobins are also involved in beer gushing [52]. Addition of *P. anomala* C565 into steeping prevented beer gushing. Results obtained with the novel competitive hydrophobin-ELISA test showed that all the *P. anomala* C565 malt samples had absorbance values >0.8. Sarlin et al. [52] reported that the risk of gushing is increased if the absorbance value of malt is <0.6. The production of gushing factors in barley and in malting is complex and still a largely unknown phenomenon. It is well known that intensive *Fusarium* growth is part of the normal malting process. However, the production of gushing factors occurs only rarely. Our results suggested that some suppression probably occurs in normal industrial practice as a result of indigenous yeasts.

It has been shown that gushing potential can be decreased during steeping, indicating that part of the gushing factors produced during the growth period of barley in the field are washed away with the steeping waters [41, 51]. However, additional hydrophobin production may occur again during germination. Production of hydrophobins is

most probably linked to variable environmental conditions and attachment of fusaria to barley surfaces. Gjertsen [20] speculated that the gushing factors were produced as a result of interactions between the barley and fungal mycelium. Munar and Sebree [41] also reported that an extract of *Fusarium* fungi grown on agar plates did not induce gushing when spiked into beer, although when *Fusarium* was grown together with barley, beer gushing occurred. These studies suggest that gushing factors arise as a result of an interaction involving viable mould and the germinating grain. Hydrophobin production may also be species related. Gushing factors formed during malting occurred under the barley husk and could not be removed by washing of the final malt [41]. Therefore, preventive actions are essential in assuring safety along the barley to beer chain.

The antifungal action of biocontrol yeasts is often due to several antagonistic mechanisms and hitherto no single mechanism has been shown to be responsible for the whole antimicrobial action. The mechanisms are poorly understood, especially in complex ecosystems. Although the mechanisms in the malting ecosystem were not studied in the present investigation and they remain to be revealed, our results indicated that *P. anomala* C565 competed with fusaria for space. As a fast-growing organism, *P. anomala* colonized the outer layers of barley and suppressed the adherence of fungal contaminants to barley surfaces. Competition for nutrients and space has often been suggested as the main mode of the action mechanism of several biocontrol agents. In addition, the antifungal action of biocontrol yeasts often includes induction of the plant defence system, mycoparatism, production of lytic enzymes such as $\beta$,1-3 glucanase or chitinase that degrade the fungal cell wall or secretion of antimicrobial compounds, such as killer proteins [28, 39, 44]. Druvefors et al. [12] suggested that the antifungal effect of *P. anomala* was probably due to the synergistic effect of ethyl acetate and ethanol produced by *Pichia* in an oxygen limited environment. Ethyl acetate was indeed detected in the gaseous atmosphere of the malting drums in *P. anomala* treated samples.

In this study, it was noticed that *P. anomala* C565 rapidly consumed the ethanol produced by the grains during the air rest. *P. anomala* can utilize ethanol as a growth substrate in aerobic conditions [32, 60]. We recently reported that the ethanol detected during the first days of malting was mainly produced by the barley embryo and the aleurone cells [69]. Fermentative metabolism and concomitant ethanol production are part of the normal grain germination. *Pichia* yeasts can utilize a wide variety of carbon and nitrogen sources for growth. Our results suggested that *P. anomala* can utilize the grain metabolites as substrate for growth, without disturbing the grain germination process.

This study confirmed previous findings that *P. anomala* had great antifungal potential [12, 19, 47, 48], and

expanded the list of potential application areas. However, there seemed to be a trend toward slightly lower wort separation when *P. anomala* C565 was applied into both steeping waters. These results need to be confirmed in pilot- or production scale, where wort separation can be more accurately evaluated. Wort filtration rate is influenced by several different factors, such as complexes formed between proteins and pentosans, $\beta$-glucans, residual starch, and lipids [40]. *P. anomala* C565 addition into the both steeping waters seemed to restrict the production of microbial $\beta$-glucanase and xylanases during malting, which might partly explain the reduced filtration rate. The microbial community, especially filamentous fungi such as fusaria, have a great influence on the malt enzyme potential and may therefore, also affect wort filtration performance [27, 51, 58, 71]. Furthermore, extracellular polysaccharides (EPS) produced by malt-derived bacteria and yeasts may also affect filtration performance [23, 31]. EPS production has been reported to occur among the yeast genera *Aureobasidium, Bullera, Cryptocccus, Pichia, Rhodotorula, Sporobolomyces, Tremella*, and *Trichosporon* [9]. Dense film formation (cream-colored film of biomass) due to intensive *Pichia* growth has been observed in the wine and beverage industry [60]. Furthermore, Kreisz et al. [31] reported that malt-derived yeast polysaccharides such as mannan and glycogen may have a significant impact on the haze level of filtered beer. Therefore, precautions must be taken when selecting biocontrol agents for malting. However, $>10^6$ cfu/g *P. anomala* has frequently been observed in the normal industrial malting ecosystem without any negative consequences [35].

The possible negative impacts of *P. anomala* on filtration performance may limit its use in malting applications alone. This study suggested that the wort filtration performance could be recovered by combining *L. plantarum* E76 treatment with *P. anomala* C565. To our knowledge, this is the first report in which *P. anomala* cultures were combined with *L. plantarum*. Our previous studies have shown that addition of *L. plantarum* E76 into the steeping notably improved lautering performance [24, 34]. The present study also confirmed our previous findings that *L. plantarum* E76 addition enhanced xylanase and microbial $\beta$-glucanase activities. Furthermore, *L. plantarum* E76 notably restricted the growth of aerobic bacteria, especially pseudomonads known to have a negative impact on wort filtration performance [23, 33]. The combination of two different microbial cultures offers a possibility to use their different properties, thus making the system more robust. However, the transfer of knowledge obtained from laboratory experiments into real complex malting processes is a challenging area which definitely needs further studies. Furthermore, experiments are needed with a wider subset of barley samples.

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In conclusion, this study clearly showed that yeasts naturally occurring in industrial maltings are capable of suppressing *Fusarium* growth and inhibiting the production of fungal hydrophobins inducing gushing. The combination of several treatments could result in a successful strategy for microflora management in complex cereal ecosystems such as malting. Well-characterized, malt-derived microbes can also be utilized as natural food-grade biocontrol agents in other cereal applications.

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| Title | **Detection and characterisation of *Fusarium* hydrophobins inducing gushing in beer** |
|---|---|
| Author(s) | Tuija Sarlin |
| Abstract | Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously foams out from its container immediately on opening. Gushing has a marked negative effect on the overall image of beer. Numerous factors causing and contributing to gushing have been reported. Two types of gushing can be distinguished based on the origin of gushing-inducing substances. Non-malt related gushing, i.e. secondary gushing, is due to faults in the beer production process or to incorrect treatment of packaged beer. Primary gushing is induced by fungal metabolites, so-called gushing factors, which are present in malt or in other cereal raw materials of beer. Particularly species of the genus *Fusarium* have been linked to primary gushing. Although gushing factors produced by fungi have been studied for decades, none of them have hitherto been fully characterised.

The hypothesis of this dissertation was that small fungal proteins called hydrophobins are one of the gushing factors inducing primary gushing. Hydrophobins are secreted, highly surface active, moderately hydrophobic proteins produced by filamentous fungi. Hydrophobins play key roles in the development and in the interactions of fungi with their environment and other organisms, particularly plants. The aim of this thesis was to isolate and characterise hydrophobins from gushing active fungi, especially from *Fusarium* species, and to demonstrate that these hydrophobins are able to induce gushing in beer. Currently, there is no practical, reliable and commercially available method for the prediction of beer gushing from large numbers of samples. The main goal of the work was to develop a test for detection of gushing potential of barley and malt by analysing the hydrophobin levels in samples. Moreover, the occurrence and fate of hydrophobins at different stages of the beer production chain were studied.

This study revealed numerous effects of *Fusarium* fungi on the quality of barley grown under Finnish field conditions and of the corresponding malt. In particular, *Fusarium* infection increased the gushing potential of malt. The results of the study indicated that the extent of the impacts is species-dependent, *F. graminearum* having more severe detrimental effects on barley and malt quality than *F. culmorum* and particularly *F. poae*.

We demonstrated that hydrophobins isolated from strains of the genera *Fusarium*, *Nigrospora* and *Trichoderma* induced beer gushing when added to bottled beer. Hydrophobin concentrations at the ppm level were sufficient for gushing induction. The gushing-inducing capabilities of the isolated hydrophobins varied probably due to their structural differences.

We generated profile hidden Markov models for the different hydrophobin classes and searched the *F. graminearum* genome database for predicted proteins with these models. The search revealed five putative hydrophobin genes belonging to both the hydrophobin classes I and II. The best matching sequences and the corresponding genes were isolated from *F. graminearum* as well as from the related species *F. culmorum* and *F. poae* by PCR and were characterized by sequencing. One each of the putative *F. graminearum* and *F. poae* hydrophobin genes were expressed in the heterologous host *Trichoderma reesei*. The proteins corresponding to the genes were purified and identified as hydrophobins and named GzHYD5 and FpHYD5, respectively. Concentrations of 0.003 ppm of these hydrophobins were observed to induce vigorous beer gushing.

An enzyme-linked immunosorbent assay (ELISA) was developed for determination of hydrophobin levels in barley and malt. A connection was found between the hydrophobin level and the gushing potential of malt, suggesting that the developed hydrophobin ELISA can be used for prediction of the gushing risk in malt.

*Fusarium* fungi were observed to produce hydrophobins during the growing period of barley in the field as well as during the malting process, especially during the steeping and germination steps. A small portion of hydrophobins originating from *Fusarium*-infected malt was shown to pass through the brewing process, ending up in the final beer where they induced gushing when present in sufficiently high levels. Addition of a selected antagonistic starter culture, the yeast strain *Pichia anomala* VTT C-04565, into the steeping water of barley was shown to suppress hydrophobin production in malting, which in turn decreased the gushing potential of the corresponding malt. |
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| Nimeke | **Oluen ylikuohuntaa aiheuttavien *Fusarium*-hydrofobiinien osoittaminen ja karakterisointi** |
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| Tekijä(t) | Tuija Sarlin |
| Tiivistelmä | Oluen ylikuohunta on ilmiö, jossa olut spontaanisti ja hallitsemattomasti vaahtoaa ulos pulloa avattaessa. Ylikuohunta jaetaan primääriin ja sekundääriin ylikuohuntaan. Sekundääriä ylikuohuntaa aiheuttavat erilaiset oluenvalmistuksen prosessitekniset ongelmat tai valmiin oluen virheellinen käsittely. Primäärin ylikuohunnan saavat aikaan homeiden tuottamat ylikuohuntatekijät, joita saattaa esiintyä maltaassa tai muissa oluen viljapohjaisissa raaka-aineissa. Yleisimmin primääriä ylikuohuntaa aiheuttavat *Fusarium*-homeet. Homeiden tuottamia ylikuohuntatekijöitä on tutkittu vuosikymmenien ajan, mutta niitä ei ole onnistuttu tarkasti identifioimaan. |
| | Tämän tutkimuksen hypoteesina oli, että pienet pinta-aktiiviset homeproteiinit, hydrofobiinit, ovat oluen primääriä ylikuohuntaa aiheuttavia ylikuohuntatekijöitä. Hydrofobiineilla on tunnetusti useita merkittäviä tehtäviä homeiden kasvussa ja kehityksessä. Tutkimuksen tavoitteena oli eristää ja karakterisoida hydrofobiineja ylikuohuntaa aiheuttavista homeista, etenkin *Fusarium*-suvun lajeista, sekä osoittaa, että nämä hydrofobiinit aiheuttavat oluen ylikuohuntaa. Lisäksi tavoitteena oli kehittää luotettava ja teollisuuden laadunvalvontaan soveltuva menetelmä ylikuohunta-taipumuksen määrittämiseksi ohrasta ja maltaasta. Tutkimuksen tarkoituksena oli hankkia tietoa hydrofobiinien käyttäytymisestä ja kulkeutumisesta "ohrasta olueksi" -tuotantoketjussa. |
| | Peltokokeet osoittivat, että myös Suomen oloissa *Fusarium*-homeet kykenivät lisääntymään haitallisesti, mikä heikensi ohran ja siitä valmistetun maltaan laatua. Etenkin ylikuohuntataipumus lisääntyi. Tutkimuksessa havaittiin eroja siinä, miten haitallisesti eri *Fusarium*-lajit vaikuttivat laatuparametreihin; *F. graminearum* heikensi laatua voimakkaammin kuin *F. culmorum* ja etenkin *F. poae*. |
| | Tutkimus osoitti, että hydrofobiinit saavat aikaan ylikuohuntaa olueen lisättyinä. *Fusarium*-, *Nigrospora*- ja *Trichoderma*-suvun homeista eristetyt hydrofobiinit aiheuttivat oluen ylikuohuntaa jo hyvin pieninä pitoisuuksina (< 1 ppm). Eristettyjen hydrofobiinien ylikuohunta-aktiivisuudet erosivat toisistaan todennäköisesti rakenne-erojen seurauksena. |
| | Käytimme tunnettujen hydrofobiinien aminohapposekvensseistä luomiamme todennäköisyysmalleja (profile HMMs) etsiessämme hydrofobiinien kaltaisia aminohapposekvenssejä *Fusarium graminearum* -homeen julkisesta genomitietopankista. Löysimme viisi hydrofobiinikandidaattia, joista valitsimme yhden todennäköisimmän jatkotutkimuksiin. Eristimme ja karakterisoimme tätä hydrofobiinia vastaavat geenit *F. graminearum* -kannan lisäksi *F. culmorum*- ja *F. poae* -kannoista. Eristetyt *F. graminearum*- ja *F. poae* -hydrofobiinigeenit siirrettiin *Trichoderma reesei* -tuottokantaan. Tuoteut ja puhdistetut proteiinit tunnistettiin hydrofobiineiksi ja nimettiin GzHYD5 (*F. graminearum*) ja FpHYD5 (*F. poae*). Molemmat hydrofobiinit aiheuttivat oluen ylikuohuntaa pitoisuutena 0.003 ppm. |
| | Hydrofobiinivasta-aineisiin perustuva immunologinen ELISA-testi kehitettiin hydrofobiinitasojen määrittämiseen ohra- ja mallasnäytteistä. Testin tulosten todettiin indikoivan maltaan ylikuohunta-taipumusta. Testin avulla tutkittiin hydrofobiinitasoja "ohrasta olueksi" -tuotantoketjussa. *Fusarium*-homeiden todettiin tuottavan hydrofobiineja ohraan kasvukauden aikana pellolla sekä mallastuksessa, etenkin liotuksen ja idätyksen aikana. Pieni osa maltaan sisältämistä hydrofobiineista kulkeutui oluenvalmistusprosessin läpi päätyen valmiiseen olueen. Lisäämällä mallastuksen luontaiseen mikrobiyhteisöön kuuluvaa *Pichia anomala* VTT C-04565 -hiivaa ohran liotusveteen saatiin vähennettyä hydrofobiinien muodostumista mallastuksen aikana ja samalla valmiin maltaan ylikuohuntapotentiaalia. |
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# Detection and characterisation of *Fusarium* hydrophobins inducing gushing in beer

Gushing is a phenomenon in which beer spontaneously foams out from its container immediately on opening. Gushing has a marked negative effect on the overall image of beer. Particularly species of the genus Fusarium have been linked to gushing. This study showed that small, highly surface active fungal proteins called hydrophobins act as gushing factors in beer. Hydrophobin concentrations at the ppm level were sufficient for gushing induction. An immunological assay was developed for determination of hydrophobin levels in barley and malt. A connection was found between the hydrophobin level and the gushing potential of malt, suggesting that the developed hydrophobin assay can be used for prediction of the gushing risk in malt. The occurrence and fate of hydrophobins at different stages of the barley-to-beer chain were investigated.