Recombinant Production of Hydrophobins HFB4 and HFB7 in *Trichoderma reesei*

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Abstract

Trichoderma reesei is a filamentous fungus long known for its secretory capacity, finding applications across several industries. The selection of a member from this genus in particular is no coincidence – *Trichoderma* are globally distributed, and therefore were some of the first well-studied fungal species. Integral to their success is the application of hydrophobins - small molecular weight proteins with high surface activity. Characterising and cataloguing the diversity of these proteins across the *Trichoderma* genus benefits research in novel medical and industrial applications, as well as our biological understanding of mycoparasitism. Thus, an acute need to develop more efficient hydrophobin production platforms exists. Alongside cataloguing the diversity of hydrophobins across the *Trichoderma* genus, overexpression models allow more realistic industrial-level yields to be achieved. In an effort to design a *T. reesei* based production system, two plasmids containing class II hydrophobin genes from *T. virens* were transformed into a *T. reesei* host. These hydrophobins were the well-known HFB4, and the recently discovered HFB7. Framing the gene of interest is a constitutive promoter, chosen to maximise secretory capacity, as well as a histidine tag for purification. As HFB4 is present in both species, it was hypothesised that *T. reesei* would produce a functionally identical HFB4 as in the native *T. virens.* The resulting overexpression mutants were therefore considered to be homologous expression systems. Proteins lacking in the parental species' genome are more unpredictable, as the organism may struggle to produce or overexpress an identical protein. For this reason, the HFB7 overexpression mutants are considered to be a heterologous expression system. The *T. reesei* hydrophobin overexpression mutants produced during the current study are of interest for continuing research, which should further characterise the overexpressed proteins to determine how they differ from those produced in cell factories based on other organisms. Fundamentally, this study provides a stepping stone into the use of *T. reesei* as a host organism for hydrophobin production at an industrial scale.

Kurzfassung

Trichoderma reesei ist ein fadenförmiger Pilz lang bekannt für seine sekretorischen Fähigkeiten von Proteinen und Metaboliten, die den Pilz industrieübergreifend interessant erscheinen lassen. Dass dieses Genus gewählt wurde ist kein Zufall – *Trichoderma* sind weltweit verteilt, und sind einige der ersten tief erforschten fadenförmigen Pilzen. Wesentlich ist die Benutzung von Hydrophobinen; Proteinen mit niedrigem Molekulargewicht und extrem hoher Oberflächenaktivität. Von der Charakterisierung und Katalogisierung der Vielfalt der *Trichoderma* Familie übergreifenden Proteinklasse würden sowohl die Erforschung von neuen medizinischen und industriellen Verwendungen, als auch das biologische Verständnis von Mycoparasitismus. In dieser Studie wurden zwei Proteinen, Hydrophobinen HFB4 und HFB7, die aus dem Pilz *T. virens* stammten, in einem *T. reesei* Vektor überexprimiert. Das Ziel war es die Funktionsfähigkeit von *T. reesei*-stämmigen Mutanten als industrielle Zellfabriken zu bewerten. Die Transformation wurde erfolgreich durchgeführt. Es konnte danach nachgewiesen werden, dass die *T. reesei* Mutanten bessere überexprimierenden Vektoren als der Wildtyp sind. In den nachfolgenden Schritten wird der Fokus auf der Erforschung des Verhaltens der Mutanten liegen, ebenso werden die Ergebnisse der Bioreaktorfermentation repliziert, um die Funktionsfähigkeit der Mutanten als industrielle überexprimierende Vektoren zu bestätigen. Die überexprimierende Mutanten sind interessant für weitere Forschung, die einen Schwerpunkt auf eine weitere Charakterisierung des hergestellten Hydrophobins, sowie einen Vergleich der Effizienz vom *T. reesei* Vektor mit deren, von anderen Zelle Fabriken herstellen. Im Wesentlichen lässt diese Studie einen Trittstein an die weitere Verwendung von *T. reesei* als Produktionsvektor für Hydrophobinen auf einem industriellen Niveau überschreiten.

Abbreviations

A – Ampere
BCA - Bicinchoninic acid
BCIP - 5-bromo-4-chloro-3'-indolyphosphate p-toluidine
BiP - Binding immunoglobulin protein
bp - Base pair
CMC - Carboxymethyl cellulose
CRISPR - Clustered regularly interspaced short palindromic repeats
Cas9 - CRISPR associated protein 9
COPII - Coat protein complex II
Da - Dalton
DNA - Deoxyribonucleic acid
ER – Endoplasmic reticulum
ERAD - ER Associated protein degradation
EtOH – Ethanol
GFP – Green fluorescence protein
GSM - Glucose synthetic medium
GTP - Guanosine triphosphate
hfb4 or *hfb7* – hydrophobin 4 and hydrophobin 7 genes, respectively
HFB4 and HFB7 – Hydrophobin 4 and Hydrophobin 7 proteins, respectively
His-tag – Histidine tag
HIS6 – Histidine tag with 6 histidines
*hfb4*OE - Hydrophobin 4 overexpression mutant
*hfb7*OE - Hydrophobin 7 overexpression mutant
HYDEN - HighlY DEgeNerate primers
LB - Lysogeny broth
MW – Molecular weight
mRNA – messenger RNA
NBT - Nitro-blue tetrazolium chloride
OE - Overexpression
PCR - Polymerase chain reaction
PD – Potato dextrose
PDA - Potato dextrose agar
PEG - Polyethylene glycol
RNA – Ribonucleic acid
RNAP - RNA polymerase
RPM – Rotations per minute
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMW – Small molecular weight
SNARE - Soluble N-ethylmaleimide-sensitive factor attachment protein
SP – Signal peptide
SRP – Signal recognition peptide
TBS - Tris-buffered saline
TCA – Trichloroacetic acid
TGN - Trans-golgi network
TUCIM – Technical University of Vienna Collection of Industrial Microorganisms
UGT - UDP-glucose:glycoprotein glucosyltransferase
UV – Ultraviolet
V – Volt
WCA – Water contact angle

Contents

1. Introduction

Genetically modified organisms have grown to play an integral role in modern day industry; a fact demonstrated by the colossal quantity of research currently being pursued on the topic. This undertaking has advanced considerably in the past decades, yet still lacks the capacity to industrially produce many compounds. This study represents another step along the path to better understand proteomic overexpression (OE) in fungal cell factories by genetically modifying an industrially relevant fungus to improve secretion of two members of a highly useful and intriguing protein class: hydrophobins.

1.1. Hydrophobins and their Applications

Hydrophobins represent a unique group of proteins secreted by filamentous fungi (1) and are present in both Ascomycota and Basidiomycota phyla. The diversity of hydrophobins has made pinpointing their biological functions difficult (2). Apart from the conserved cysteine residues, homology across hydrophobins is remarkably low, nor is cross-hybridisation possible (3, 4). Hydrophobin profiles are unique enough that one may differentiate patterns at the isolate (sub-species) level (5). Nonetheless, they have been implicated in the morphogenesis of aerial hyphal structures and fruiting bodies (6), as well as in interactions with other organisms - in parasitic as well as mutualistic conditions (7, 8).

They are generally single domain proteins with eight conserved cysteine residues. They are ca. 96-157 amino acids in length, and have usually between two and four hydrophobic patches (9, 10), which comprise around 20% of the surface (11). They are also the most surface active proteins known (12), with a tendency to assemble as monolayers in a highly stable manner, however only at specific sites (hydrophobic patches) obliging the largely hydrophilic surface to be shielded away (13, 14).

Hydrophobins are divided into two general classes, class I and II (3). The class distinction was initially developed and to organise hydrophobins based on the relative clustering of hydrophobic and hydrophilic regions (8). In recent years, many intermediary hydrophobins have been discovered which poorly fit into the original categories (15), as visually represented in Figure 1. Complementing the original class partition with hydropathy plots and spacing measurements between conserved cysteines of the α-helix allow hydrophobins to be more accurately clustered (8).

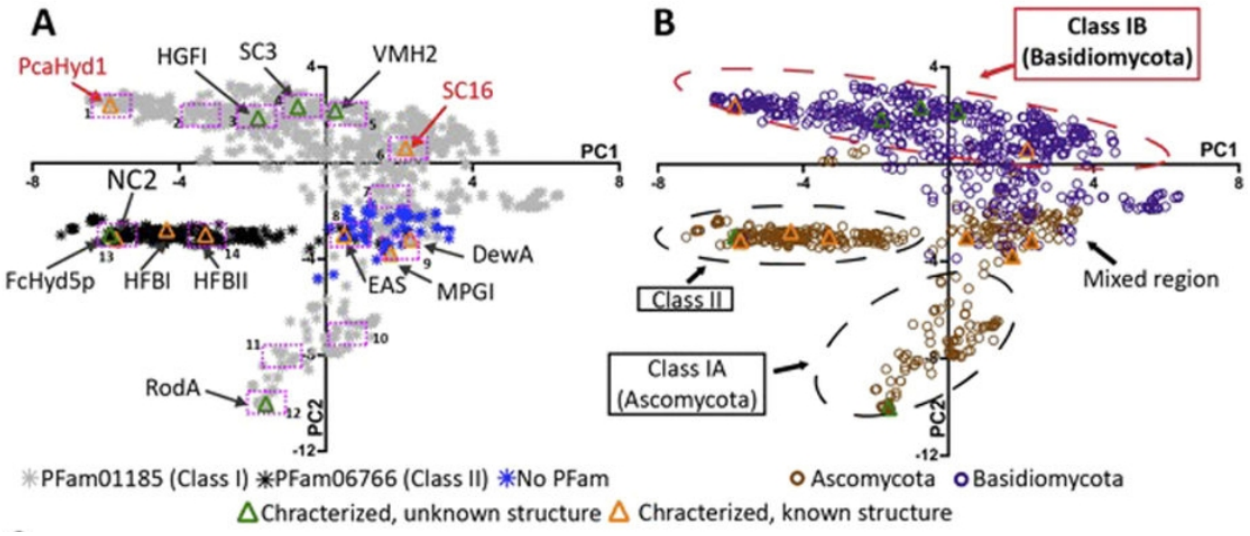


Figure 1. Principle component analysis based on the sequences of 1052 hydrophobin sequences. Of particular note is the mixed region, indicating that hydrophobins not fitting the classification paradigm are more common than a few outliers (16).

Structurally, both classes exhibit an α-helix in the middle of the protein's primary structure consisting of eight cysteine residues forming four disulphide bridges (16). All bridges are symmetrically located on the same plane, spanning the length of the molecule (14). The disulphide bridges are not directly involved in assembly (17), however they do keep monomers soluble in solution (18), and are integral to the stability of the molecule (19). Hydrophobin solubility is also mediated by the role of auxiliary agents (20, 21) and other hydrophobins (22). Point mutations in the hydrophobin HFB2[1] indicate that there is no association between oligomer association in solution and surface properties at the interface (23). Knockout mutants with the cysteine residues replaced with alanine substitutes had difficulty secreting the affected hydrophobin, as seen via the lack of a five nanometer rodlet sheet on the surface of Δ*mpg1* conidia (*Ustilago maydis*) The conidial rodlet layer was still observed in a few samples, indicating that the substitution did not completely prevent self-assembly(24).

Class I hydrophobins form insoluble interfaces, which precipitate out of solution upon disintegration of the assembly (e.g. sonication of the sample) (25). These structures disassociate into monomers only in the presence of strong acids (e.g. formic acid and TFA) (12). A leucine zipper domain was identified in the *Pleurotus ostreatus* class I hydrophobin HYD (26, 27), a common motif in protein binding. This could help contribute to the class's increased stability. Another factor is an increased number of hydrophobic residues in class I members compared to class II, notably found in a longer unstructured loop between the 1st β-hairpin segment between the 3rd and 4th

[1] HFB2 may be alternatively written as HFBII, following the convention initially described by Nakari-Setälä et al. (4).

cysteines (Cys_{3-4}) (28). Unique to class I HFBs, the segment contains several aliphatic residues, coordinating the orientation of the patch (29).

The variable region responsible for diversity across hydrophobins is largely attributed to the size and orientation of β-hairpins located at the N and C terminals. In class I hydrophobins, the presence of a loop is considered pivotal to the self-assembly structure of the molecule, extending as part of the macrostructure backbone along the hydrophobic side of the interface upon assembly (30). Comparisons between class I hydrophobin from *Neurospora crassa*, EAS, and the class II hydrophobin from *T. reesei*, HFB2, further reveal two stranded β-sheets are formed from the loop in class I, whereas the region is an α-helix in class II hydrophobins (28).When 15 of 25 residues in the Cys_{3-4} loop were deleted in EAS, rodlet formation and surface activity were severely impaired, yet the physiochemical properties of the molecule (e.g. core structure) were unchanged (31). Representations of both classes are provided in Figure 2.

Class I hydrophobins tend to have longer and more varied cysteine spacing and are thus more structurally diverse, resulting as well in conformational changes upon coagulation (32, 33). They tend to form highly robust β-sheet structures stable at high temperatures and pH (34-36). In contrast to the robust class I assemblies, class II hydrophobins may be dissociated in 60% EtOH or 2% SDS (12). They are also more susceptible to the influence of salts and pH (18). Class II hydrophobins have regular cysteine intervals, and form weaker monolayers (32) (37). They tend to also be more compact, rigid and shape-persistent (38). Although class II hydrophobins do not form class-I-like rodlets, they do create fibrillar assemblages at high protein concentrations (13, 39). Whereas class I rodlets tend to be ca. 5 nm in width and 20 nm in length, the class II fibrillar assemblages are larger with widths around 1 μm and lengths from 10 to 100 μm (40). Fibrils may be described as protein aggregate islands, effectively creating a bilayer structure with the monolayer immediately around them. The aggregates and monolayer exist in a concentration dependent equilibrium, favouring fibrillar formation at higher hydrophobin concentrations (41). Class II hydrophobins are more uniform as a group than their class I counterparts (13), attributed to their smaller size which allows less room for variation. Implicitly, it is thereby easier to identify which residues impart the greatest contribution to structure and assembly by comparing members within the class.

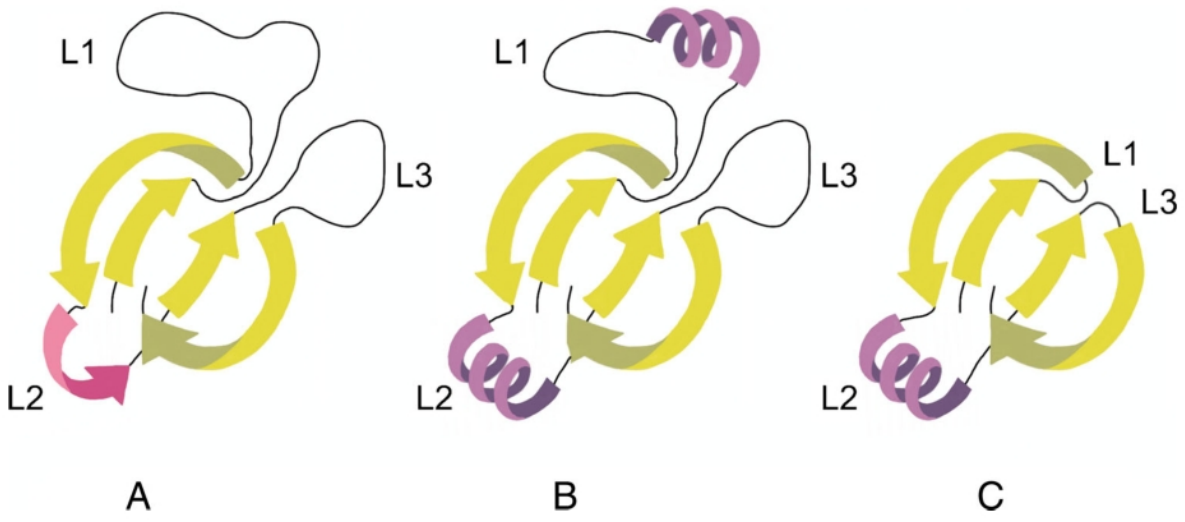


Figure 2. 3D representation of the more variably structured class I (A, B) and the compact class II (C) hydrophobins. The β-barrel core is conserved, consisting of eight cysteine residues creating four disulphide bridges (42).

The tendency of hydrophobins to aggregate along surfaces also represents one of the most difficult aspects of their production and is explained by their conformational equilibria. Hydrophobin conformation states are split between soluble monomer or multimers, less ordered interfacial assemblies and more ordered interfacial assemblies (43). As the proteins naturally exist in an aqueous milieu, the most energetically favourable orientation necessitates shielding of the hydrophobic region in the form of aggregates interacting at the hydrophobic patches (14, 44). Individual monomers in solution are not surface active below a minimum concentration required for interface assembly, the critical concentration (18). This threshold is hydrophobin-specific and determinable by interpretation of the hydrophobin's absorption isotherm (45). Moreover, whether hydrophobins assemble into dimers or tetramers in solution is concentration dependent (30, 46). Gyration radii of monomers is significantly larger than tetramers (ca. 24 Å vs ca. 65 Å), emphasising how aggregation decreases surface exposure to the surrounding solution (39). It is difficult to determine the degree of hydration for hydrophobins in solution due to changes in the electron cloud as well as x-ray contrast created by the complex inner folding of the molecules (47).

Only at very dilute concentrations (< 10 µg x mL^{-1}) do hydrophobins exist mainly as monomers (39). They otherwise have a tendency to aggregate as dimers or tetramers in solution, and as rodlet layers along surfaces (3). While class I hydrophobins tend to form films, class II may often form globular oligomers in solution (22, 38). Both responses effectively occur to protect the hydrophobic patches of the hydrophobin protein from water. Class I and class II assemblies do not integrate, however the presence of a class II hydrophobin inhibited the precipitation of a class I hydrophobin, thus indicating that some interaction may occur (22).

Ultimately, the diversity of hydrophobins is a result of a rapid evolution of the protein across filamentous fungi (Figure 4). It is difficult to categorise each hydrophobin by its biochemical role due to overlapping expression and function (48). This complicates tremendously the phylogenetic analysis (Figure 3). It has previously been determined that within the phylum Ascomycota, the class Sordariomycetes has a disproportionally large amount of hydrophobin genes (49). The *Trichoderma* genus (Hypocreales, Ascomycota), is host to the greatest known diversity of hydrophobins (50), and is predominately mycoparasitic and saprotrophic (49). Within this genus is the extensive evolution and diversity of the hydrophobin genes (figure 3). which likely developed through a death-and-birth mechanism, i.e. the evolution of hydrophobin paralogs, which continued to evolve divergently or be decommissioned as pseudogenes(49, 51). The rapid mutation rate within hydrophobin genes visible within the Ascomycota phylum by the fact that hydrophobins diversified significantly after the branching off of the *Trichoderma* genus (49). It is likely that the affinity of hydrophobins for hydrophobic-hydrophilic boundaries promoted the innovation observable among hydrophobin secreting filamentous fungi (52). Around 40% of hydrophobins have been identified as results of gene duplication events (53), overtime resulting in highly similar genes but with different expression profiles (54). This is in line with the birth-death evolution theory, whereby duplicates are "purified" in the sense of either continuing to evolve (specialise) or are silenced (2). The process is well exhibited in the *Trichoderma* genus hydrophobin phylogenetic tree, where higher evolutionary rates are estimated in some clades compared to the others on the phylogeny tree (55).

Combined with a propensity for diversity, the capacity of hydrophobins to be secreted in large quantities highlights their integral role at the interface (56). In general, hydrophobins may be expressed in hyphae or in reproductive structures (57-59), in different sexual stages of the fungus' life cycle (60, 61), and under different stress conditions (4), representing a high functional specificity (12).

Most applications of hydrophobins are based on their amphiphilic nature. Due to the assembly models of class I and class II hydrophobins, the former is optimal for long duration coating, while the second for transient coating of materials (37). The affinity a liquid maintains to a solid surface, referred as the wettability of the surface, is increased by adding hydrophobins into the liquid (42). Water contact angle (WCA) measurements have shown that the surface tension of water decreased from 72 mJm^{-2} to 24 mJm^{-2} upon application of 20 μgmL^{-1} of HFB2 (62). The binding of the hydrophilic residues to hydrophilic surfaces ensures that the hydrophobic residues of the protein are exposed (42). This practically renders a hydrophilic surface hydrophobic, a property which may be well exploited. Modifying the surface may facilitate immobilisation of molecules to a surface (63), or the binding of medically relevant substances to their respective substrates (64). Hydrophobins have already been found to bind enzymes to surfaces such as glass and various plastics during laboratory experiments. The difficulty arises

in ensuring that the HFB layer retains in ability to form ordered layers without compromising the activity of the enzymes it binds (65).

Reducing the wettability of surfaces is the desired goal of kitchen products such as Teflon, or in creating waterproof materials (66). Binding patters however, differ wildly across hydrophobins. HFB1 and HFB4 are known to stably immobilise fusion proteins to a hydrophobic surface, whereas HFB2 is more easily washed off, and the recently discovered HFB7 appears to bond poorly to hydrophobic surfaces (50).

Hydrophobins are already present in our food, although not necessarily by human design. Fungus-fermented foods as well as edible filamentous fungi (mostly Basidiomycota mushrooms, some Ascomycota) have taken part in the human diet for centuries, and thus easily prove the safety of hydrophobins in human diet. The amphipathic nature stabilizes air bubbles in foam. One study found that a foam with HFB2 and a thickening agent present was stable with minimal phase loss up to 2.5 years (67). More recent experiments have demonstrated that emulsion-stabilizing particles may further contribute to the hydrophobin foam stability (68).

The medical potential of the protein takes advantage of its immunological inertness. The average cubic meter of air contains 10^9 spores. Of these, spores some spores are directly pathogenic to humans, in particular during cases of compromised immune systems (69-72). The majority of spores, however, pass freely through the lungs without interacting with the host immune system (73). Investigations into this have pointed to hydrophobins being the mechanism rendering fungal spores invisible to the immune system as they are breathed by animals (42, 74). One innovative use of hydrophobins is as a coat complex for the serum insulin regulator protein GLP-1. Normally, GLP-1 degrades quickly in the blood, however when packed in a hydrophobin (HGF1, *Grifola frondosa*) fusion protein, it may serve as a long term diabetes control mechanism, working more efficiently than current methods (75). As research in the field increases, hydrophobins will certainly be explored as drug-carriers in the body, as well as in functionalising hydrophobic surfaces (42).

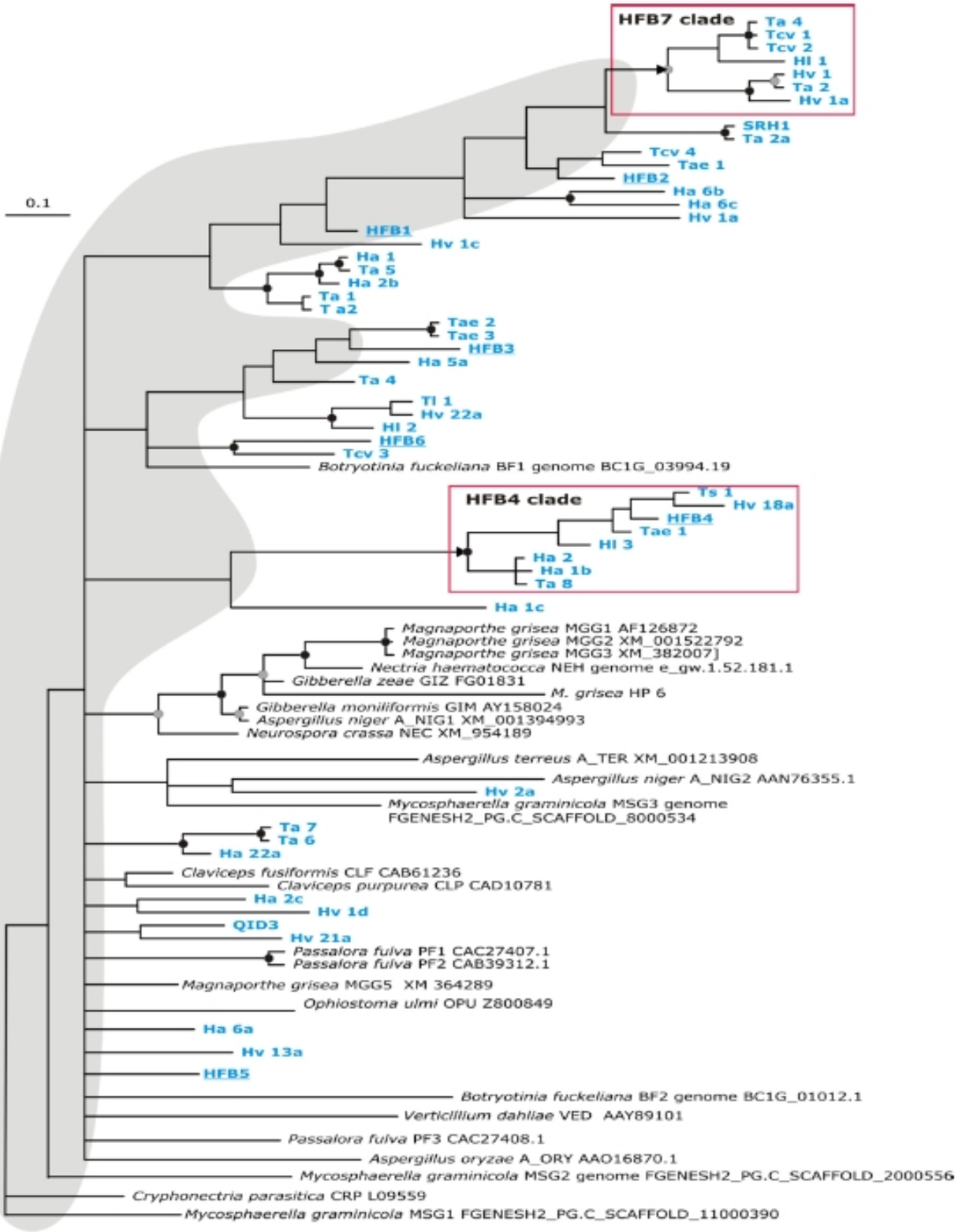


Figure 3. Bayesian phylogram derived from the amino acid alignment of hydrophobins across multiple members of Ascomycota. *Grey regions represent areas of unresolved internal structure of the tree. The clades outlined in red are of particular interest to this study (50). Blue type represents hydrophobins present in* Trichoderma.

1.2.Recombinant Protein Production

A gene of interest is overexpressed when the transcription of a desired gene artificially increased. There are many ways to construct the overexpression vector, as well as insert it into the genome.

The gene encoding a selected protein may originate homologously, meaning that the protein exists in the organism prior modification, or heterologously, meaning that it was not originally present in the host genome. In many closely related species, genes may be orthologous to one another. Thus, the two genes have the same ancestral sequence, but have diverged after the species separated into two species. Depending on the time lapsed and which environmental pressures are present, the genes may diverge faster or slower. Furthermore, the gene's location along a chromosome will also determine the rate of evolution (76). Technically, any expression of a foreign-derived gene in a species should be considered heterologous expression. However, if two orthologous are sufficiently close to one another, they may be considered to be homologous.

Integral to the insertion of a new gene is the selection of a promoter and terminator, which play a role in the sense of being the punctuation for how the gene is transcribed. Another consideration is the copy number - how often should the desired gene be present in the genome, which will affect the frequency that the gene may be transcribed. Although procedures such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 gene editing are representative of the most modern and precise techniques to locally knock out genes (77), they have only recently been successfully implemented in filamentous fungi. The success rate of CRISPR/Cas9 transformations remains low, unoptimized, and costly (78, 79).

Random insertions of the gene of interest are therefore continue to be a viable technique to investigate overexpression systems. A common genetic engineering tactic involves a shock therapy of the protoplast organism in a solution containing a linearised DNA fragment. Successful transformants are then chosen via subsequent screening among the surviving mutants for the desired traits (i.e. high secretion capacity) (80). In this way, the copy number of the inserted gene is unpredictable, as well as the specific location of the uptaken DNA within the genome. The gene of interest is brought into the host organism via an insertion cassette, which fundamentally consists of at least three components: the promoter, the gene of choice, and a terminator.

The first decision is whether an inducible promoter or constitutive promoter is more appropriate for the expression goals. Inducible promoters receive the transcription cue depending on the environment (81). The promoter of the cellobiohydrolase I gene (*cbh1*) is one of the most widely used inducible promoters, and has recently been paired with *cbh2* gene to allow simultaneous heterologous expression by two separately regulated promoters (82). The *cbh1* gene is induced by cellulose as well as the industrially convenient lactose. It is easily negatively regulated

by catabolic repression when biomass growth alone is preferred (81), which occurs to the extent that 90-95% of cellulases and proteases are suppressed by high glucose concentrations (83, 84). Inducibility is advantageous when one wants protein expression to occur at specific instances. Nonetheless, inducible promoters may be leaky if their regulation is not only dependent on the inducer, or protein synthesis may be less efficient than if under a constitutive promoter (85, 86). The second type of promoters are therefore constitutive. They initiate transcription of the gene of interest at all stages of the organism's development. If the copy number of the insertion cassette is too high, the ability of the fungus to grow is significantly hindered, in the sense that the tax of bearing the overexpression prevents efficient expression of necessary proteins for survival (87). With an optimal copy number, secretion may be higher than under an induced promoter. Some examples of constitutive promoters include the translation elongation factor gene *tef1*, pyruvate kinase-encoding gene *pki*, and *cdna1* (88). The *cdna1* promoter is widely considered to be constitutive, in the sense that there it is seen to be active in all growth conditions (89), however its function is unknown. The *cdna1* promoter is considered the most effective of the three (90).

The transformed gene, a.k.a. the gene of interest comes immediately afterwards. To complete the cassette, a terminator is needed. Terminators are codon sequences which disassociate the mRNA from the genome, arresting the transcription of the DNA. As with the promoter, ideal terminators originate from the organism into which the gene of interest in being transformed (91, 92). The choice of terminator is less vital than that of promoter. The *cbh1* terminator, originating from the original *cbh1* promoter and terminator combination used commonly in *T. reesei* expression systems (89) was chosen for the study.

Often, a tag is added to more easily purify the target protein. Two common tags for *Trichoderma* are glutathione S-transferase (GST) and histidine tags (his-tag). The former is often used in conjunction with pull-down assays, as the GST molecule displays a high affinity to glutathione (93). The latter consists of a variable amount of histidine residues and may be more efficient at large scale protein production due to the small size of the tag (50, 94). Here, six histidines were used, hence it may be referred to as a 6x histidine (HIS6) tag. As several class II hydrophobins exhibit long N-terminal hydrophilic extensions, varying the length on the N terminus by adding tags should not affect the functionality of the protein (50). Due to its small size, the his-tag can be considered minimally invasive to the secretion pathway of the organism. Furthermore, his-tags do not significantly alter the elution conditions, nor are they affected by strongly ionic or chaotropic agents and detergents (95).

Fundamentally, expressing a foreign protein in an expression vector is similar to loading a computer program in an operating system for which it was not explicitly designed for - it may not work at all, and if it does work, there

may be unanticipated problems along the ways. Thus it is logical that heterologous overexpression has generally not been as efficient as homologous expression (96).

1.3.Filamentous Fungi as Cell Factories

Enzymatic production via filamentous fungi represented a €1.5 billion market in 2000, and a €3 billion market by 2013, encompassing technical, chemical, and medical applications (97) (Figure 4). The modern food industry has incorporated fungal proteins and metabolites (i.e. citric acid, milk proteins, enzymes) into many food products as cheaper substitutes for naturally derived products (98). Modern industrial scale fungal fermentations can expect > 40 gL^{-1} (99), although secretion quantities will vary wildly depending on the organism and how the product protein or metabolite interacts with the secretory pathway available.

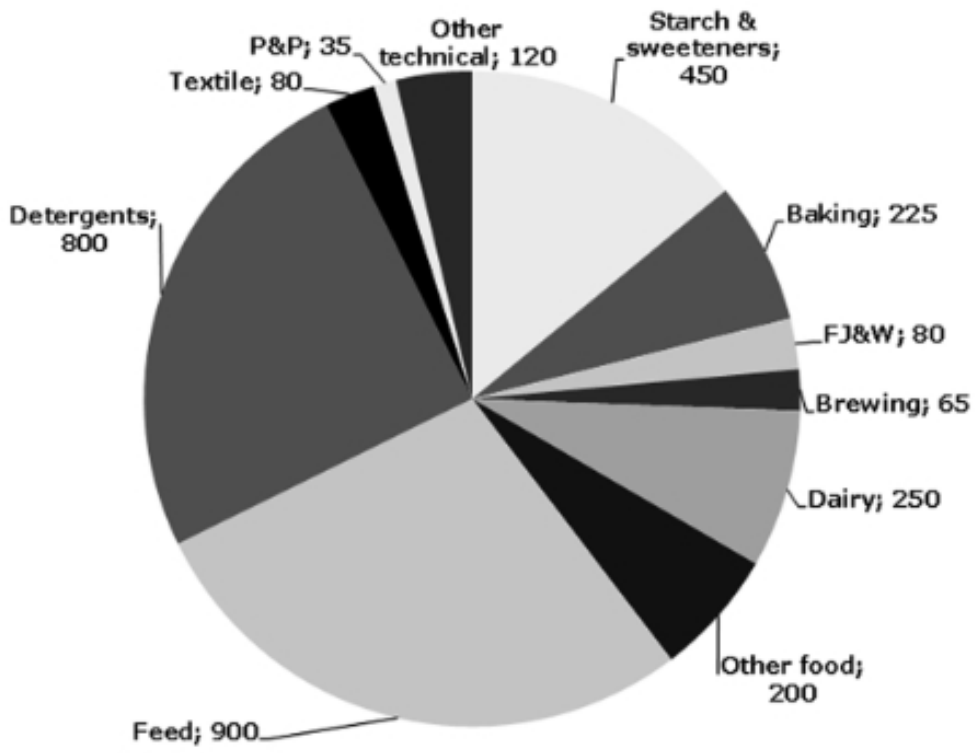


Figure 4. Percentage breakdown of enzymatic production using filamentous fungal vectors. Starch & sweeteners, baking, FJ&W (fruit juice & wine), brewing and dairy segments represent food applications while detergents, textile and P&P (paper & pulp) are technical applications. In more general terms, technical enzymes represent 65 % of production, the food industry represents 25 %, and animal feed enzymes represent 10 % (100).

The term *cell factory* is used to describe genetically modified organisms which are used to produce desired proteins or metabolites. The following section will review considerations which must be taken into account when

designing a cell factory, namely which bottlenecks exist along the fungal secretory pathway for a target protein being expressed in industrial quantities.

The main goal of reviewing the stations along the secretory pathway is to prevent inadvertent limitations in production due to protease activity or a bottleneck along the production line. For example, it has not been fully elucidated how the choice of codons present in an overexpressed gene influences mRNA stability and translation efficiency (100). The protein folding efficiency may be impacted by the availability of specific codons, the temporary lack of which could cause translation errors. Each organism will inevitably have a codon usage bias - certain codons will be present in the nucleus in greater concentrations that others during transcription. An overuse of an uncommon codon may induce mutations at the translation phase (101). Gosser et al. (102) found that increasing Binding Immunoglobulin Protein (BiP) up to a threshold could improve protein expression, likewise protein disulphide isomerase (PDI) increased to a threshold improved protein overexpression (103). The folding of the protein represents a fundamental mystery in modern biology and chemistry, as it is currently unexplainable how proteins fold so quickly (<1 ms) and efficiently (104). Heterologous expression in yeast or bacteria may induce inclusion bodies, i.e. nuclear or cytoplasmic aggregates. In filamentous fungi, these often arise as vacuoles containing partially folded proteins (105). Otherwise, the properly folded protein may be mislabelled and subsequently removed as a misfolded protein by the host organism (106).

Departing the ER, another potential pitfall is represented by the Golgi apparatus. Despite active research identifying and manipulating key secretion bottlenecks within the Golgi apparatuses of filamentous fungi (107, 108), it is still impossible to explain the diversity of the organelle's shape across organisms (109), and even more how this could influence posttranslational modifications or the final destination of the proteins. Furthermore, glycosylation, mostly done within the Golgi apparatus, differ in patterning across species (103). If a protein designated for human use (i.e. insulin) is expressed in a cell factory imparting a non-human glycosylating pattern, it may be attacked by the human immune system as a foreign body. As proteins are often stored and concentrated in vacuoles (110), it is possible that the desired product is delayed at this step. If the amount of protein expression begins to overwhelm the cell, endosomes (111) and proteasomes will more likely degrade the target protein (103).

Finally, when considering that apical expansion is fueled by the steady source of secretion at the hyphae tips (107), it is worthwhile to investigate how to maintain optimal conditions for maximum secretion yet minimal biomass growth. This normally arises in starvation (more generally: stress) conditions. Depending on the product desired, factors such as pH, carbon source, and other nutrient needs of the fungus must be taken into consideration. Lactose has gained popularity as a minimal carbon source which allows the fungus to meet its metabolic requirements yet does not allow extensive biomass growth.

1.4.Fungal Secretory Pathways

The capacity of filamentous fungi to secrete high quantities of proteins is explained by the cell machinery involved in protein expression. Identifying where bottlenecks along the path may be. In general terms, a secreted protein will be translated, folded, modified, and finally transported for secretion (Figure 5). The pathway will be reviewed here with an emphasis on mechanics applicable to filamentous fungi.

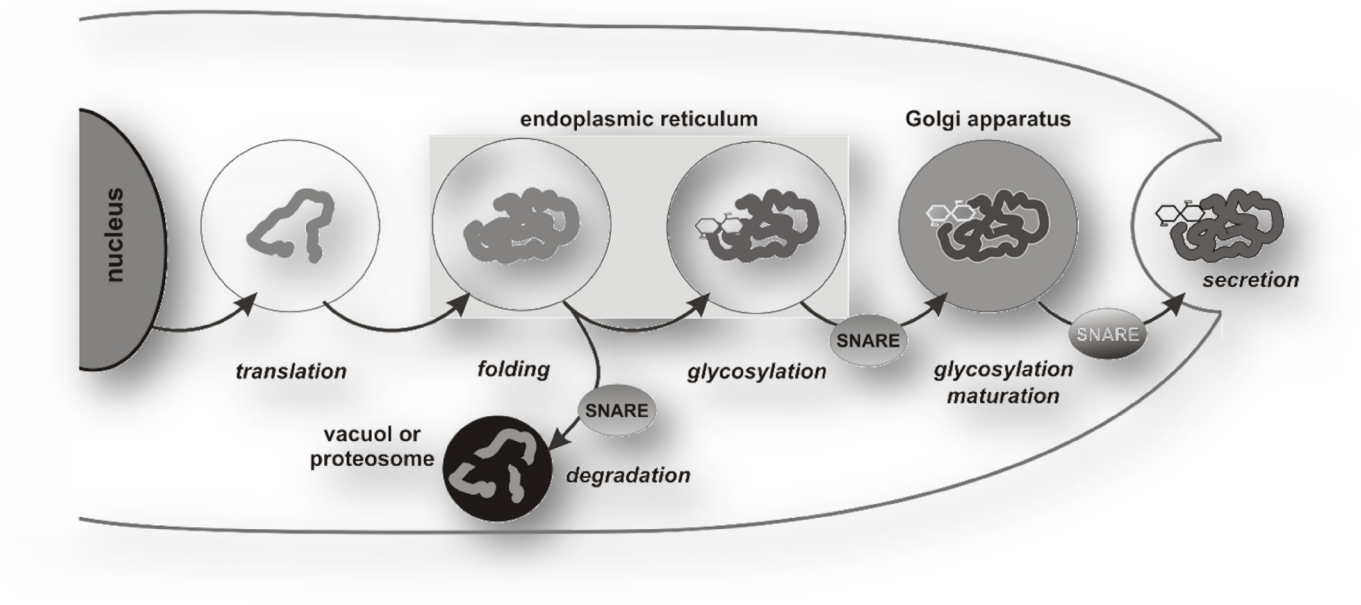


Figure 5. An overview of the evolution of a DNA strand into a protein along the fungal secretory pathway (103).

The transcription of the gene from DNA into mRNA is a universal initial step for all proteins. Initiation begins as the promoter is targeted by an RNA polymerase (RNAP), consisting of a core element paired to a holoenzyme structure (127, 128). Promoters may be strong or weak depending on their specific affinity to the RNAP. Furthermore, promoters may be inducible or constitutive - attributes discussed in greater detail in section 1.2. As the RNAP moves along the DNA strand, the corresponding messenger RNA (mRNA) elongates, linked at the 3' end to the polymerase molecule. Finally, a terminator dissociates the RNAP from the DNA strand, thus concluding transcription. Intron splicing prepares the mRNA strand before it is ready for translation into a peptide. This includes removing introns from the sequence, as well as adding a 5' cap and a 3' poly-adenosine tail. The exons composing the resulting strand represent the coding mRNA regions (103, 127).

In the cytosol, certain ribosomes bind to the mRNA, beginning the process of translation into a peptide chain. The first point of differentiation occurs by the selection between one of two translocation mechanisms from the cytosol into the endoplasmic reticulum (ER). The first path, cotranslational translocation is a signal recognition

Particle (SRP)-dependent mechanism. The N-terminus of the protein is preceded by a signal peptide (129) containing 3 conserved domains (Figure 6). Once the SP is detected by the ribosome, the peptide elongation is paused, and the entire complex is directed to an SRP receptor (130). Necessary for the docking as well as the following steps is the heat shock class protein HSP70 chaperone, as well as Binding Immunoglobulin Protein (BiP) found in the ER lumen (131). BiP with its entourage of chaperones, is recruited to the ribosomal tunnel to mediate the folding of the polypeptide chain. On the opposing side of the ER membrane is a Sec61 translocon complex, through which the peptide must travel to reach the intermembranal ER space (103).

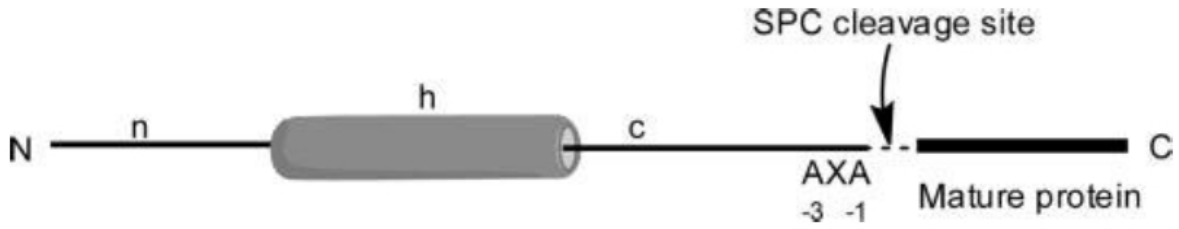


Figure 6. A signal peptide, with emphasis on the cleavage site from the rest of the protein. Conserved domains are distinguished by n (terminal domain), h (hydrophobic domain), and c (carboxy-terminal hydrophilic domain) (103).

The second possible path is posttranslational translocation, which was discovered only after the former and the specificities of which are thus less well known. Here, cytosolic molecular chaperones Hsp70 and Hsp40 bind to the peptide as it emerges from the ribosome, maintaining the correct form and ensuring solubility of the peptide in the cytosol. The ER chaperone BiP with co-chaperones translocate the peptide to a heterotrimeric membrane protein complex, and ultimately pass the peptide through a Sec61 translocon complex into the ER (103).

Once inside the ER, the peptide undergoes further folding and possible glycosylation, creating an N-linked glycoprotein. Glycosylation is the process of adding sugars to specific sites in the protein, which help increase solubility and decrease the tendency for aggregation (103, 121). Another ER chaperone molecule, calnexin, facilitates further folding of the protein. Before being released from the ER, a UDP-glucose: glycoprotein glucosyltransferase (UGT) complex assess proper folding by removing 3 terminal glucoses from a previously inserted core glycan ($GlcNac_2Man_9Glc_3$). If the N-linked glycoprotein concentration is too high, α-mannosidase attaches to one of the mannose groups, which may signal BiP to bond to the hydrophobic patches of the malformed glycoprotein (132). The BiP mediated pathway rarely occurs if the protein is correctly translated. In this way, it may be regarded as the destination of chronically misfolding proteins (133). Ultimately, prolonged binding to either of the ER chaperones will trigger the ER associated protein degradation (ERAD) pathway, which retrotranslocates the proteins into the cytosol, where they are degraded by ubiquitin and proteasome dependent

mechanism (103). Stress factors upon the cell, as well as an accumulation of misfolded proteins within the ER will retroactively regulate gene expression via the Unfolded Protein Response (UPR) pathway (134) (135).

Along the ER membrane, coat protein complexes (COPII) generate vesicles to transport the mature proteins to their target via a process called biogenesis (103). Mature proteins are sent to the adjacently located *cis*-cistern of the Golgi apparatus (107). Received proteins undergo posttranslational modification including glycosylation (136) and changes to lipid composition (137). If the Golgi apparatus exists as a singular entity in the cell, the opposing side of the organelle, the trans-Golgi network (TGN) functions as the main station for protein sorting. This is generally a site handling endo- and exocytosis (137), although the precise mechanism is not fully elucidated for multicellular fungi (103). One particularity of the Golgi apparatus among filamentous fungi is the decentralisation of the organelle throughout the cell (138). A lesser understood mechanism for translocation out of the ER is known as unconventional trafficking, whereby proteins are transported to the plasma membrane directly (139).

Ultimately, all secreted proteins must reach the plasma membrane and the exterior space. This is achieved through the colossal feat of 600 secretory vesicles per second fusing at the apical hyphal plasma membrane (140). It is likely that the hyphal apexes are the point of maximum protein secretion (120), although vacuoles are also used to store proteins before release (103). The integration of vesicles into the plasma membrane is facilitated by exocysts, binding to regions of active hyphal growth (141). The docking is facilitated partially by vesicle traffic events regulators, a family of GTPases (103). The secretory proteins are directed to the plasma membrane and outside the cells by a class of chaperones called soluble N -ethylmaleimide-sensitive factor attachment protein receptors (SNARE). It is likely that a multitude of distinct secretion pathways exist in filamentous fungi due to the formation of various SNARE complex facilitated pathways (142). The apical region is populated by a mix of small tubular and spherical vacuoles, 10 – 30 μM from the apex tip. After the first septum back, the more typical larger, spherical vacuoles appear (140). The transportation from the Golgi apparatus to vacuoles is facilitated by endosomes, which further play a role in recycling proteins and constituents to and from the plasma membrane (111). In addition to a larger number of vacuoles, the ER as well as Golgi apparatuses of apical hyphae are significantly expanded compared to cells towards the centre of the mycelium (125). In this sense the fungus is able to continuously expand in the direction of its food, while not abandoning until necessary the mycelia from previously exploited nutrition sources.

1.1.Nutrition in Filamentous Fungi

The term filamentous fungus describes the organisation of fungal growth in the form of hyphae – tubular, sometimes multinucleated cells with hemiellipsoidal apical regions (107). Together they create a network called the mycelium. Mycelial macrostructures are resilient, allowing the fungus to grow over a variety of terrain in solid and liquid substrates.

Filamentous fungi digest their food externally via various secretory agents, mining carbon and nitrogen from macromolecules insoluble to the fungal cell wall (112). This encompasses an array of enzymes, metabolites, and proteins (112). The plethora of digestive enzymes means that in additional to being saprotrophic (decomposers), some fungi are biotrophic (plant pathogenic) (113, 114). Logically, the smallest and simplest nutritional compounds accessible to the fungus are digested first, followed by more complex molecules. Thus glucose will inhibit cellulase production as it is energetically cheaper for fungi to catabolise (115).

The ability of filamentous fungi to live in virtually every habitat on earth (116-118) arises from their capacity to draw upon an arsenal of highly specialised and potent enzymes. This diversity is described by the secretome, i.e. the organic and inorganic secretory capacity of an organism. It is influenced by the substrate, temperature, as well as growth phase (119). This arsenal is particularly effective at decomposing the plant cell wall, which constitutes one of the major bioavailable carbon sources on Earth (119).

The plant primary cell wall complex is composed mainly of cellulose, hemicellulose. Other polymers such as lignin, pectin or cutin may be attached to or embedded within plant cell walls (103, 120). These constituents are efficiently arranged similar to a defensive perimeter protecting the interior. For example, the crystalline structure of the cellulose renders the structure difficult for degrading enzymes to access (119). Additionally, the plant cell wall contains monomeric substituents or larger side chains around the polymeric backbone, which inhibit the binding of proteases (121). To tap into the wealth of nutrients behind this palisade, an array of hydrolytic enzymes such as cellulases, hemicellulases, and pectinases act as key factors in the decomposition of the plant cell wall (121). Oxidative enzymes are prevalent especially with lignin (119), as are a variety of proteases in dismantling the cell wall matrix (122). An exhaustive summary of relevant enzymes is difficult in so far as filamentous fungi are concerned, and is made more complicated by accessory proteins, which facilitate the binding of the enzymes to the substrate (121). For even model species such as *T. reesei*, there exists still a significant amount of unknown proteins (123).

Another significant auxiliary faction to the enzymatic vanguard consists of other small molecular weight (SMW) proteins, employed by the fungus in the initial penetration of the cell wall. Repellents are small proteins with

significant hydrophobic regions, first identified in *U. maydis* (56), which are also a necessary component for the formation aerial hyphae (57). This has lead to speculation that repellents may integrate with the hydrophobin coating. Disabling the repellent peptide precursor gene kex2 prevented aerial hyphae formation in the subsequent mutant, despite hydrophobin genes remaining intact (58). A similar protein found in *Streptomyces coelicolor* appears to function similarly (59) and was even able to complement SC3 and REP1 in deletion studies (57). Schizophyllans, a class of high molecular weight cell wall polysaccharide produced by *Schizophyllum commune*, was found to act as a viscosity modifier on hydrophobin multimers in aqueous solution (60). This may prevent premature self-aggregation or may decrease the tendency to assemble sufficiently long enough to allow lateral reorganisation along the interface. In addition to schizophyllans, cell wall polysaccharides (β(1–3),β(1– 6)-glucan) and β(1–3)-glucan are known to promote SC3 assembly in a concentration dependent manner. Several polysaccharides have been identified which do not induce amyloid formation, and likely function to prevent hydrophobin aggregation beyond the tetramer size. One small polysaccharide, paramylon, was able to co-immobilise SC3 on polytetrafluoroethylene (PTFE). Which residues are responsible for the HFB-polysaccharide conjugation remains unknown (61). Investigations into glucose-hydrophobin complexation using Vmh2 from *P. ostreatus* as a model revealed the formation of a chemically stable biofilm. Major differences included monolayer thickness (thinner in the Glucose-Vmh2 monolayer), an increased wettability of the surface (explained by polar residues in the sugar), as well as changes in rodlet structure (62). Another protein from *S. commune*, SC15, was found to mediate aerial hyphae and attachment in the absence of the hydrophobin SC3. Lacking any cysteine residues, SC15 is not a hydrophobin. Deletion mutants lacking both SC15 and SC13 cannot produce aerial hyphae and lack the capacity when isolated to decrease water tension (63).

In nature, however, the extensive diversity of species and environmental conditions renders accurate cataloguing of actively secreted enzymes a near impossible task. Two further relevant considerations are protein modification along the fungal secretory pathway and auxiliary agents such as metabolites and proteins. This will be investigated in more depth throughout the following sections. Taken together, it may be generalised that secretion in the wild represents the interaction site between the fungus and the exterior world: detecting food sources, measuring pH, etc. (124). Harnessing this capacity for industrial uses is a major focus of this thesis and an ongoing highly active field of research.

The secretory mechanisms used in the fungus' digestion fit conveniently into many industrial applications. Industrially modified *Aspergillus* or *Trichoderma* strains in ideal conditions may reach 100 gL^{-1} protein secretion (99). This property is already well exploited across several domains: agriculture, medical, food processing, and manufacturing industries fit primarily into the scope (64, 125). Exploring biofuel production has been a hot topic

since the 1970's. The filamentous fungus *Mucor circinelloides* reached a biomass concentration with lipid accumulation of up to 28 wt% at 120 h of cultivation. Ethyl or methyl esters derived from the lipids are the raw material for producing for biodiesel generation (126), a socially popular and less environmentally damaging fuel source.

1.2.The Trichoderma Genus

The *Trichoderma* genus (previously also referred to under the name of *Hypocrea*) compromises of a group of highly opportunistic fungi, likely having evolved from a purely mycotrophic ancestor which expanded to also colonise wood (114, 124). *Trichoderma* represents the most extensively used biocontrol agent in the world and is an effective bio fertiliser, apart from being as well an efficient industrial producer of enzymes and secondary metabolites (85). The same secretory capacity exploited in industrial applications has been applied to bioremediation of toxic waste (121). Their choice for these tasks is no coincidence – *Trichoderma* are believed to be globally distributed (143) – a feat possible only due to their propensity to succeed and dominate against other fungi, as well as metabolise diverse substrates.

Within the sphere of plant/fungal/bacterial interactions with *Trichoderma*, the fungus elicits use of compounds seemingly tuned for each individual confrontation. *Trichoderma* spp. produce hundreds of volatiles organic compounds in response to nearby plants, other fungi, or bacteria (144). As a mycoparasite, *Trichoderma* invades its host by wrapping its own hyphae around the prey hyphae. The mechanism of this action is induced by the detection of lectins originating from the prey (145). Concurrently, a combination degrading enzymes (chitinases, glycanases, proteases) penetrate the cell wall (146), sometimes by additionally using a ram in the form of glycerol to increase turgor pressure (147) (145). *In situ* experiments have described *T. atroviride* mycoparasitising common soil plant pathogenic fungi. Spores of *T. atroviride* which came in contact with the host mycelium adhered to the hyphae, germinated on, and began to colonise the prey (148). Although voracity and choice of prey may vary, the general trend of mycoparasitism is constant across the genus. Additionally, there is a discourse existing between fungi and plants. The first fungal cysteine-rich surface-active protein known to directly be associated with pathogenesis on plants was cerato ulmin (CU), produced by the fungus *Ophistoma novo-ulmi* and closely related species (149). Long thought to be the responsible pathogenic compound in Dutch Elm Disease (150), the compound was later acquitted as a toxin, although nonetheless found to play an integral role in virulence (151), specifically in fungal attachment to the plant cell wall (10) and appresorium formation (152). Knockout mutants (*Δmpg1* from *U. maydis* and *Δcu1* from *O. novo-ulmi*) were both found to be less virulent than the wild type and especially in comparison to hydrophobin overexpression (OE) mutants (150, 152). "Less" is here the

key word, as virulence was not eradicated. Expressing the *cu* gene in the non-pathogenic strain *Ophistoma quercus* resulted in the fungus being able to cause symptoms of the disease (153). Furthermore, pathogenicity was found to be directly correlated to *cu* transcription in the wild type (154). Hydrophobin interfaces are impermeable to large molecules (> 200 kDa) as long as there are free hydrophobins in solution to reinforce the membrane. This provides a defensive mechanism against antifungal agents during infection and prevents the identification of fungal antigenic molecules by the host while allowing the continuous uptake of low molecular weight nutrients (155).

The heightened virulence in OE strains alongside decreased but not eliminated virulence in deletion strains could be explained by the role of the appressorium in modulating plant transcriptomic regulation through other agents alongside hydrophobins (156). The *M. grisea* hydrophobin MPG1 was found to be most expressed during the initial appressorium formation (157). Hydrophobins could play a role enforcing the cell wall, and thus increase turgor pressure on the host cell during penetration (158).

Hydrophobins influence plant expression, functioning as elicitors rather than toxins (12). This is further supported by evidence that some hydrophobin genes are upregulated in the presence of plant cell wall components, such as cellulose (159). Transcription of hydrophobins *hydpt-1* and *hydpt*-2 from *Pisolithus tinctorius* increased fourfold during ectomycorrhizal contact (160), a phenomenon observed in other fungi during plant colonisation (156, 161). Purified hydrophobin *hytlo1* from *Trichoderma longibrachiatum* displayed antifungal properties, solicited microbial-associated molecular pattern pathways, and plant growth promoters. Tomato plants heterologously expressing *hytlo1* were observed to have epinastic deformations, but as well enhanced formation and development of roots (162).

It is well known that an extensive degree of communication is possible between *Trichoderma* spp. and plants (163). Secreted proteins from the *Trichoderma* elicitors are known to be imported by plants, in some cases reprogramming host gene expression (164). An acute term to describe the relationship between *Trichoderma* spp. and plants is as an opportunistic plant symbiont (165). Thus although superficial penetration of root tissue does occur, plant disease is rare, and an endophytic relationship usually develops (164). This appears to be facilitated through proteomic and metabolic signaling, triggering an induced systematic response in the plant (165).

The answer as to why *Trichoderma* is such an effective mycotroph likely rests within the cadre of the previously mentioned protein class, hydrophobins (124). The genus boasts the most expansive hydrophobin count, with *T. atroviride* having the most amount presently known at 10 hydrophobin encoding genes (49). Hydrophobins have been implicated in cell wall breaching, as they may delay or prevent a concerted immune response of the host

(166). Outside of mycotrophy, hydrophobins have been implicated in the activation of various secreted enzymes to facilitate digestion of a substrata (I. Druzhinina, Personal Communications, 2016).

Extracellular secretion therefore compromises a broad spectrum of compounds including cellulases, hemicellulases (99), peptaibols, mycotoxins, over 100 known antibiotic metabolites (85), and thousands of other secondary metabolites (167). The high secretory capacity of *Trichoderma* strains must therefore be weighed against this background (168).

1.3.Recombinant Hydrophobin Production in Trichoderma

Trichoderma reesei has a long history of recombinant production and strain improvement, while other *Trichoderma* species are gaining popularity in recent years (169). There are several important factors related to strain optimisation which must be intimately considered when developing a recombinant production vector.

In terms of modifications to the strain or the protein, target protein production can be facilitated in a few ways. By creating deletion mutants, protein secretion of desired mutants may be ameliorated in a process which effectively frees up space in the fungal secretory pathway (170). For example, the enzyme cellobiohydrolase I (CBH1) from *T. reesei* is considered responsible for ca. 60% of the protein secretion capacity (171), thus by knocking out the respective *cbh1* gene, the cell factory may focus on producing the protein of interest (assuming that the knocked out gene will not significantly inhibit the survival capacity of the organism). This may be exploited to better streamline hydrophobin production, for example by utilising as a starting point certain optimised strains such at RUT-C30 (172). Given the role of the ER in protein folding (*vide supra*), increasing the ER content in some cases has improved protein secretion yield (170). Structural changes in the ER have been observed as secretion pulses, describing a phenomenon where protein yield fluctuates over the course of fermentation (170). It is important to select the proper promoter when handling taxonomically remote organisms. Research by Schmoll et al. (2010) showed that using an *hfb2* promoter (originating from *T. reesei*) succeeded in successfully expressing *dewA* (*S. commune*) while a *cbh1* promoter could not provide similar secretion levels (173). Glycosylation, a post-translational modification affecting half of eukaryotic proteins (174), should not be overlooked as it plays a role in enzyme activity, as well as more generally in aggregation and thermal stability in proteins (170). It has already been explored in enzymatic production and in modifications of the protein may work alongside strain improvement to increase yield (175).

Fusion genes have been used effectively in *Trichoderma* spp. to increase protein yield. For example, the native C-terminal carbohydrate binding module (CBM) may be replaced by a catalytic module, a linker, or a new CBM. In comparative studies between fusion proteins and their unaltered counterparts, these modifications have been

associated with increased mRNA stability, ER import, and passage through other segments of the secretory pathway (176).

Of the many *Trichoderma* species, one, despite lacking any outstanding capacity for secretion, mycoparacitism, or unique secondary metabolites, emerged as the standard for industrial applications: *T. reesei*.

1.4.Recombinant Hydrophobin Production in *T. reesei*

T. reesei was first discovered by the US Army in the World War II in the Solomon Islands (177). Despite being identified as a pest in the 1940's, the fungus was re-evaluated as an effective depolymerising agent for cellulose after further research was conducted in the following three decades. It was at this time that the wild type isolate QM 6a, initially identified as *T. viride*, was later attributed to a new species, *T. reesei*. However, it was only in the 1970's that the cellulolytic quality was again reviewed for the production of biofuels (177). The mutant QM 9414 was derived from random mutagenesis coupled with intensive selection screening, exhibiting a cellulose production two to four times that of the wild type (178). Another group at this time developed RUT-C30, an even better cellulase producer (179). Since then, *T. reesei* mutants have found their way into many industries and have defined themselves as a gold standard in filamentous fungi cell factory use.

This study has also chosen to use *T. reesei* as the expression host. This was decided firstly because of its industrial relevance, as findings produced during the study may be scaled up fermenter production. Correspondingly, keeping to a standard organism allow easier comparison of findings and techniques to other groups working on the same species (180). Secondly, *T. reesei* is capable to grow quickly on simple media, and thirdly, it is well-known for its secretory capacity among industrial filamentous fungi. It also contains a relatively low diversity of hydrophobin encoding genes (six have been identified from the genomic sequence (181)); instead of 10-12 in some other species (I. Druzhinina and F. Cai, Personal Communications, 2016). Given the extracellular digestion mechanism seen in filamentous fungi, it understandable that they produce a more diverse array of enzymes alongside the desired protein, in comparison to their bacterial or yeast counterparts. Nonetheless, *T. reesei* comes with less extra genomic baggage than other members in the *Trichoderma* genus, necessitating fewer purification steps post-fermentation (Table 1). Lastly, several strong promoters within the genome of the fungus have been identified, providing the framework for efficient homologous or heterologous protein expression using a constitutive promoter (182).

Table 1. A comparative genome and secretome analysis of Trichoderma genomes (N>15) and a screening of EST libraries for a few other species showed that the genus Trichoderma contains the highest number of hydrophobins found so far in Ascomycota (182).

	T. reesei	*T. atroviride*	*T. virens*
Total genes	9143	11 865	12 518
Genes with signal peptide	826	1030	1096
Extracellular proteins	747	968	947
Glycosyl hydrolases (GH)	114	140	139
Proteases	61	81	63
Oxidases	24	32	28
SSCPs	174	258	250
Unknown genes	213	264	253
Orphan genes	50	78	63
Other genes	111	115	151

1.5.HFB4 and HFB7 Clades

Due to their properties and characteristics for basic research and in industry, the hydrophobins HFB4 and HFB7 were chosen as the protein of interest in the current study. The clades within which HFB4 (100-102 amino acids) and HFB7 (93 amino acids) reside were first identified in 2013 by researchers investigating enzymatic modification of synthetic polymers with hydrophobins (50). Hydropathy profiles comparing HFB4 and HFB7 to previously studied hydrophobins (namely HFB2) revealed that divergences in the primary sequence would lead to changes in surface binding behaviour of the proteins. HFB4 was found to increase WCA on glass and PET, whereas HFB7 was inactive on the former, and only increased WCA on PET (50).

A sequence alignment of the orthologous *hfb4* sequences originating from *T. virens* and *T. reesei* shows that the two genes are highly similar (See Supplementary Materials Vector Sequences). The overexpression of the *T. virens* HFB4 gene in *T. reesei* is therefore being regarded in the current study as homologous expression. Existing divergences will be apparent after comparing the *T. reesei* native HFB4 alongside the *T. virens* HFB4 expressed in the *T. reesei* cell factory. If the two proteins are physically and functionally alike, it may be concluded that the divergence is not significantly important to the protein.

In contrast to the universally present HFB4 (in *Trichoderma*), HFB7 is present only within the genome of species from closely related *Harzianum* and *Virens* clades (50). The two hydrophobins compliment each other here and will provide insight into the expression a non-native hydrophobin in T. reesei.

1.6. Aims of This Study

Considering the secretion potential of the *Trichoderma* genus, and the potential to bypass additional folding/refolding steps as in yeast or bacterial cell factories, *T. reesei* was chosen as the overexpression system. One of two hydrophobin genes were selected, dividing the overexpression transformants into two groups: those containing the hydrophobin gene *hfb4*, considered here to be a homologous transformation (*vide supra*), and those containing the heterologous hydrophobin gene *hfb7*. The rationale behind inserting *hfb*4 was to create a more efficient strain for producing specifically HFB4 amongst other hydrophobins. The *hfb*7 gene originated from *T. virens* and is not natively present in *T. reesei*[2].

The goal of the current study was to assess the viability of *T. reesei* as a host for homologous and heterologous hydrophobin production of the selected hydrophobin genes using a specifically designed construct containing a strong, constitutive promoter and an N-terminal HIS6 tag. This encompasses several steps.

- The homologous overexpression of HFB4 from *T. virens* Gv29-8 in *T. reesei* QM 6a using a constitutive promoter.
- The heterologous overexpression of HFB7 from *T. virens* Gv29-8 in *T. reesei* QM 6a using the same constitutive promoter.
- Physiological characterisation of the overexpressing mutants.

[2] The universal distinction between the gene and corresponding protein is writing the name of the gene italicized, and the protein capitalised and non-italicised. The acronym HFB2 therefore represents the hydrophobin protein, while *hfb2* represents the hydrophobin gene.

2. Materials and Methods

2.1. Microbial Strains

Plasmid propagation prior to fungal transformation was completed using the cloning vector *Escherichia coli* strain JM109 (183). The *T. reesei* wild type strain QM 6a used in the study (184), as well as the *T. virens* strain Gv 29-8 from which the heterologous genes originated (114), were obtained from the Technical University of Vienna Collection of Industrial Microorganisms (TUCIM). TUCIM samples are stored at -80°C, as agar plugs or spore solutions in 50% glycerol stock for long term storage.

T. reesei (https://genome.jgi.doe.gov/TrireeStandDraft_9_FD/TrireeStandDraft_9_FD.info.html) and *T. virens* (https://genome.jgi.doe.gov/TrivirGv298_FD/TrivirGv298_FD.info.html) wild type genomes are available to the public via the Joint Genome Institute (JGI) website.

2.2. Preparation of Cultures

A standard lysogenic broth (LB) medium was used during the plasmid propagation phase, to provide a suitable growing environment for *E. coli* JM109. This medium consisted of 10 g casein-derived peptone, 10 g NaCl, and 5.0 g yeast extract, diluted to 1 L deionised water. Contents were autoclaved at 120°C for thorough sterilisation before use. The LB medium was supplemented with 15 gL^{-1} agar-agar prior to the sterilisation step to create a solid medium for petri dish cultivation of the *E. coli* vector if needed.

Two liquid fermentation media and two solid agar media were used during the cultivation of the *T. reesei* overexpression strains. The first liquid medium was designed for shake flask cultivation (Table 2). The carbon source used was glycerol. Peptone is included to provide the amino acid source for the organism. The non-ionic surfactant Tween-20 was added as a dispersing agent. The rest of the components consist of physiological salts and necessary trace elements. The bioreactor fermentation broth was also based on a 4% glycerol carbon source, but with different amounts of the other ingredients (Table 2).

Table 2. Component lists for the shake flask fermentation (left), bioreactor fermentation (right).

	Substance (g/L)	
	Shake Flask	**Bioreactor**
Glycerol	40 mL	40 mL
casein-derived peptone	0.35	4
KH_2PO_4	0.68	5
K_2HPO_4	0.87	-
$(NH_4)_2SO_4$	1.7	2.8
$MgSO_4 \cdot 7H_2O$	0.2	0.5
$CaCl_2 \cdot 2H_2O$	0.2	0.5
KCl	0.2	
$FeSO_4 \cdot 7H_2O$	0.02	0.25
$MnSO_4 \cdot H_2O$	0.02	0.08
$ZnSO_4 \cdot 7H_2O$	0.02	0.07
$CoCl_2 \cdot 2H_2O$	-	0.10
Tween-20	0.175	-

Solid medium was prepared by diluting 39.0 g potato dextrose agar (PDA) in 1 L tap water and autoclaving at 120°C. After the medium has reached a temperature below 60°C, 200 µL L^{-1} of the antibiotic hygromycin-B was added if necessary. The antibiotic was only used when growing mutants after transformation to provide an extra security against contamination by non-transformed strains of *T. reesei*, other fungal spp. or bacteria. During the first stage of mutant growth post transformation, 1 M saccharose was combined with the PDA before autoclaving, and no hygromycin-B was added post autoclaving.

Media for a static 24-well cultivation experiment were glucose synthetic medium (GSM) and potato dextrose (PD). GSM was prepared prior to use as per Table 3.

Table 3. Components for GSM medium used in Static 24-well Cultivation.

	Substance (g/L)
	Liquid GSM
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.05
$MnSO_4 \cdot H_2O$	0.016
$ZnSO_4 \cdot 7H_2O$	0.014
$CoCl_2 \cdot 2H_2O$	0.02
KNO_3	1.0
Glucose	100.0
KCl	0.5

2.3. Plasmid Construction and Primer Design

The backbone plasmid used during the study was the pUC19 cloning vector (185). This was chosen due to the large amount of cloning sites and its well documented use as a cloning vector (183). Screening among *E. coli* mutants is facilitated by a resistance gene in the plasmid to the antibiotic ampicillin. The pUC19 plasmid was provided by TaKaRa Bio Inc (Kyoto, Japan). To achieve the goal of a highly productive overexpression strain, the constitutive promoter cDNA1 (90, 186) was integrated into the cloning vector selected (Eurofins Genomics). Cellobiohydrolase *cbh1* (*cel7a*) originating from *T. reesei* Qm 6a (*tcbh1*) was used as the terminator in the current study. The combination of the *cdna1*P and *cbh1*T has been previously published as an effective expression cassette construction (89, 187). A sequence encoding hygromycin B phosphotransferase (*hph*) expression cassette was also inserted into the plasmid to provide a simple screening protocol for successful mutants.

The plasmid cassette consisted of the hygromycin-B resistance gene, the cDNA1 promoter, the hydrophobin gene, and the terminator, and a histidine tag (his-tag) giving a total size of 4.5 kb. The modified pUC19 vector, including the hydrophobin cassette with relevant restriction sites, is labelled in Figure 7, with sequences provided in the

support materials. Restriction site availability was screened using the program NEBcutter 2.0 (New England Biolabs, USA) (188).

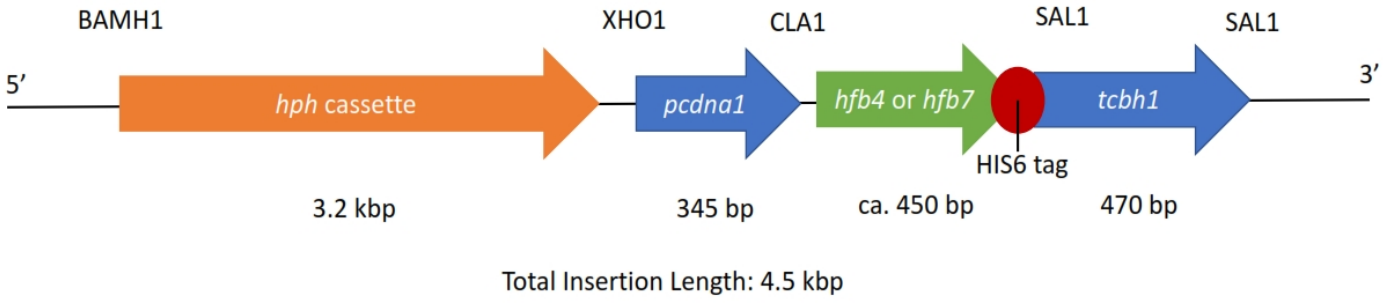


Figure 7. The hydrophobin insertion cassette within the pUC19 plasmid. Segments and restriction sites are annotated. Once expressed, HIS6 tag should be attached to the N-terminal of the translated protein.

A cassette containing all constituents save the terminator was previously designed by the TU Vienna Microbiology Lab. The current study therefore focused on the insertion of the terminator into the existing cassette (Figure 7). An initial step after the design of the new cassette was to design primers to facilitate the addition of the *cbh1*T terminator.

Primer design was driven by size requirements and consideration of the restriction sites available. A primer too short would impart an intolerable generality, while one too long would require exceptionally high melting temperatures during the polymerase chain reaction (PCR), influencing the other reaction ingredients. HighlY DEgeNerate primers (HYDEN) Software was used to design optimal primers to fulfill these requirements. This resulted in the two primer sets for *hfb4* (Table 4) and *hfb7* (Table 5). Forward primers overlapped the relevant hydrophobin gene, the GTCGAC restriction site, and the beginning of the terminator sequence. The 5' to 3' GTCGAC restriction site is cleavable using the SAL1 restriction enzyme. Reverse primers overlapped the end of the terminator sequence, the GTCGAC restriction site, as well as ca. 15 bp of the following pUC19 plasmid. The PCR specifications determined appropriate for the amplification of the both genes of interest with their respective primer is in included in Table 4 and Table 5. The inserted sequences for *hfb4* and *hfb7*, as well as a multiple sequence alignment for homology comparison between *hfb4* in *T. reesei* and *T. virens* are included in the supplementary materials Vector Sequences/ CLUSTAL 2.1 Multiple Sequence Alignment.

Table 4. Primer specifications for the hfb4 gene PCR amplification. Green represents overlap with the pUC19 plasmid, yellow represents the restriction site, and red represents overlap with the terminator. The forward primer is written as 5' to 3', the reverse primer as 3' to 5'.

***hfb*4 primer sequences**				
	Primer Sequence	GC Content (%)	Length	Melting Temperature (°C)
Forward Primer (5' → 3'):	CACCATCACCACTAAGTCGACAGCTCCGTGGCGAAAGCC	59	39	61.3
Reverse Primer (3' → 5'):	CGCCAATTGGTAATGACCCATAGGGAGGTCGACCTGCAGGCATGCAAG	56.3	48	61

Table 5. Primer specifications for the hfb7 gene PCR amplification. Green represents overlap with the pUC19 plasmid, yellow represents the restriction site, and red represents overlap with the terminator. The forward primer is written as 5' to 3', the reverse primer as 3' to 5'.

***hfb*7 primer sequences**				
	Primer Sequence	GC Content (%)	Length	Melting Temperature (°C)
Forward Primer (5' → 3'):	CACCATCACCACTAGGTCGACAGCTCCGTGGCGAAAGCC	61.5	39	61
Reverse Primer (3' → 5'):	GTAATGACCCATAGGGAGGTCGACCTGCAGGCATGCAAG	56.4	39	61

Table 6. PCR program for hfb4 and hfb7 amplification using associated primer. It was found that the same running specification produced acceptable results for both hfb4 and hfb7.

PCR program to amplify gene of interest using above primers:		
Step	Time (min)	Temperature (°C)
Initial Denaturation	1	95
Amplification: 30 cycles	1	95
	1	53
	1.5	72
Final Extension	5	72
Storage	-	4

2.4. Plasmid Propagation Using an *E. coli* Vector

The initial phase of the study involved the propagation of the modified pUC19 plasmid using the *E. coli* strain JM109 as a host. This was done in preparation of the insertion of the *tcbhI* sequence into the modified pUC19 plasmid (Figure 7). This was done for the *hfb*4 as well as the *hfb*7 plasmids.

JM109 competent cells are a well growing modified *E. coli* species, lacking the native K restriction system and carry an endonuclease A mutation; both which prevent interference with the episomal replication of the plasmid as well as discourage recombination with the host DNA (183).

2.5. *E. coli* Transformation

A JM109 competent cell culture was transformed with the modified pUC19 plasmid via a heat shock treatment in the following manner. A PCR amplified the *tcbh* terminator sequence from a sample containing the terminator in a plasmid vector as according to figure 7. The *E. coli* vector containing the modified pUC19 plasmid was incubated with the PCR product containing *tcbh* sequence for 30 min on ice, followed by a 45 sec heat shock at 42°C. The sample was then transferred onto ice for 2 minutes, before a one-hour incubation at 37°C and 300 Figure 5 in a Thermomixer.

Culture plates containing the transformed *E. coli* colonies were then incubated for 16 h at 37°C on LB plates. Using a pipette, 10 samples from the colony plate were chosen, and transferred to a growing plate containing a solid LB medium with ampicillin at 100 g/L. Each pipette tip was disposed of in a falcon containing liquid LB medium supporting the same quantity of ampicillin and left to incubate at 37°C for 24 h in a water bath.

2.5.1. Plasmid Extraction

After the incubation, bacterial culture samples were removed from the water bath and centrifuged at 8000 RPM for 5 minutes at room temperature. Cell pellets were collected on ice and processed using the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific). Cells were resuspended in 250 µL resuspension solution containing RNAse. A lysate was added to the resuspended cells. The samples incubated 5 min at room temperature before the solution was neutralized via a lysate neutraliser and centrifuged at 11 000 RPM for 5 minutes. The supernatant was filtered via filtration centrifugation to concentrate the plasmid onto the membrane. 500 µL of an ethanol-based washing solution was used to de-salt the DNA samples. This step was done twice. The plasmid was eluted with 50 µL of an elution buffer, after allowing a 5 min incubation phase at room temperature of the DNA in the elution buffer. The concentration of the resulting purified DNA samples was measured using a Nanodrop Spectrophotometer ND- 1000 (Peqlab). Purified plasmid samples were prepared for PCR amplification.

2.5.2. Polymerase Chain Reaction (PCR) Amplification of the Constructed Cassette

The following PCR protocol was used for a sample of 5 µL purified DNA (10 ng μL^{-1}). The DNA sample used in this situation was the transformed plasmid from the *E. coli* fermentation. This sample was combined with a PCR master mix, composed of 10 µL of 1M PCR buffer (ThermoFisher Scientific), 2.5 µL of 3.0 x 10^{-3} M $MgCl_2$, 4 µL of 1.6 x 10^{-4} M deoxynucleotide triphosphates, 2 µL each of the designed forward and reverse primers at concentration of 2.5 x 10^{-7} M each, 0.25 µL of 0.5 M Taq polymerase (ThermoFisher Scientific). The master mix was then diluted to 50 µL in ddH_2O. To ensure consistency across samples, a master mix for all samples was prepared before the DNA added as a final step before initiating the PCR. In addition to the samples, a positive control of isolated *hfb*4 or *hfb*7 gene sequences was included. In this way the quality of the PCR mix could be checked, i.e. a negative result can not be attributed to a poor-quality master mix if the hydrophobin genes in the positive control are successfully amplified. A negative control of ddH_2O was used in lieu of the sample, to ensure that no components of the master mix were giving a falsely positive signal.

A PCR program was designed to amplify the constructed plasmid cassette was designed as summarised table 6. An initial denaturation step was set for 1 min at 94°C. The amplification consisted of a 1-minute denaturation phase at 95°C, an annealing phase of 1 minute at 53°C, and an extension phase of 90 s at 72°C. This cycle was repeated in total 30 times. Once terminated, a final extension phase at 72°C for 5 minutes was included before cooling the PCR samples for storage at 4°C until use. PCR amplification was done using the Peqstar apparatus (Peqlab). Results from the PCR were initially screened based on their size by separating the samples with an electrophoresis on 1.5% agarose gel. SYBR® Safe DNA Gel Stain dye (ThermoFisher Scientific) renders the

PCR amplified DNA luminescent under UV light. The running specifications for the electrophoresis are 80 V, 200 mA, over 30-45 min. The exact time is dependent on the size of the gel used, and the detail of separation desired.

A PCR was run to amplify the gene of interest, i.e. *hfb4* or *hfb7*. The sample designated for the PCR amplification originated from the purified DNA retrieved from the mutant monospore cultures. The primers used to amplify the *hfb*4 gene are described in Table 4, and those used to amplify the *hfb7* gene are found in Table 5.

The length of the PCR-product corresponds to the length of the amplified sequence, thus ca. 1.8 kb for either gene of interest. Before the amplified sequence could be sequenced, it had to be purified from the PCR master mix. This was done using the mi-PCR Purification Kit (Metabion). The samples were sent for sequencing (Microsynth, Austria) to confirm the proper insertion and orientation of the terminator gene into the pUC19 plasmid. Sequencing results were aligned using the GeneDoc tool (189) to check the completeness of the terminator sequence and the orientation within the plasmid. Two confirmed constructs containing the correctly inserted terminator for the overexpression of HFB4 and HFB7 were chosen as the stock for later transformation into *T. reesei*. The post-transformation sequencing was necessary because directional cloning was not an option during the current study, as only one restriction site, SAL1, was available for the insertion of the terminator.

2.6.Transformation of *T. reesei*

With the terminator gene of interest having been successfully integrated into the modified pUC19 plasmid (183), the construct was ready to be transformed into the *T. reesei* host.

2.6.1. Plasmid Linearisation

Before transformation, the plasmid was linearized by cleaving the SAL1 restriction site. 1 µL of purified plasmid was combined with 0.2 µL of the SAL1 restriction enzyme (Fermentas), and 1 µL of the corresponding 10x O buffer to the enzyme (Fermentas). The mixture was diluted with 8 µL of water to make 10 µL of digestion mix. Linearisation is done to facilitate the uptake of the foreign plasmid into the host organism, giving a 5 fold improvement in DNA assimilation compared to non-linearized plasmids(190, 191).

2.6.2. *T. reesei* Protoplast Preparation

The wildtype strain QM 6a was grown for 16 h on cellophane coated PDA plates. The mycelia-coated cellophane sheets were collected in a large glass petri dish, where it was incubated with an osmotic balancing solution,

solution A (8.3), and lysing enzymes from *T. harzianum* (Sigma-Aldrich). After 100 min of incubation, the cellophane sheets were removed and the mycelia was filtered through glass-wool to remove large hyphal clumps. The suspended cell solution was collected in a 50 mL falcon tube and centrifuged. At this point, the hyphal cells have a reduced cell wall, and are correspondingly greatly weakened. Solution B (Supplementary Materials: Protoplasting Media**)** was added to the suspended cell solution to increase the porosity of the membranes while maintaining an electrolytic balance.

2.6.3. *T. reesei* Transformation

200 µL primed *T. reesei* protoplasts were immediately incubated on ice in a 15 mL falcon tube for 20 min with 10 µL linearized DNA at 10 ng/µL and 50 µL 25% polyethylene glycol (PEG). After the incubation period, 2 mL of 25% PEG and 3 mL solution B were added. The mixture was immediately plated in 0.5 mL increments onto 1 M saccharose-PDA plates. The plates were left to incubate at 28°C for 16 h, whereupon a PDA layer containing hygromycin-B was overlaid. The plates were left to incubate at 28°C until mycelial growth became visible on top of the overlay. This provided an initial screening criterion for mutants, as only mycelia which have taken up the resistance gene should be growing on the upper layer.

Mycelia from these colonies were cut out and replated onto new, medium-sized (90 mm) hygromycin-B-PDA petri dishes and left to incubate at 28°C until sporulation. The mutants were at this point characterised between slow, medium I, medium II, and fast growers depending on the time they required to achieve full sporulation. Fast growers sporulated within 48 h, medium II within 96 h, medium I within one week. Slow growers encompassed all mutants which required more than one week to sporulate.

2.6.4. Verification of the mitotic stability of the mutants

Once the mutants had sporulated, the plates were washed with 2 mL sterile dH_2O. Non-sporulating mutants were discarded. Spores were collected in 2 mL Eppendorf tubes. From these spore collections, samples containing 1:100, 1:1000, and 1:10 000 factor dilutions were prepared. The spore dilutions were plated onto PDA plates containing hygromycin-B at a concentration of 200 µL L^{-1}. After overnight incubation in darkness at 37° C, plates were considered to have an ideal dilution if individual hyphae were visible and not overlapping. It was determined that 1:10 000 dilutions provided the optimal amount of monospores for replating purposes. Monospores were left to incubate at 28°C until sporulation, at which point the same washing procedure was done as before. Spore samples were divided into 1 mL samples 50% glycerol solutions for long term -80°C storage, and 1 mL samples for immediate further use, stored at 4°C. Examples of the process are seen in Figure 8.

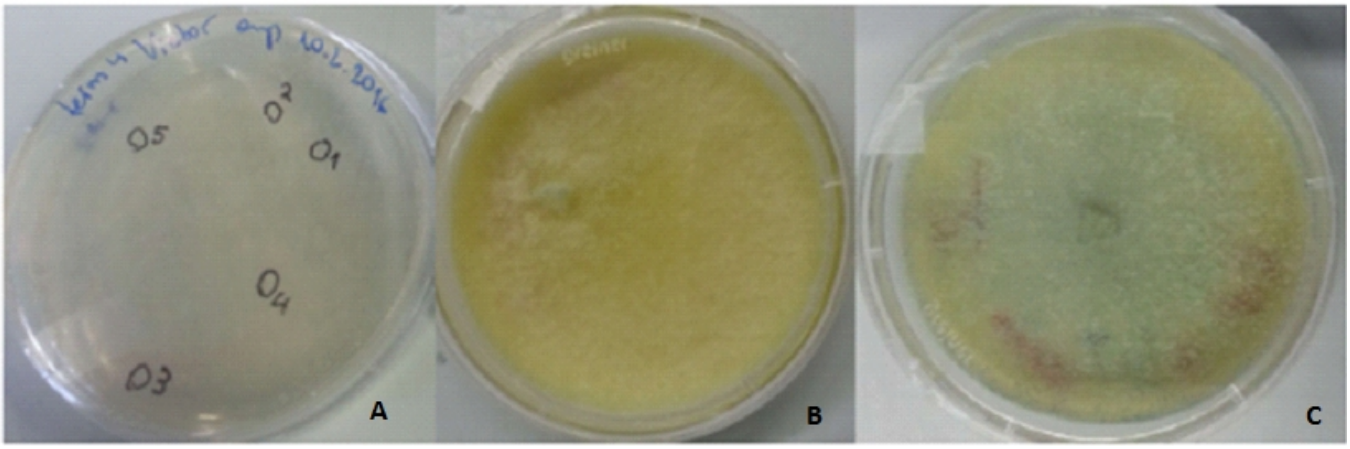


Figure 8. Of the several transformed E. coli colonies, only a few were selected for screening, i.e. growth, plasmid purification, and sequencing confirmation of the amplified cassette (left). A similar process of circling and labelling the colonies was used when choosing individual monospores during the T. reesei transformation. A juvenile hfb4OE mutant (centre). A mature, e.g. sporulated, hfb4OE mutant (right).

2.6.5. Extracting DNA from Fungal Samples

Before continuing with further characterisation of the mutant strains, it was necessary to confirm the success of the transformation. Thus, DNA needed to be extracted from the monospore cultures to be sent for sequencing (Microsynth). Preparation of the samples for sequencing occurred in two steps: preparing the monospore samples for DNA extraction and extracting the DNA from fungal mycelia.

Prior to inoculation, PDA plates were covered with a layer of cellophane in sterile conditions. Cellophane enables the fungi to access the nutrients within the PDA while allowing the mycelium to be easily removed. Spores were washed off of monospore plates using sterile water and stored in 2 mL Eppendorf tubes. Spore samples were ca. 10^7 spores per mL. Samples containing 20 µL of suspended monospores were placed centrally onto a medium sized petri dish containing the cellophane/PDA. After a couple of days of growth, the juvenile mutants completely covered the cellophane, indicating they were ready for DNA extraction.

DNA extraction from the mycelia was conducted using the DNeasy Plant Mini Kit (Qiagen). Mycelial samples were collected off the cellophane layer with a microspatula in sterile conditions and transferred into a 2 mL Eppendorf tube containing glass beads (0.75 mm, 1.5 mm, 3 mm). 400 µL of buffer APA1 containing 4 µL RNase A were added. The mixture was vortexed and incubated for 10 min at 65°C. 130 µL buffer P3 was added to the Eppendorf, which was subsequently incubated for 5 min on ice. The mixture was centrifuged for 5 min at 14,000 RPM. The lysate was transferred to a spin column and centrifuged for 2 min at 14,000 RPM. The flow through was transferred to a new Eppendorf, whereupon 1.5x volume of buffer AW1 was added. After mixing, the sample was transferred to a new spin column and centrifuged for 1 min at 8000 RPM. After passing through the entire

sample, the column was transferred to a 2 mL Eppendorf, and washed twice with 500 µL buffer AW2. It was then centrifuged for 1 min at 8000 RPM. After the collection tube was replaced with a fresh Eppendorf, 100 µL ddH_2O was added, and the sample was incubated for 5 min at room temperature. The extracted DNA was finally eluted by centrifuging for 1 min at 8000 RPM.

The concentration purified DNA was measured using a Nanodrop (Peqlab) and regularised to 10 ng μL^{-1} before further use. DNA extraction from mycelium was done following the Rapid Miniprep of Fungal DNA procedure (192). Using the aforementioned PCR specifications, the transformed hydrophobin genes were amplified via PCR. Special attention was given to the primers used. For *hfb*4OE mutants, it sufficed to use primers corresponding to the *hfb*4 gene. The hydrophobin genes were sequenced to confirm that an identical sequence and orientation was preserved from the original pre-transformation plasmid.

The post-PCR product was purified using the mi-PCR Purification Kit (Metabion). To prepare each mutant sampled for sequencing (Microsynth), 12 µL of the purified PCR product, i.e. the amplified gene, was placed in a 1.5 mL Eppendorf. Additionally, forward and reverse primers for the *hfb4* and/or the *hfb7* gene amplification were included, measuring 5 µL per singular directional sample, and a minimum of 20 µL per order.

2.7.Shake Flask Fermentation

To characterise the growth patterns and hydrophobin secretion rates of the mutant strains, a 96 h shake flask fermentation was designed. In the first fermentation, six *hfb4*OE mutants as well as the QM 6a wild type as a control were selected. The second fermentation had eight *hfb*7OE mutants. In both sets, duplicates of each mutant and the wild type was used.

Incubation was maintained at 28°C with flasks shaken at 200 RPM. A preculture consisting of 100 mL shake flask fermentation medium and 50 µL spore suspension in a 500 mL shake flask was initially prepared. After 24 hours, 10 mL of the preculture was transferred into a 1 L shake flask along with 180 mL dH_2O and 20 mL of a 10x shake flask fermentation medium (Table 2).

A sample of ca. 15 mL was removed from each replicate daily. Samples were centrifuged at 8000 RPM and saved as solid biomass as well as supernatant samples in falcon tubes. Biomass samples were dried out using a vacuum pump. Pre-weighed filter paper was placed on top of a funnel, and the vacuum was turned on. As the biomass was added, the liquid supernatant was pulled through. The biomass covered filter papers were wrapped in aluminium foil and dried out at 80°C over the course of a week. Supernatant samples were stored at -20°C for further use in protein quantification, SDS-PAGE, and western blot assays.

2.8. Bioreactor Fermentation

A bioreactor fermentation was necessary to test the mutants in an industrially relevant environment. Candidate mutants for the fermentation were chosen based on their performance during the shake flask fermentation and resulting quantification analyses. Of the four bioreactors available, one was reserved for the wild type as a control and the three mutants.

The fermentation was set up as per the following procedure. Spore solutions corresponding to each of the fungus were adjusted to contain 10^7 spores per mL in 10 mL solution. 6.25 mL of this stock was added into 250 mL of bioreactor fermentation broth. This preculture was then left to incubate for 48 h at 28°C and 250 RPM in 500 mL shake flasks.

Before the incubation period was over, the bioreactors had to be prepared for use. This consisted of an extensive washing of all equipment, as well as equipment calibration. Fermentation broth medium was prepared into five parts to maximise homogeneity across the mutants, as certain components of the medium are poorly soluble and would precipitate everything was mixed together. These five parts consisted of the carbon source (4% glycerol), 80 mM $MgSO_{4(aq)}$, 100 mM $CaCl_{2(aq)}$, the remaining buffer salts and trace elements, as well as dH_2O to dilute the components to the correct concentration. After dividing the segments equally across the four bioreactors, the pH and pO_2 sensors were calibrated and the entire apparatus was autoclaved. Once complete, the apparatus was left to run for 7 h without preculture inside to provide an initial test for faulty machinery. The fully assembled Bioreactor is shown in Figure 9.

After the 48 h preculture incubation was completed, the contents of the preculture flasks were transferred directly to the bioreactors. Acid (15 M ortho-phosphate) and base (1 M NH_3OH) were added to each bioreactor to regulate pH to 5.0 during the fermentation, an acidity deemed ideal for *Trichoderma* growth. Temperature was kept constant at 28°C due to the action of a heating pad and a cooling finger. Aeration was maintained at 3 L min^{-1} to keep the dissolved O_2 > 30 %. Agitation was provided by a large stirring mechanism and maintained at 500 RPM throughout the fermentation. An antifoaming agent Struktol J647 was added at the amount 1 mL per 10 L, and kept at hand in case the mutants were to begin rapidly foaming. Sterility was maintained by the use of metal clamps on all outlet and inlet tubing, as well as a backpressure in the chamber itself which automatically expelled medium through an outlet to facilitate sampling.

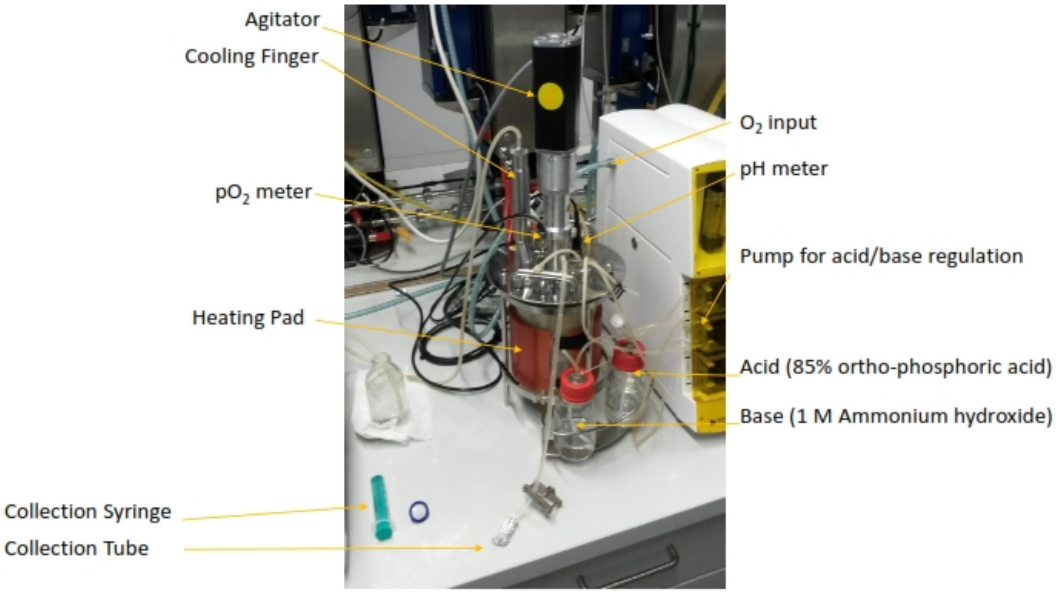


Figure 9. The fully assembled Bioreactor used during the fermentation. pO2, pH, and agitation were automatically regulated by a computer program. The reactors were samples regularly using the collection tube and syringe.

Sampling was carried out periodically over the 60 h of the fermentation run. Before each sample was taken, the area was sprayed with 85% EtOH. 10 mL of supernatant was allowed to pass through the collection tube before the sample was taken to prevent contamination of the samples. After initial incubation and sampling at 0 h, a 9 h growth period was allowed, as no significant changes were expected in this time. From the 9 h to 27 h time point, sampling was conducted every 3 h. For practical scheduling reasons, a 6 h gap was included at this point, and then the 3 h sampling was resumed from 33 h to 51 h. As the fermentation was reaching an advanced stage of maturity, a 9 h gap was made, whereupon a final 60 h time point was made. The end point was logical, as the medium was becoming incredibly dense due to the amount of biomass, eventually preventing sampling. Samples, as well as the remaining ca. 3 L of the final biomass at 60 h, were stored at - 20°C until use.

2.9.Strain Characterisation

The following analysis techniques were employed during the quantification and characterisation of the mutant strains and produced protein.

2.9.1. Biomass Assessment

During the shake flask fermentation, the biomass of the wild type replicates and mutants were characterised by their growth characteristics. Macromorphology of the mycelium, the colour of the cultivation broth and the foam formation were recorded.

Biomass collection during the shake flask fermentation was carried out by collecting 10 mL of sample from each replicate. 20 mm Ø Whatman filters were weighed prior to use. The supernatant was pulled through the filter via vacuum filtration and discarded. Biomass samples were labelled and wrapped in aluminum foil before being stored at 80°C for 1 week to dry out. Biomass collection during the bioreactor fermentation took place directly in the Eppendorf tubes. Samples were initially centrifuged at 8000 RPM for 10 min. The supernatant was collected separately for further experiments. The remaining biomass was stored at 80°C for 1 week to dry out.

2.9.2. Quantifying Supernatant Protein Concentrations

When applicable, the following procedure was used to concentrate supernatant samples from the fermentations (193). A 100% w/v trichloroacetic acid (TCA) solution stored at 4°C and a 100% acetone solution stored at -20°C were prepared in advance. The TCA solution was added to the samples in a 1/10 ratio to sample volume in 15 mL falcon tubes, and mixed. Samples were then incubated overnight at -20°C. Upon thawing, samples were centrifuged at 8000 xg, 4°C, for 10 min. The samples were decanted, centrifuged in the same manner again, and left to air dry. The pellet was resuspended in 1 mL HPLC grade H_2O, and mixed with 10 mL 100% acetone solution. The tubes were vortexed in total four times over one hour, with 15 min incubation periods at – 20°C in between, before being incubated at - 20°C overnight. The centrifugation and air-drying steps were repeated to create a dry pellet. This pellet was suspended in 5x β-mercaptoethanol sample buffer as described in the section above before use.

Protein Quantification was conducted using the bicinchoninic acid (BCA) assay technique (Pierce™ BCA Protein Assay Kit, Thermofisher Scientific). A supplied bovine serum albumin (BSA) stock was used to make a standard protein dilution of 2 gL^{-1}, 1.5 gL^{-1},1.0 gL^{-1}, 0.75 gL^{-1}, 0.5 gL^{-1}, 0.25 gL^{-1}, 0.125 gL^{-1}, and 0.025 gL^{-1}. This formed the standard curve which allows conversion between the measured absorbance of the sample and their actual protein concentration. 25 µL of standard and sample solutions were added to each well of the microplate.

Secondly, two supplied reagent stocks were mixed in a 1:50 ratio, as per the provided protocol. Once 200 µL of the reagent mix was distributed to all samples laid out on a 96 well plate, an incubation period of 30 minutes at 37°C is required to achieve a detectable, but not oversaturated level of activity. Absorbance was measured after the incubation period using a spectrophotometer (λ = 510 nm) (ThermoFisher Scientific). Finally, absorbance results were plotted in excel for visual interpretation. It was necessary to use BCA as opposed to other protein concentration assays (e.g. Bradford assay) due to the intrinsic nature of hydrophobins, which otherwise are poorly detectable by the assay.

2.9.3. SDS-PAGE Analysis

Samples for the Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot originated from two sources: fermentation supernatant and cell wash. Supernatant samples were combined with a 5x β-mercaptoethanol sample buffer in a 5:1 ratio. Composition of the sample buffer is provided in the supplementary materials section 8.4. Protein denaturation was carried out in an incubator at 95°C for 5 min immediately prior to use. Cell wash was done by mixing 500 mg biomass with 500 µL 1x SDS solution. The mix was then shaken for 10 min, 200 RPM, at room temperature. Afterwards, the samples were centrifuged and the resulting supernatant treated with β-mercaptoethanol in the manner described above.

SDS-PAGE is useful for comparing the quantity of hydrophobins produced across the mutants and to the wild type strain. The protein of interest was easily identifiable as the 10 kDa band. Of course, given the nature of filamentous fungi to produce a spectrum of hydrophobins all around the same size, the SDS-PAGE assay cannot however definitely prove that the specific OE HFB is being expressed.

The PageRuler™ Unstained Protein Ladder (ThermoFisher Scientific) was used to create a standard scale for size comparison across samples. SDS-PAGE was run parallel to the western blots. This was done to keep running conditions similar, and decrease variation arising from the sample itself.

Acrylic gels for SDS-PAGE was cast shortly before use, using vertical glass plates for the mould. Ingredients to make a single gel are included in the supplementary materials section SDS-PAGE Composition and Buffer Solutions. Casting occurs in two steps, first for the separating gel, and secondly for the stacking gel. All steps in the following procedure occur at room temperature.

The separating gel was cast and overlaid with isopropanol for half an hour. Isopropanol is required to allow the separating gel to polymerise without irregularities and the introduction of bubbles in the contact point between the two gels. After the incubation, the isopropanol was removed and the stacking gel was overlaid and left to incubate for a 30 min once again. A comb provided the proper spaced wells necessary for sample introduction, as well as the air-free environment required as before. Additionally, a running buffer is required to facilitate the electric current and maintain the required pH (8.3). All gel and buffer components are provided in the supplementary materials. The power supply (Biorad) was set to deliver 180 V and 50 mA per gel inside. The electrophoresis run is completed when the dye has passed through the gel, i.e. is at the bottom of the glass plate. Thus, a run time of 1 h was pre-set.

Post electrophoresis, the gels were developed using the SilverQuest™ Silver Staining Kit (ThermoFisher Scientific). The procedure was followed as per the proportions suggested, but with a total volume of 30 mL per gel instead of 100 mL. First, the gel is transferred to a container, and incubated for 20 min in a fixative solution (methanol, acetic acid, deionised water). The fixative solution removes any excess running buffer or interfering ions present, as well as helps restrict the movement of proteins out of the gel. After a 10 min. incubation of the gel in a washing solution (30% ethanol), a sensitising solution (30% ethanol, 10% sensitiser, 60% deionised water) is applied, to improve the contrast of the silver stain. The gel is incubated for 10 min in the sensitising solution. Once removed by two successive washing steps (10 mL each of 30% ethanol, then deionised water), incubated for 10 min each, a silver ion solution is added to bind the proteins in the gel. The ion solution is left to incubate for 15 min. After a 30 s rinse with deionised water a developer solution (10 mL developer and 1 drop of a developer enhancer, diluted to 100 mL in deionised water) is applied to view the results. The developer reduces the silver ions to metallic silver, which become visible at the location of their binding (i.e. the proteins). It was left to react until bands became sufficiently visible. Finally, a stopping solution is added, complexing any free silver ions to prevent them from further staining the gel. Silver staining, as opposed to Coomassie blue, was necessary once again due to the nature of the hydrophobins being studied. Using silver staining and a denaturation step before loading the gels, the proteins could remain denatured long enough to migrate down the gel as monomers.

2.9.4. Protein Immunoblot Assay

A HIS6 tag attached to the protein of interest allowed use of a protein immunoblot (a.k.a. western blot) assay to allow more specific protein identification. The procedure initially is identical as to the SDS-PAGE, differing only in the choice of standard ladder used. However, after the gel run, a second electrophoresis in conducted instead of following the silver staining procedure.

The electrophoresis unique to the western blot uses a different buffer (see supplementary materials: Western Blot Buffer Solutions), and a blotting sandwich (Figure 10). Furthermore, the standard used for each gel was a PageRuler™ Prestained Protein Ladder (ThermoFisher Scientific). After the electrophoresis the membrane is blocked with a 4% bovine serum albumin (BSA) diluted to 20 mL in dH_2O for 1 h. A washing step is required afterward to remove the excess BSA from the membrane. This is done via three washes of 15 min with Tris-buffered saline (TBS) buffer (pH 7.5). The blot sheets were then incubated for 1 h in 15 mL with anti-his C-term AP antibodies (ThermoScientific) in a 1:2000 dilution in water. The antibodies bind to the his-tag, but not to the BSA. After a second TBS washing phase, the addition of a substrate solution of nitro-blue tetrazolium chloride

(NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) reacts with the enzyme conjugate alkaline phosphatase (ThermoScientific), on the antibodies. This reaction creates a visible blue-purple band, whose location and intensity reveals the size and relative production of the transformed protein. All gel and buffer components are provided in the supplementary materials.

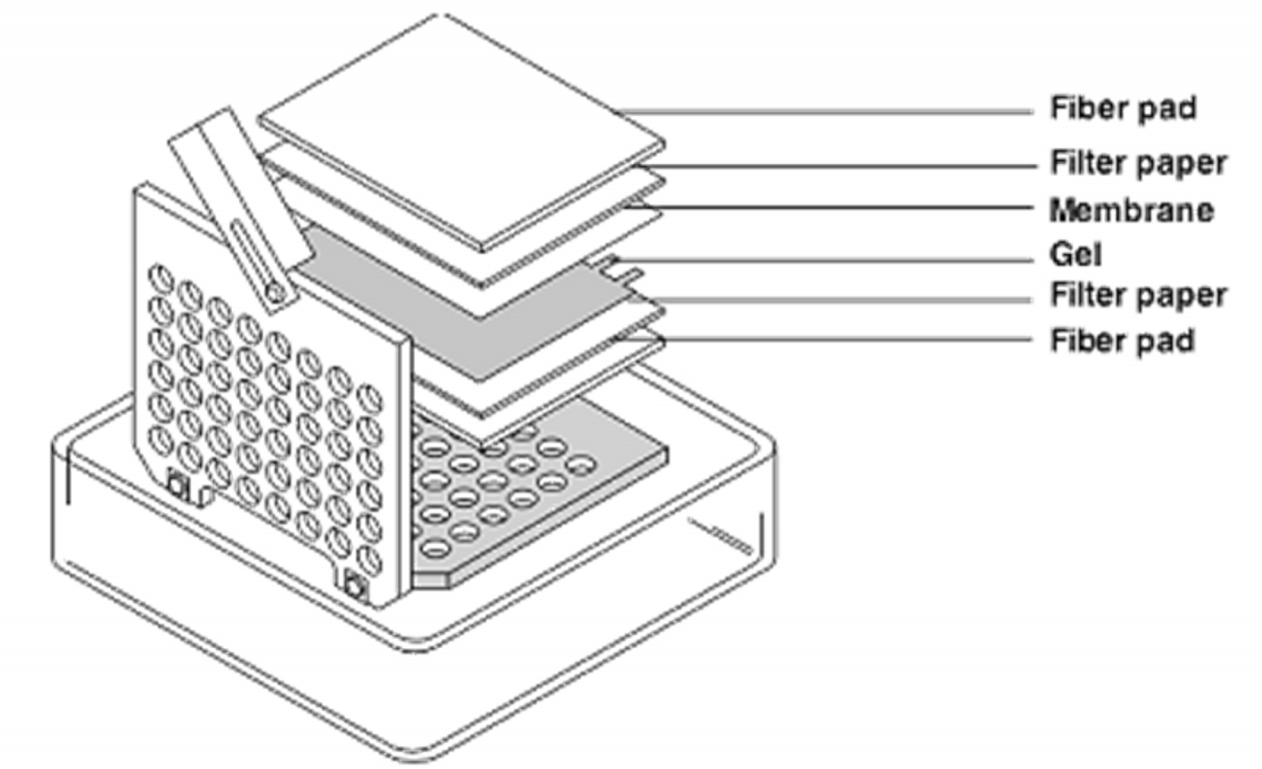


Figure 10. Western Blot sandwich. The current passes horizontally through acrylic gel onto the blotting membrane, leaving a coloured ladder and the proteins embedded on the surface of the material. Instead of a fiber pad, a plastic mesh pad was used. (http://www.radio.cuci.udg.mx/bch/EN/Western.html).

2.9.5. Static 24-well Cultivation

Two media were selected to assess the growing patterns of the mutants against the wild type in static liquid media. The first is a Glucose Salt Medium (GSM), the second potato dextrose (PD) medium. GSM is stored in four stock solutions, which are combined upon use. The first component is the salt medium (KNO_3, KH_2PO_4, KCL, $MgSO_4 \cdot 7H_2O$). 44 mL of GSM was combined with 5 mL of a 10x glucose solution, 0.5 mL of a 100x $CaCl_2$ solution, and 0.5 mL of a 100x trace element solution, to create a total volume of 50 mL. This was divided into 500 µL per well. Two plates were prepared and incubated with QM 6a and five mutants, with four replicates per sample. Regularised spore solutions were used for the inoculation of the wells (5 µL spores at 10^7 spores per mL). PD 24-well plates were prepared in the same manner.

Plates were left to incubate at 28°C in an incubator hosting a 12 h day-night cycle. The plates were observed daily, and pictures were taken once the samples had grown out of the liquid and began to sporulate.

2.9.6. Cell-bound Hydrophobin Removal

The following techniques were implemented to extract cell-bound hydrophobins. A 2% hot (95° C) SDS wash was used. In a second, 8 M urea, and in a third, a combination of 70% EtOH, 1% Triton X, and 29% 9 M urea. Following each procedure, the supernatant and extraction solution was precipitated and resuspended in sample buffer using the procedure outlined in 2.9.2.

3. Results

The purpose of the current study was to investigate the applicability of *T. reesei* as a hydrophobin cell factory. This was done by expressing two hydrophobins from the original *T. virens* host in *T. reesei*. The transformed *hfb4* gene originates in *T. virens*, although *T. reesei* produces a homologous HFB4. The latter HFB7 was a product of heterologous expression from the same *T. virens* source and does not have a homolog in *T. reesei*. Both transformations were completed successfully.

3.1. *E. coli* Transformation

Prior to the current study, a pUC19 plasmid was modified to include a hygromycin-B resistance cassette, a PCDNA1 promoter, and each one of the two *hfb* genes. Thus, an initial step in this study was to complete the cassette by adding additionally a *Tcbh* terminator. The native pUC19 plasmid contains 2,686 base pairs (bp) (188), (185). The *hfb*4 gene cassette and the analogous *hfb*7 cassette were 1,872 bp and 1,879 bp, respectively. Thus, the inserted modified pUC19 in both transformations sized at ca. 4.5 kbp.

The resulting transformation was first confirmed by a gel electrophoresis of the PCR amplified cassette (figure 11). Positive results are larger, i.e. have a greater number of base pairs. The post-PCR product was purified and sent for sequencing. From the confirmed mutants, *hfb*4OE-2, *hfb*4OE-5, *hfb*7OE-3, and *hfb*7OE-5 were chosen as the plasmid propagators for the *T. reesei* transformation.

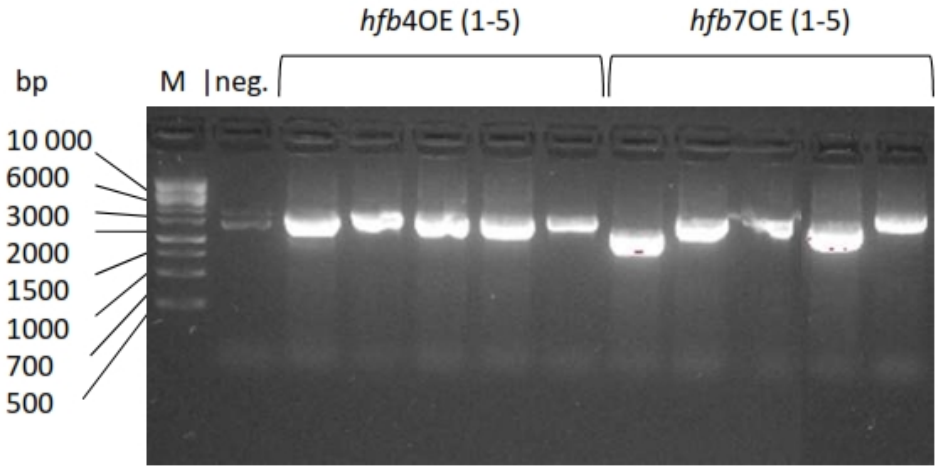


Figure 11. PCR results showing an amplification of the plasmid vector. Size differences between the bands indicate the presence of the terminator sequence (ca. 500 bp), or lack thereof. The terminator is present in samples hfb4*OE-2,* hfb4*OE-5,* hfb7*OE-3,* hfb7*OE-5, as visually indicated by being closer to the top of the gel image (4.5 kb band). Results were later confirmed through sequencing of the purified PCR samples. It is possible that other positives exist, however only two from each group were needed to create a sufficient stock of the modified plasmid cassette. M represents the GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific), neg. is the negative control (dH_2O).*

3.2. *T. reesei* QM 6a Protoplasting

Creating protoplasts from the QM 6a wild type was carried out in the minimum possible time to ensure the greatest survival rate of the cells. Despite being suspended in solutions to maintain osmotic and electrolytic balance in completely sterile conditions, the fragility of the cells necessitates steady and timely handling. The success of the protoplasting technique would only become apparent after the purified DNA from the isolated monospore mutants was sequenced. For this reason, the transformation process ended up being repeated in total three times for the *hfb4OE* group, and five times for the *hfb7OE* group, until an adequate collection of OE mutants could be amassed.

The modified pUC19 cassette (hygromycin-B resistance gene, PCDNA1 promoter, relevant *hfb* gene, *tcbh* terminator) was linearized prior to incubation with the protoplast cells (figure 12). This allows the cells to more easily integrate the plasmid into their genome.

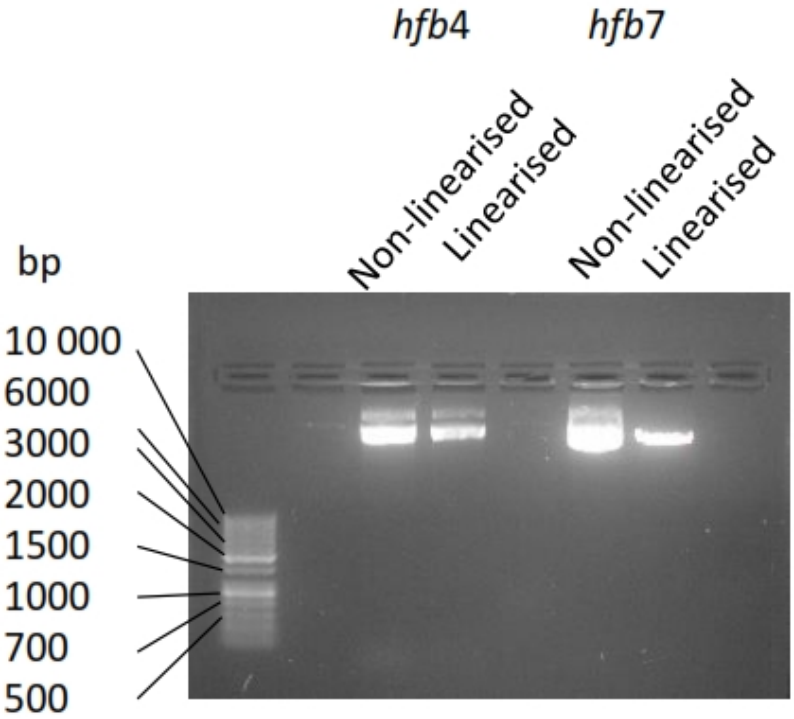


Figure 12. Gel electrophoresis displaying non-linearised plasmids from the pUC19 hfb4OE and the pUC19 hfb7OE vectors next to their linearised counterparts. Linear plasmids were cut at the SAL1 restriction site.

3.3. *T. reesei* Homologous Transformation

After the transformation, positive mutants were isolated as from single spores. DNA was collected from the mycelia of these monospores. A PCR was run to amplify, the cassette originating from the modified pUC19 plasmid. This was followed by a gel electrophoresis of the PCR product to tentatively confirm the insertion based

on the change in construct size (expected size: 1283 bp). The plasmid at this point is integrated into the *T. reesei* genome, although the position within, as well as the copy number, could not be predicted. Table 7summarises growth patterns exhibited by the OE monospores.

The homologous transformation in this study describes the insertion of a *hfb*4 gene from *T. virens* into *T. reesei*, which is similar, but not identical to the native *hfb*4 gene already present in *T. reesei*. To confirm the presence of the non-native *hfb*4 gene, a PCR was used to amplify the predicted sequence (figure 13). The PCR product of the positive samples were subsequently sent to be sequenced.

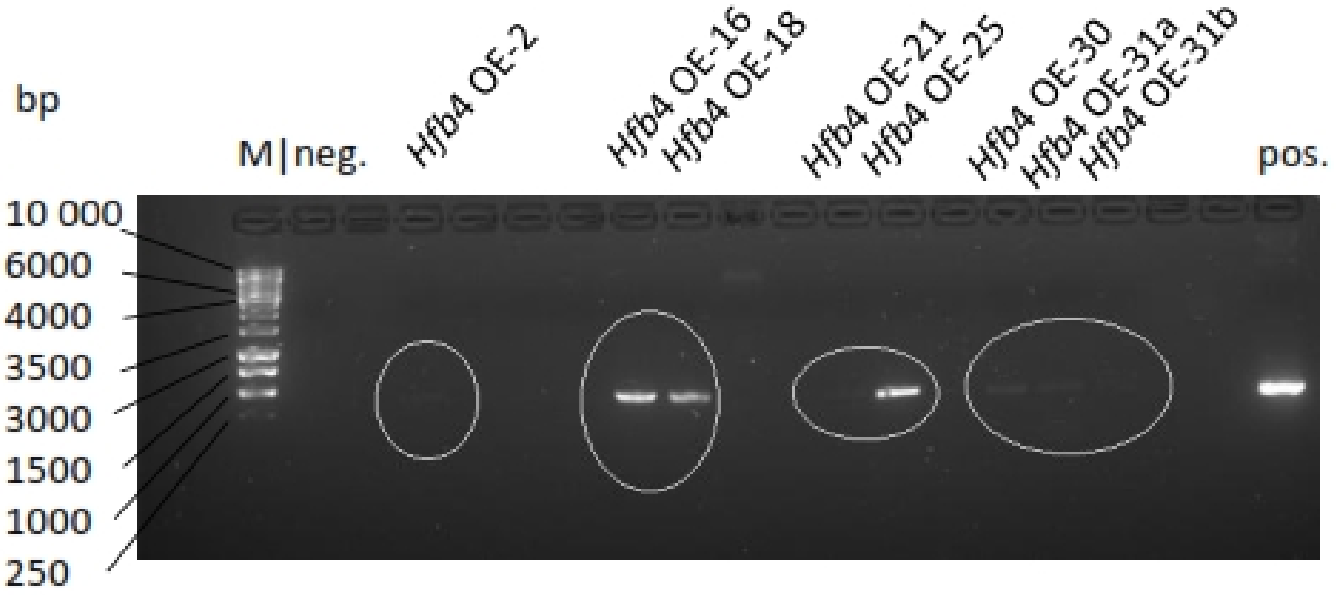


Figure 13. Gel electrophoresis indicating successful hfb4OE *mutants (circled). The transformed* hfb4 *gene was amplified by the PCR. Although some bands appeared fainter than others, the size corresponded to the prediction, hinting that the mutants may be true positives. Sequencing of the amplified hfb4 gene later confirmed the results. M represents the GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific), neg. is the negative control (dH2O), and pos. is the positive control (the pUC19* hfb4OE *plasmid). Transformants* hfb4OE-31a *and b represent two monospores isolated from the same strain. Ultimately only 31a was chosen for further investigation.*

3.4.*T. reesei* Heterologous Transformation

The heterologous transformation in this study describes the insertion of a *hfb*7 gene from *T. virens* into *T. reesei*, which is not native to *T. reesei*. To confirm the presence of the *hfb*7 gene, a PCR was used to amplify the predicted sequence (figure 14) using the forward primer from the cDNA1 promoter and the reverse primer from the *hfb*7 gene. A nested PCR, i.e. a PCR amplification of the previous PCR product, clearly displayed the predicted 1.3 kb band corresponding to the *hfb7* gene. As before, the post-PCR product of the positive samples was sequenced to confirm proper insertion.

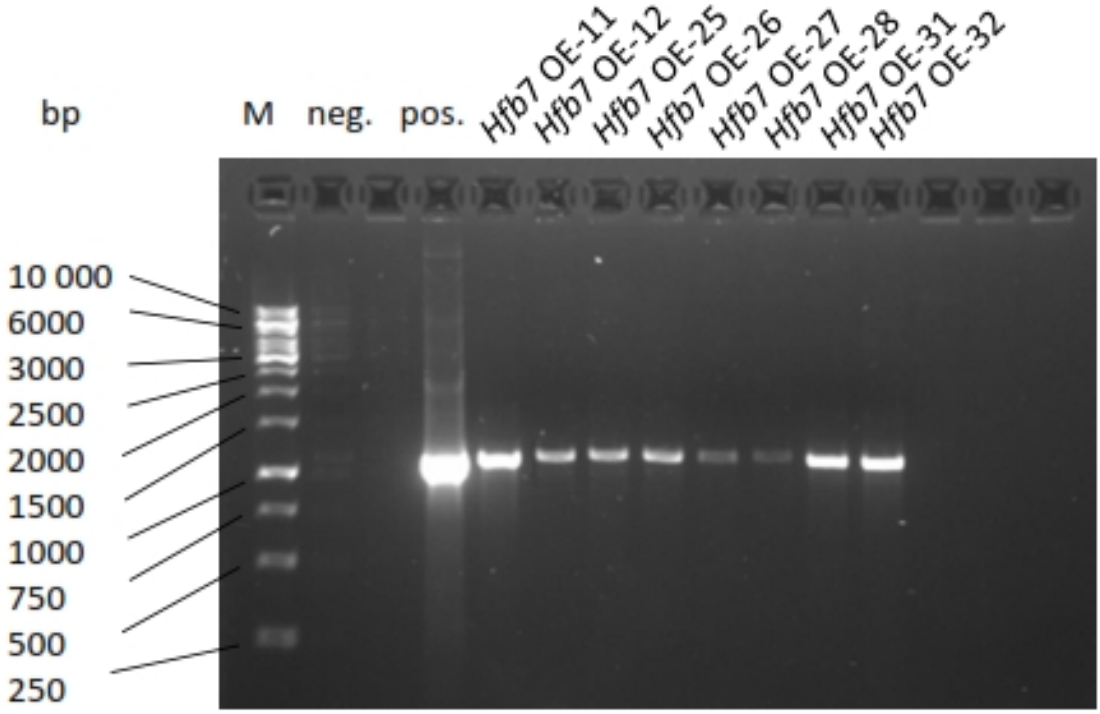


Figure 14. Gel electrophoresis indicating successful hfb7OE *mutants (expected band at 1283 bp). The transformed hfb7 gene was amplified by the PCR. Sequencing of the amplified hfb7 gene later confirmed the results. M represents the GeneRuler 1 kb DNA L*

3.5.Shake Flask Fermentation

Given the quantity of positive mutants, it was important to be able to distinguish between the OE mutants in a more profound way. Thus, a shake flask fermentation was planned, so as to compare the growing habits and production levels of hydrophobins of the different mutants against themselves and the wild type in liquid media. The shake flask fermentation medium was prepared and divided between all the flasks equally, before being combined with the individual precultures from each replicate. It was of note to see that the mutants always produced more foam that the wild type, in the *hfb4OE* and *hfb7OE* fermentations alike.

After the termination of the fermentation, a BCA assay, as well as SDS-PAGE and western blot assays were made. The goal was to determine which of the OE mutants have the potential to produce the most amount of HFB4 or HFB7. The selected *best* mutants would then be fermented in a bioreactor, which allows a more direct conjecture to be made as to how the mutant profiles will appear in industrial applications.

3.5.1. Analysis of the *hfb4OE* mutants

During the fermentation, a characteristic profile of each mutant was observed. Mutants *hfb4OE*-28, *hfb4OE*-25, and *hfb4OE*-16 had above average foaming. The wild type has particularly low foaming, strengthening the

observation (figure 15). Clumping to various degrees was observed across all mutants but less so in the wild type. Greater clumping activity was observed in *hfb4OE*-25, *hfb4OE*-16, and *hfb4OE*-18.

Table 7. Positive hfb4OE mutants and their respective growth types. S = slow; sporulation at one week with assistance from 12 h alternating day-night cycle stimulation, M1 = medium 1; sporulation within 1 week, M2 = medium 2; sporulation within 96 h, F = fast; sporulation within 48 h.

Mutant	*hfb*4OE-2	*hfb*4OE -16	*hfb*4OE -18	*hfb*4OE -21	*hfb*4OE -25	*hfb*4OE -30	*hfb*4OE -31
Growth Type	M1	F	F	S	S	M1	M1

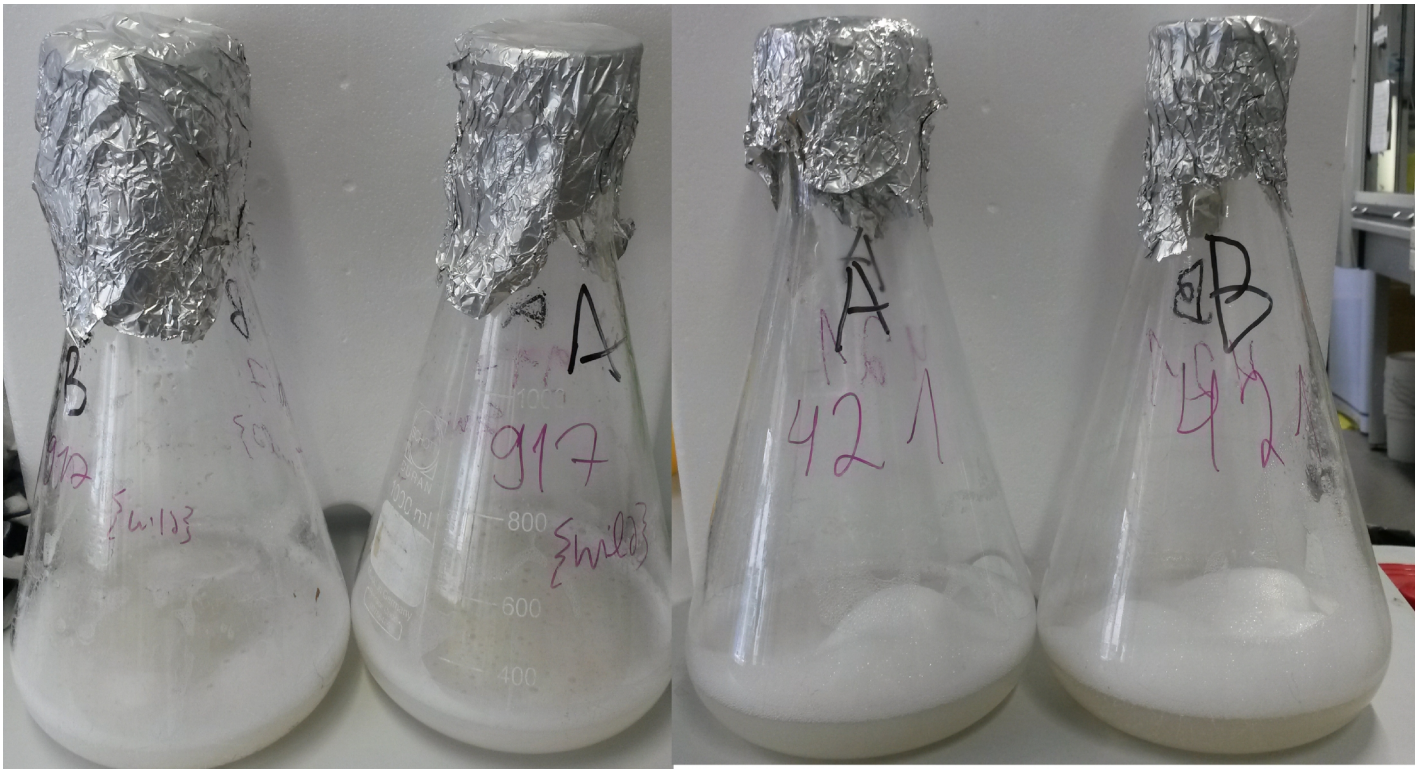
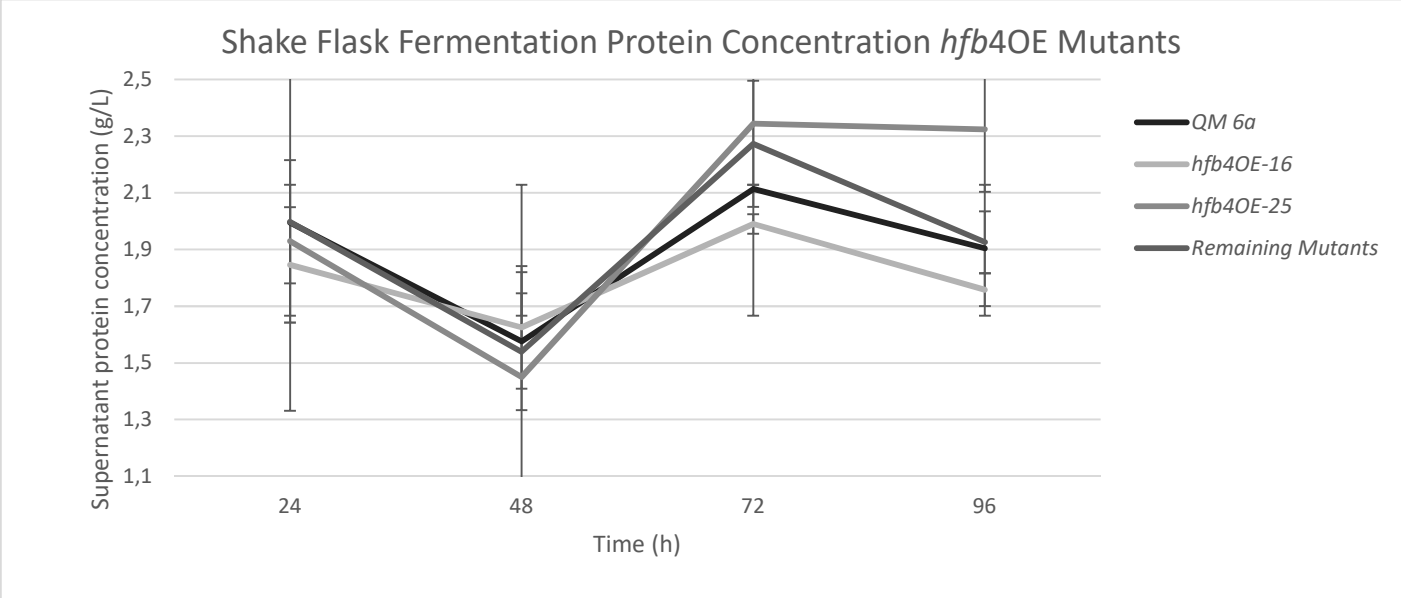


Figure 15. Comparison of hfb4OE-*2 with the wild type QM 6a. The wild type (first and second flasks from the left) exhibits visibly less foam – a trend seen consistently throughout the fermentation.*

3.5.1.1. Protein Quantification

The BCA assay indicates which mutants have secreted the most of proteins into the medium. Thus, it can be determined whether a strong OE mutant produces a large or small amount of background proteins in relation to the desired HFB, by comparing the BCA results to the SDS-PAGE and western blot assays. The highest two producing strains were *hfb*4OE-25 and *hfb*4OE-2, a trend visible in both replicates (figure 16). Mutant *hfb*4OE-16 in contrast, performed so poorly that it also became a sample of interest. Its protein production resembled the wild type, however sequencing of the PCR product confirmed that it contains the gene of interest. It was

interesting to further investigate the mutant, to determine if it produces naturally a *cleaner* product, viz. having fewer background proteins next to the desired HFB4.



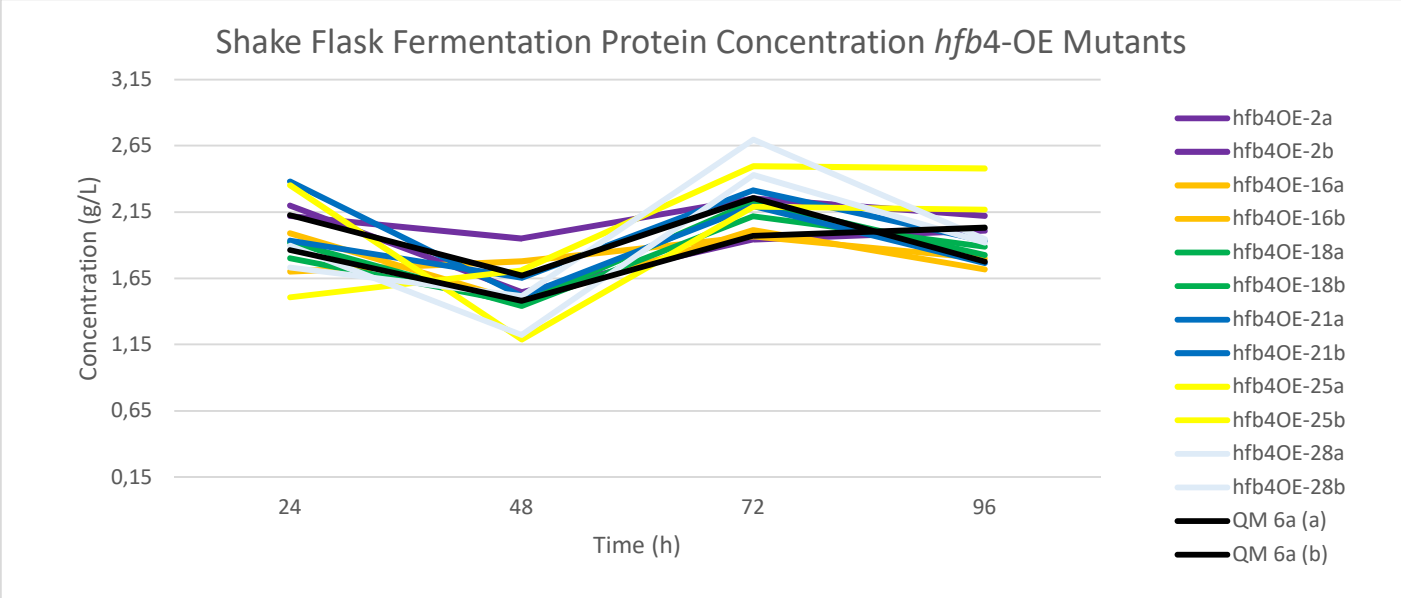


Figure 16. Comparative BCA results taken from shake flask supernatant during mutant fermentation at 24 hour intervals. Results indicate that mutant hfb4OE-*25 is a high protein producer.* hfb4OE-*16, which foamed readily during the fermentation, has a lower protein production amount than the wild type (QM 6a).*

3.5.1.2. SDS-PAGE Analysis

SDS-PAGE was run for each time point (24 h, 48h, 72h, 96h), across all the OE mutants and the wild type QM 6a (figure 17). There is a noticeable difference across the mutants, which is likely explained by the copy number of the inserted *hfb*4 gene, and the location of insertion in the *T. reesei* genome.

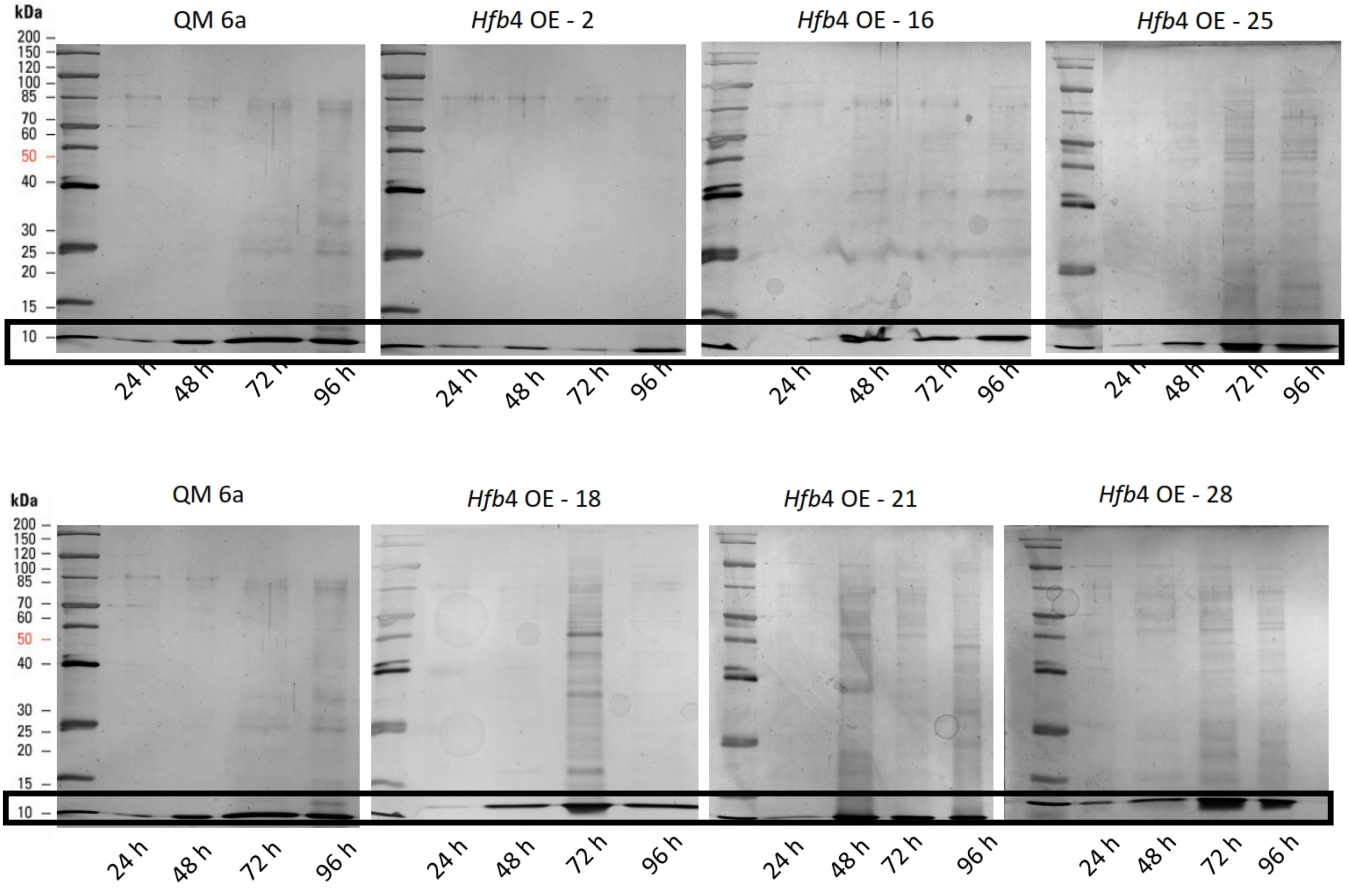


Figure 17. SDS-PAGE for the hfb4OE *mutants compared to the wild type. All mutants have noticeable activity at the 10 kDa region.*

3.5.1.3. Immunoblot Analysis

The immunoblot (western blot) assay confirms the presence of a tagged protein in the sample, by allowing the HIS6 tag to be visually detected on an immunoblot. Hydrophobin aggregation could not be avoided, thus it is unrealistic that the intensity of the expected 10 kDa band is representative of the HFB4 quantity in the supernatant. It can still be used effectively for comparative purposes, as was done in this case to discern which *hfb4OE* mutants exhibit the most ideal production characteristics. An immunoblot assay is important here, as there are many SMW proteins around 10 kDa in size, and therefore an SDS-PAGE is alone not enough to confirm the presence of the hydrophobin protein of interest. Figure 18 shows the band in the 10 kDa region which indicated the presence of

the HIS6 tagged protein. The supernatant was concentrated into a pellet before beinmg resuspended in a denaturing loading solution, as per the procedure in section 2.8.2. Therefore it was concluded that the protein of interest was located free flowing in the supernatant, however the protein concentration in the unconcentrated form was too low to be detected.

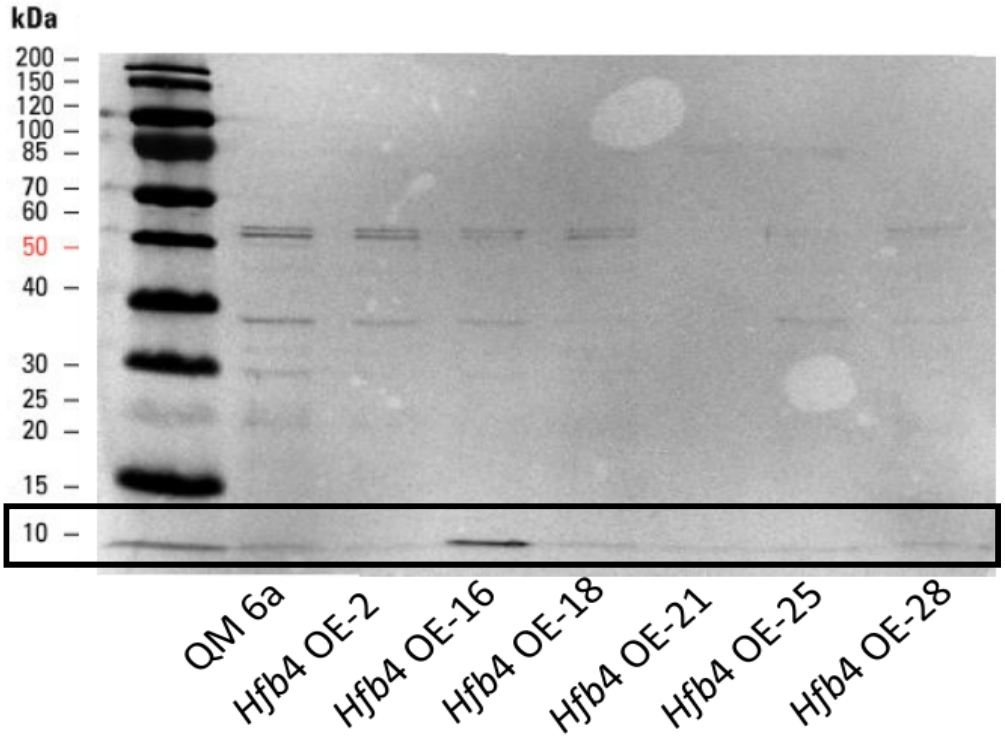


*Figure 18. Western blot of concentrated (from pellet, section: 2.8.2: **Quantifying Supernatant Protein Concentrations**) supernatant samples at 96 h from the hfb4OE shake flask fermentation. The dark bottom band appeared within 20 minutes. The lighter upper and lower bands appeared later. The last bands to appear, likely non-specific bonding, appeared after several hours. They may be attributed to HFB4 aggregation.*

3.5.1.4. Selection of *hfb4OE* Mutants for Bioreactor Fermentation

From the preceding images, it became apparent that the most interesting mutants are *hfb4OE* - 16 and *hfb4OE* - 25. The western blot assay indicated the target protein has been secreted into the medium. The SDS-PAGE strengthened this conclusion, with distinct bands visible at the 10 kDa range. The BCA results however, indicated that *hfb4OE* - 25 produces a significantly greater amount of protein sections than *hfb4OE*- 16. Finally, the growth type of *hfb4OE* – 16 was fast (sporulation at 48 h), while for *hfb4OE*- 25 is was slow (sporulation at 1 week). The two complement each other for research: the one producing a large quantity of all proteins, and the second seeming to produce the desired HFB4 in a greater proportion to other protein secretions.

3.5.2. Analysis of the *hfb7OE* mutants

Of the *hfb*7 group, no mutant had an exceptional amount of foaming. Mutants foamed more that the wild type, but not at levels seen during the *hfb*4 fermentation. Clumping was especially significant in *hfb*7 OE - 11 and *hfb*7 OE - 12. This resulted in a megastructure consisting of almost the entire biomass aggregating by 48 h. Clumping was also prevalent in *hfb*7 OE - 28, *hfb*7 OE - 31, and *hfb*7 OE - 32, while the remaining mutants developed more homogenous solutions. The mutant growth patterns are listed in table 8.

Table 8. Positive hfb7*OE mutants and their respective growth types. S = slow; sporulation at one week with assistance from 12 h alternating day-night cycle stimulation, M1 = medium 1; sporulation within 1 week, M2 = medium 2; sporulation within 96 h, F = fast; sporulation within 48 h.*

Mutant	*hfb*7O*E*- 11	*hfb*7OE– 12	*hfb*7OE– 25	*hfb*7OE– 26	*hfb*7OE– 27	*hfb*7OE– 28	*hfb*7OE– 31	*hfb*7OE– 32
Growth Type	F	M2	M1	M1	S	M1	F	M2

3.5.2.1. Protein Quantification

In terms of protein production, *hfb7OE* – 11 and *hfb7OE* – 12 lead the group, with the wild type producing comparatively little (Figure 19).

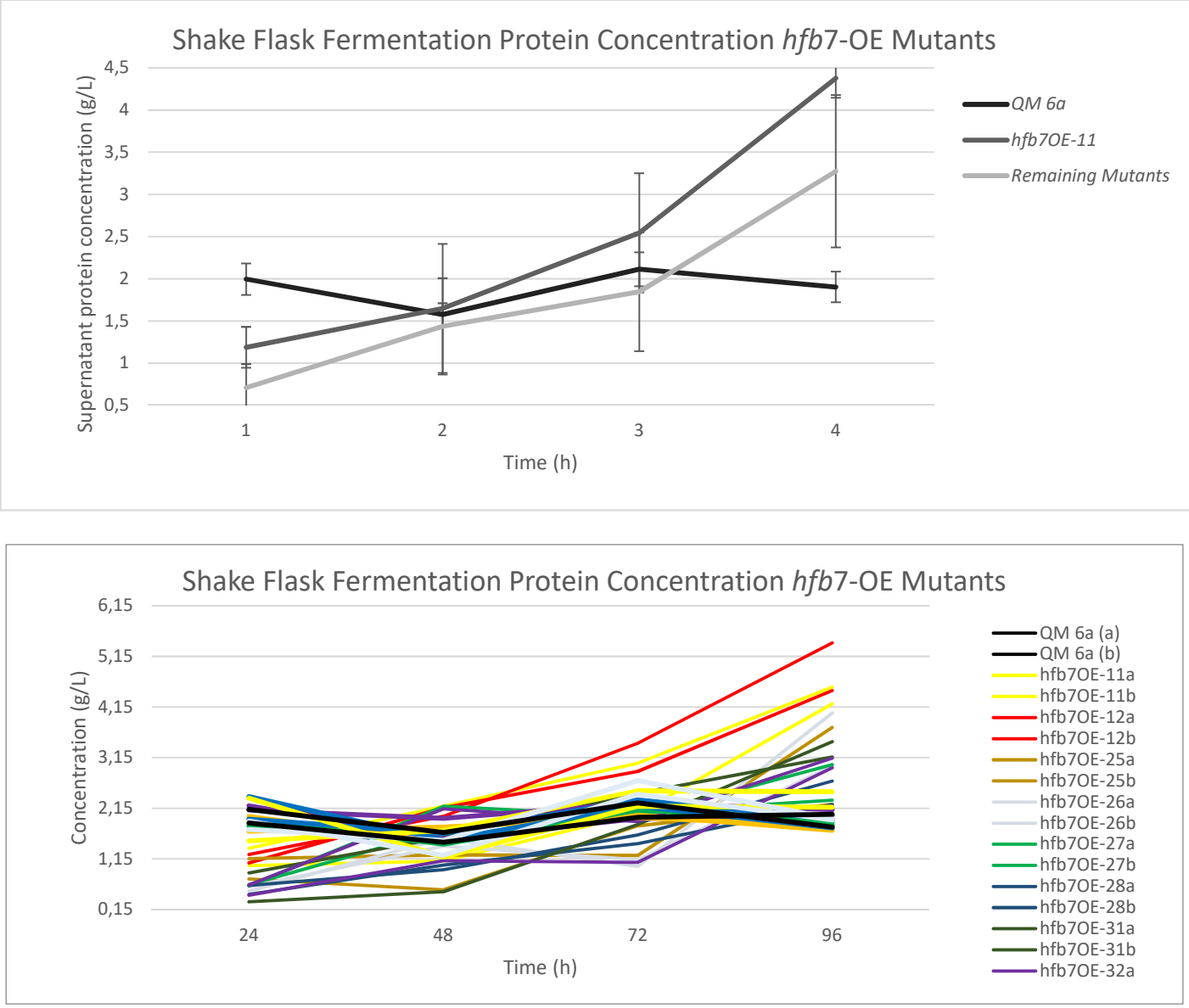


Figure 19. Comparative BCA results taken from shake flask supernatant during mutant fermentation at 24 hour intervals. Results indicate that mutants hfb7OE-*11 and* hfb7OE-*12 produce exceptionally high protein amounts, at almost all time intervals. QM 6a in black is the wild type for comparison.*

3.5.2.2. SDS-PAGE Analysis

SDS-PAGE results further supported *hfb7OE* – 11 and *hfb7OE* – 12 as being significantly better HFB7 producers compared to the other OE mutants and the wild type. More intense bands are visible in the mutant strains at the 10 kDa region, in comparison with the wild type (Figure 20). The band likely corresponds to monomeric hydrophobins in most samples, however bands associated with dimerism are also possible, as seen in the top row mutants *hfb7OE*-11, *hfb7OE*--12, and *hfb7OE*--25.

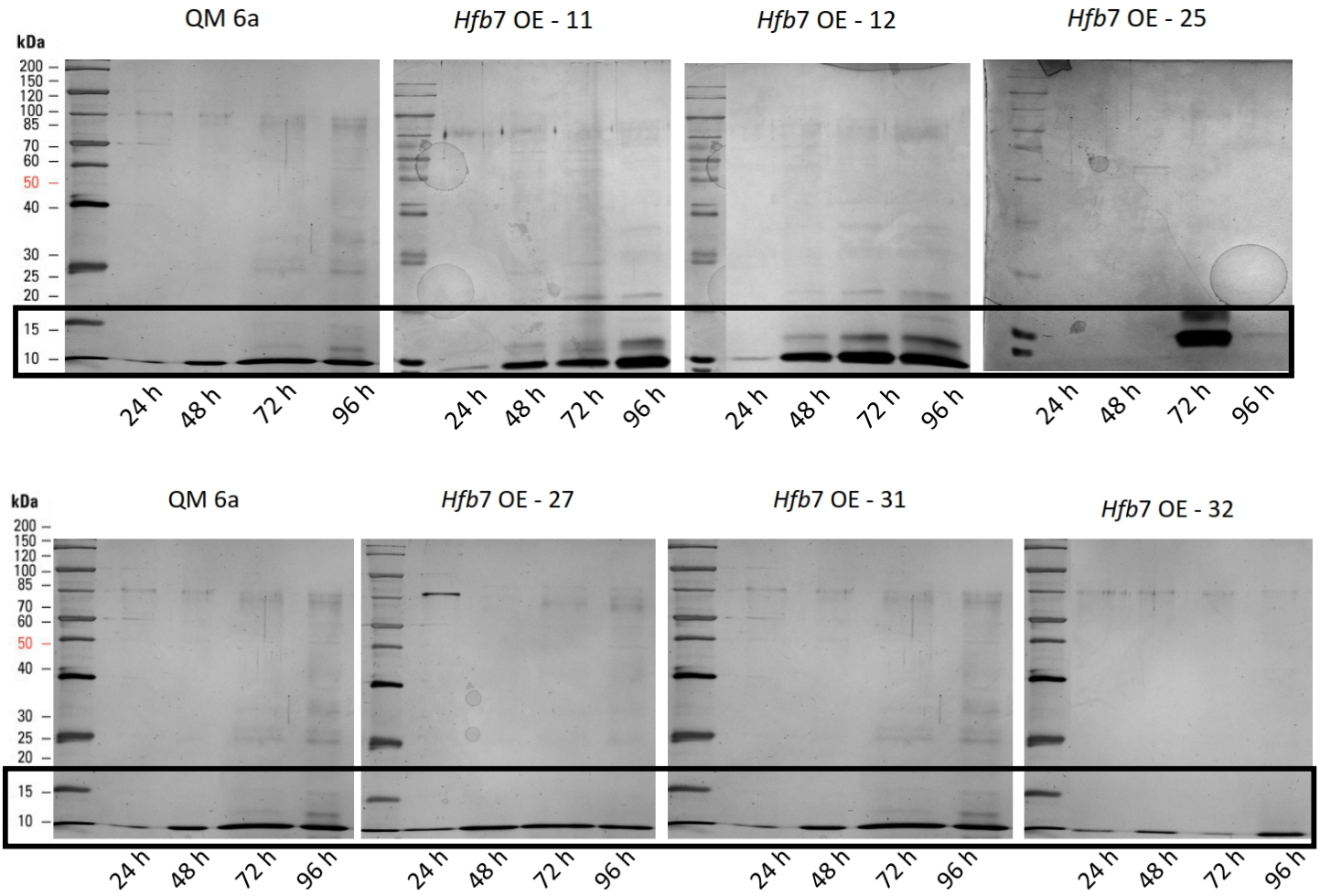


Figure 20. SDS-PAGE for the hfb7OE *mutants compared to the wild type. The activity of* hfb7OE *– 11 and* hfb7OE *– 12 stand out compared to other mutants.*

3.5.2.3. Immunoblot Analysis

The western blot assay was done in the same manner as with the *hfb4OE* strains, i.e. by concentrating the 96 h time point supernatant sample into a pellet, and resuspending the purified protein, as per the procedure outlined in 2.9.2. Bands at the expected size of 10 kDa were visible in several mutants (figure 21).

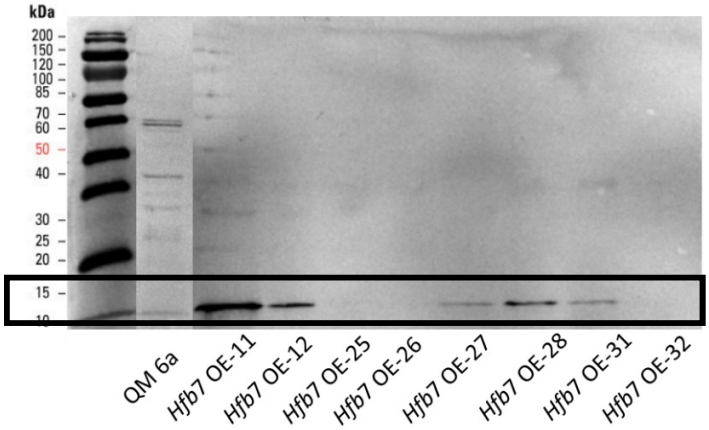


Figure 21. Western Blot assay of the hfb7OE *concentrated supernatant samples at 96 h. Dark bands appeared within 20 minutes of substrate incubation, fainter bands (non-specific binding) appeared around one hour later.*

3.5.2.4. Selection of *hfb7OE* Mutants for Bioreactor Fermentation

The *hfb7OE* strains 11 and 12 had more biomass, and more prominent SDS-PAGE bands at the 10 kDa region than the other OE strains. They both produced strong bands in the western blot assay. Considering these two factors, both mutants were strong candidates for the bioreactor fermentation. This led to the conclusion this strain is the best representative of the *hfb7OE* group, and thus was chosen for the Bioreactor.

Shake flask fermentations are not ideal representatives of industrial fermenters, however suffice for proof of concept work, i.e. showing that the mutants grew differently than the wild type. As *T. reesei* grows in the medium, it begins to acidify the environment. At some point this will begin to affect hydrophobin secretion, as well as the continual survivability of the fungus. Without sufficient agitation, the mycelium forms into pellets which decreases the O_2 and nutrient supply to the inner mycelia. As the fermentation was conducted in batch, the cells will also starve towards the end of the fermentation. It is difficult to predict how these factors would influence the overexpressed hydrophobin production across the fermentation. Conclusively, it was necessary to perform a bioreactor fermentation to show an up-scalable, industrial example.

3.6. Bioreactor Fermentation

The bioreactor analysis was conducted over 60 hours, with a successful outcome. No contamination was ever detected, and both mutants and wild type grew in a predictable manner as per their known characteristics. Of the four bioreactors available, one was reserved for the QM 6a wild type. Two more were designated for the *hfb*4 complementary pair (*hfb4OE* – 16 and 25), and the final was designated for *hfb7OE* – 11.

3.6.1. Analysis *of hfb4OE* mutants

The *hfb*4OE mutants chosen represent two types of mutants, one being a high producing strain and the other a very low producing one. It is industrially advantageous to select a low producing strain capable however, of efficiently producing the desired protein, as it is thus more efficient at converting the carbon source input into the desired product. However, it is also possible that the mutant possesses a poor secretory capacity with regards to all proteins, and thus the desired HFB4 is proportionally equal in comparison to the background protein concentration.

3.6.1.1. Protein Quantification

As with the shake flask fermentation, a BCA assay was done to analyse the change in protein concentration over time (figure 19). The average protein concentration is significantly less that during the shake flask fermentation.

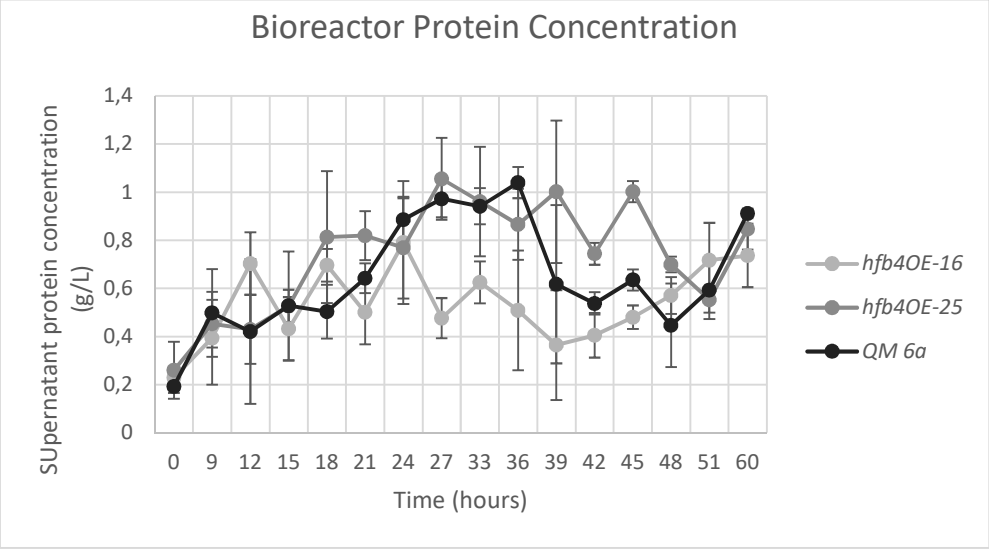


Figure 19. Comparative BCA results taken from bioreactor supernatant from hfb4OE *mutants and wild type.*

3.6.1.2. SDS-PAGE

SDS-PAGE results confirmed that *hfb*4OE-16 is a poor secretor for all types of proteins, including HFB4 (Figure 20). The lowest band on the gel marks the 10 kDa small molecular weight proteins, which includes HFB4. The strain *hfb*4OE-25 shows a capacity for high protein production, which makes it a favourable candidate for future research as a production host.

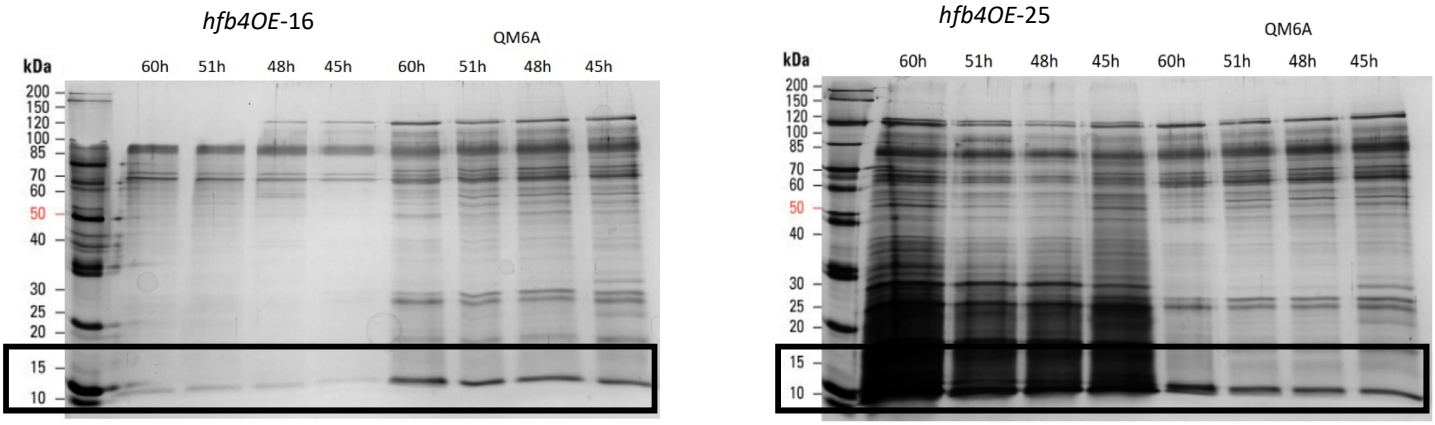


Figure 20. SDS-PAGE of the selected hfb4OE *mutants for bioreactor fermentation. As before in the shake flask fermentation,* hfb4OE *– 16 is a low protein secretor, while hfb4OE – 25 is a strong protein secretor.*

3.6.1.3. Immunoblot Assay

The Immunoblot assay confirmed the successful secretion into the supernatant for two of the three mutants (Figure 20). The bands were only weakly visible, and so the membranes were left overnight to reveal which non-specific bands appear first. Non-specific bands are not indicative of successful secretion in themselves, but may indicate aggregation, if the molecular weight corresponds to the expected size.

The mutant *hfb4OE*-25 produced clearly visible bands, however *hfb4OE* – 16 did not. The protein was either not produced in a detectable amount, or not secreted into the medium. As a band was seen in the shake flask fermentations, the former possibility is likely. SDS-PAGE nonetheless suggests that some proteins at the 10 kDa size are being produced, however this could easily be a background of other SMW molecules. Western blots of the cell wash did not reveal a band in the 10 kDa region for any of the samples.

hfb4OE-16

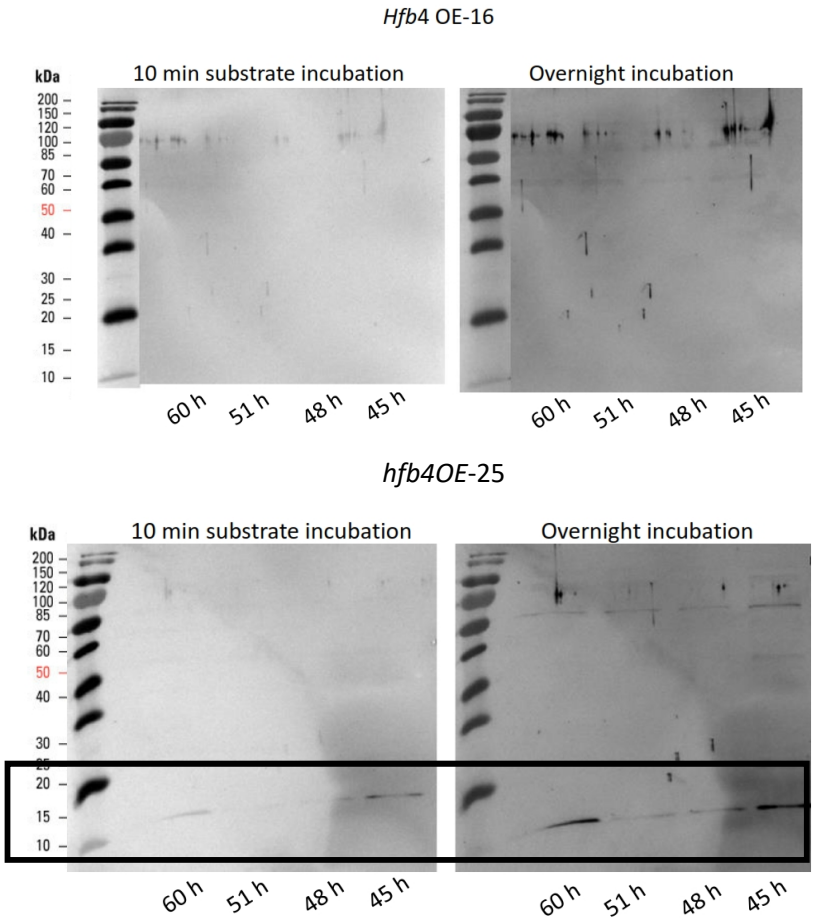


Figure 21. Western blot assay results of bioreactor samples at late fermentation time points. The lack of bands in the hfb4OE-*16 mutant indicate that either the target protein was not secreted into the medium, or it was secreted but undetectable.*

3.6.1.4. Biomass Comparison

Samples containing 10 mL of biomass were collected periodically throughout the fermentation via a sterilised syringe. It was expected that there would be a steady rise, and then a plateau effect. It is visible here that the plateau appears around 30-40 h. Also noticeable is a second climb at the 60 h time point, which is visible in all mutants but not the wild type (Figure 27).

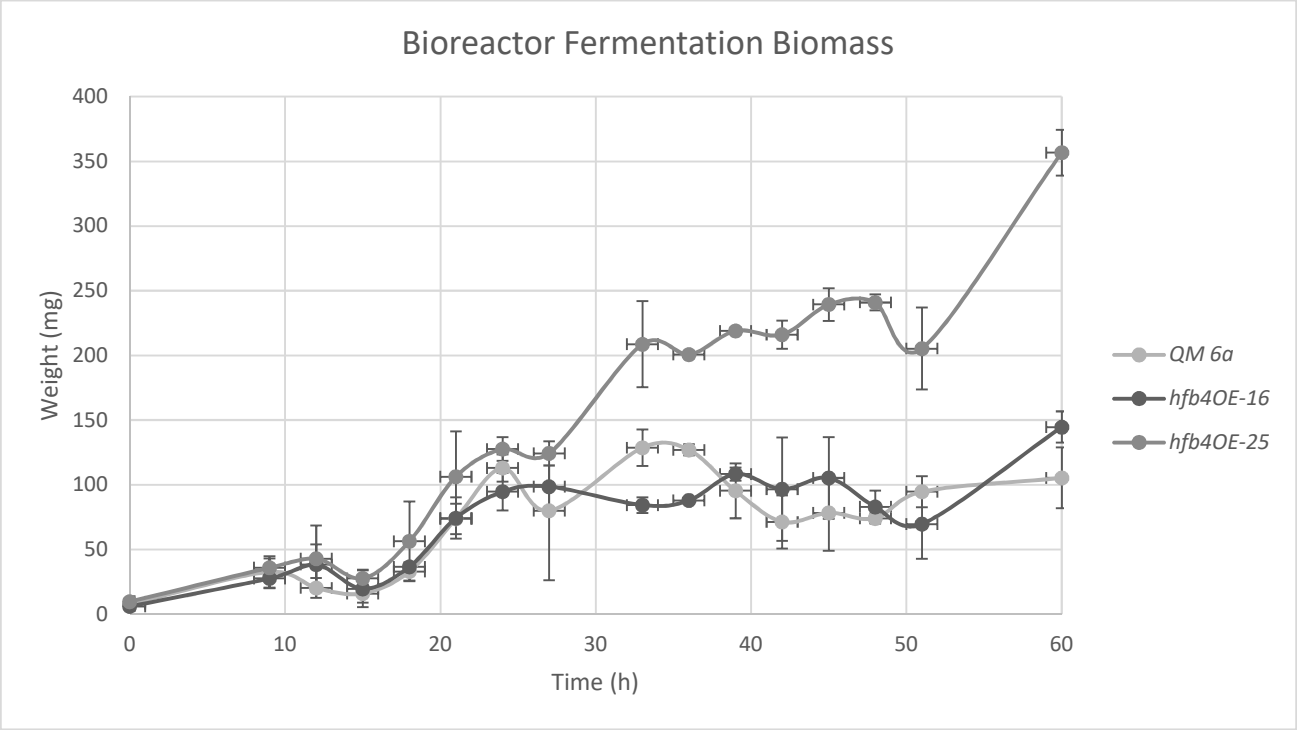


Figure 22. The biomass was collected, dried, and weighed for all time points. This was done to create the following chart showing the growth of the mutants over time. The transformant hfb4-*25 grew exceptionally over the course of the fermentation.*

3.6.2. Analysis *of hfb7OE* mutants

As there was only one *hfb*7 mutant used during the bioreactor fermentation, it could only be compared to the wild type and the *hfb*4 mutants. Nonetheless, *hfb*7OE – 11 exhibited many similar characteristics to *hfb*4OE -25, in terms of growth and protein production.

3.6.2.1. Protein Quantification

As with the shake flask fermentation, a BCA assay was done to analyse the change in protein concentration over time (Figure 23figure 23). The average protein concentration is significantly less that during the shake flask fermentation.

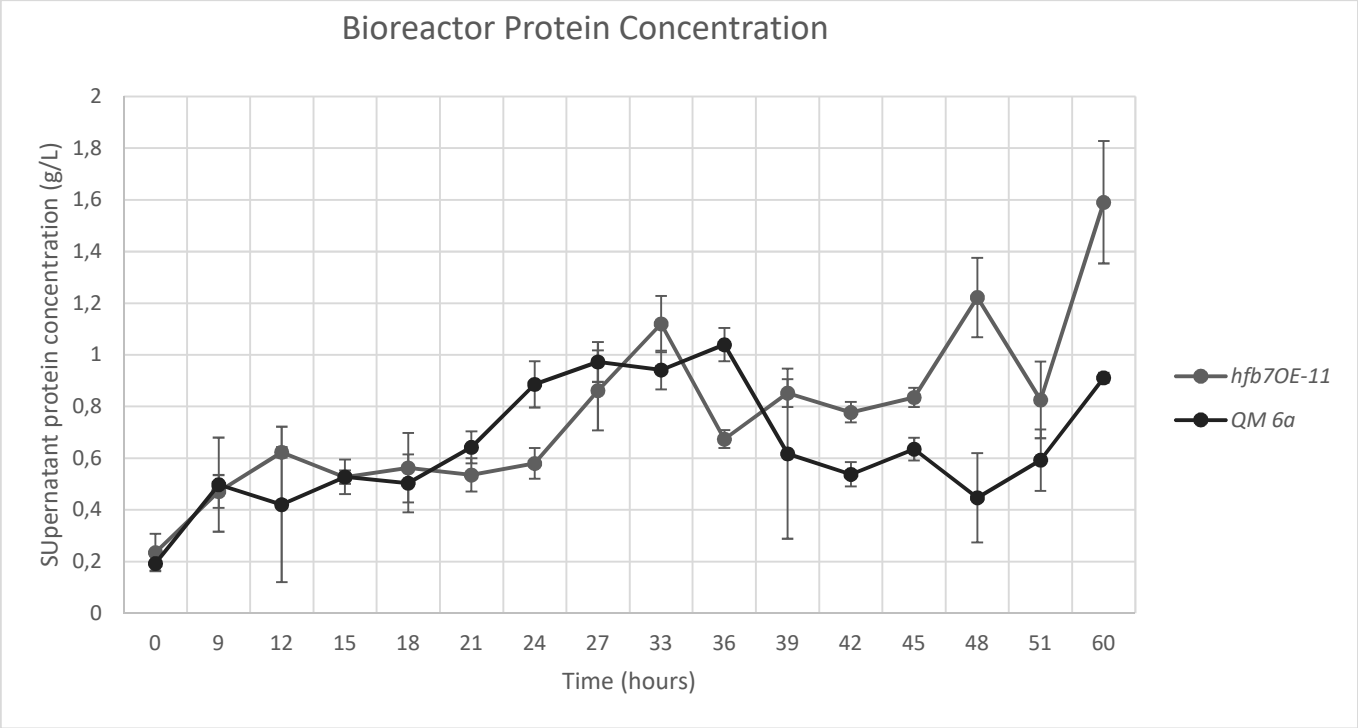


Figure 23. Comparative BCA results taken from bioreactor supernatant from hfb7OE mutant and wild type.

3.6.2.2. SDS-PAGE

The transformant *hfb7OE-11* clearly out-produced the wild type in terms of secretion, with the 60 h growth spike also visible (figure 24). The lowest dark band at the 10 kDa size corresponds to small molecular weight proteins, including the target protein, HFB 7. The band is stronger in the mutant than the wild type, indicating that the heterologously expressed protein is truly being overexpressed.

hfb7OE-11

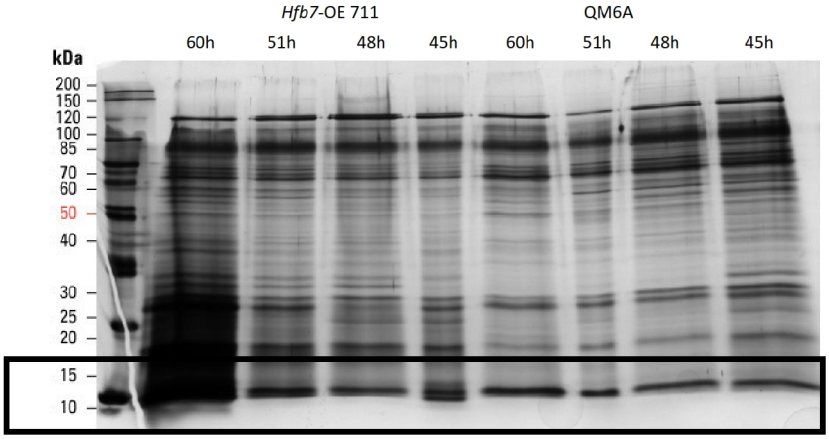


Figure 24. SDS-PAGE of hfb7OE-*11 during the bioreactor fermentation. It exhibited greater protein production than the wild type, as expected.*

3.6.2.3. Immunoblot Assay

As with the *hfb*4 group, a western blot was run using supernatant samples and cell wash. Bands were visible in supernatant derived samples, but not those from cell-wash (figure 25).

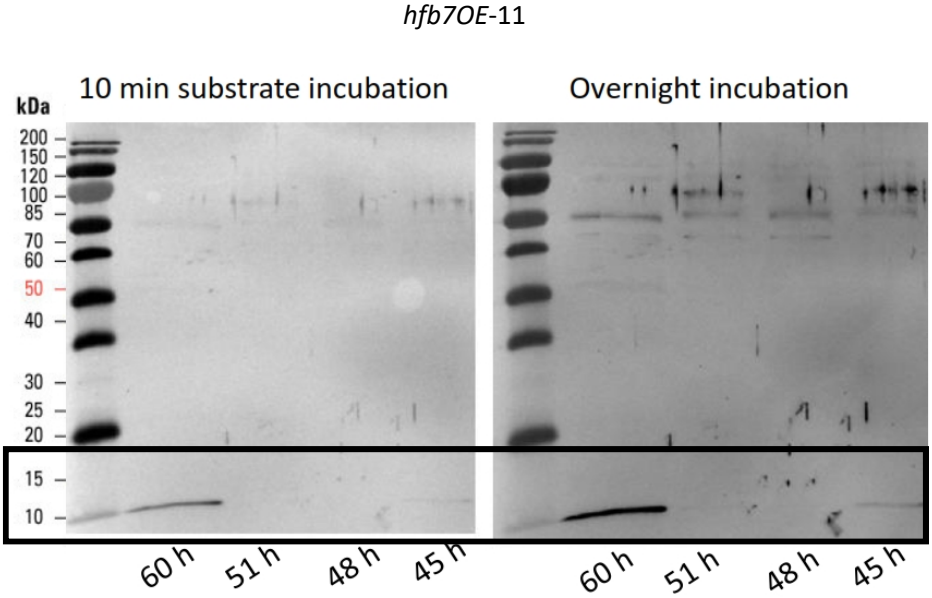


Figure 25. Western blot of hfb7OE-*11 at late fermentation time points. It is of note that only at 60 h was the band highly visible, with 51 h and 48 h missing even after overnight exposure.*

3.6.2.4. Biomass Comparison

The biomass comparison between *hfb*7OE-11 and the wild type shows a similar story as with the *hfb*4OE mutants: an increase around 30-40 hours up to a plateau (figure 26). The wild type saturated the medium at 60 h, following a similar trend as *hfb*4OE-25.

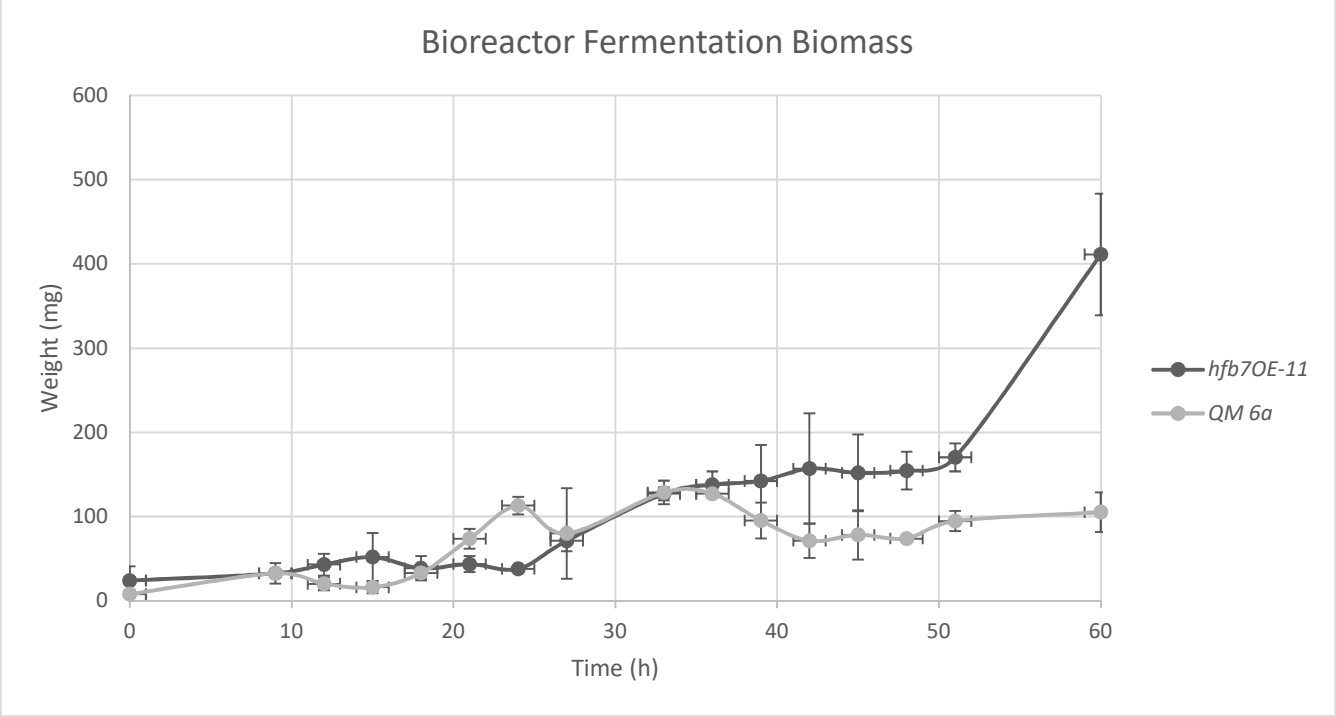


Figure 26. BCA assay to determine protein concentration of the supernatant in hfb4OE samples as well as the wild type during the bioreactor fermentation. The biomass of hfb7OE – 11 dramatically exceeded the wild type, in a similar fashion to hfb4OE – 25.

3.7. Macromorphology in Static Culture

As hydrophobins in the solution are though to assemble in a monolayer in interface, the overexpressing mutants may have altered ability to low water surface tension and form aerial colonies. Strains were grown in both GSM and PD medium for 1 week (Figure 27). However, activity was only visible on the GSM plates. Surprisingly, *hfb*4OE–2 grew the fastest out of the liquid medium; a mutant which hitherto did not display any exceptional characteristics. The other mutants extended out of the medium over the following days, with the incubation finally terminated when the wild type itself escaped the liquid. Most interestingly, the mutant strains exhibit visibly different mycelial morphology than the wild type. Without knowledge on the location of the genes in the genome, as well as their copy number, it is difficult to explain the cause for the mycelial diversity.

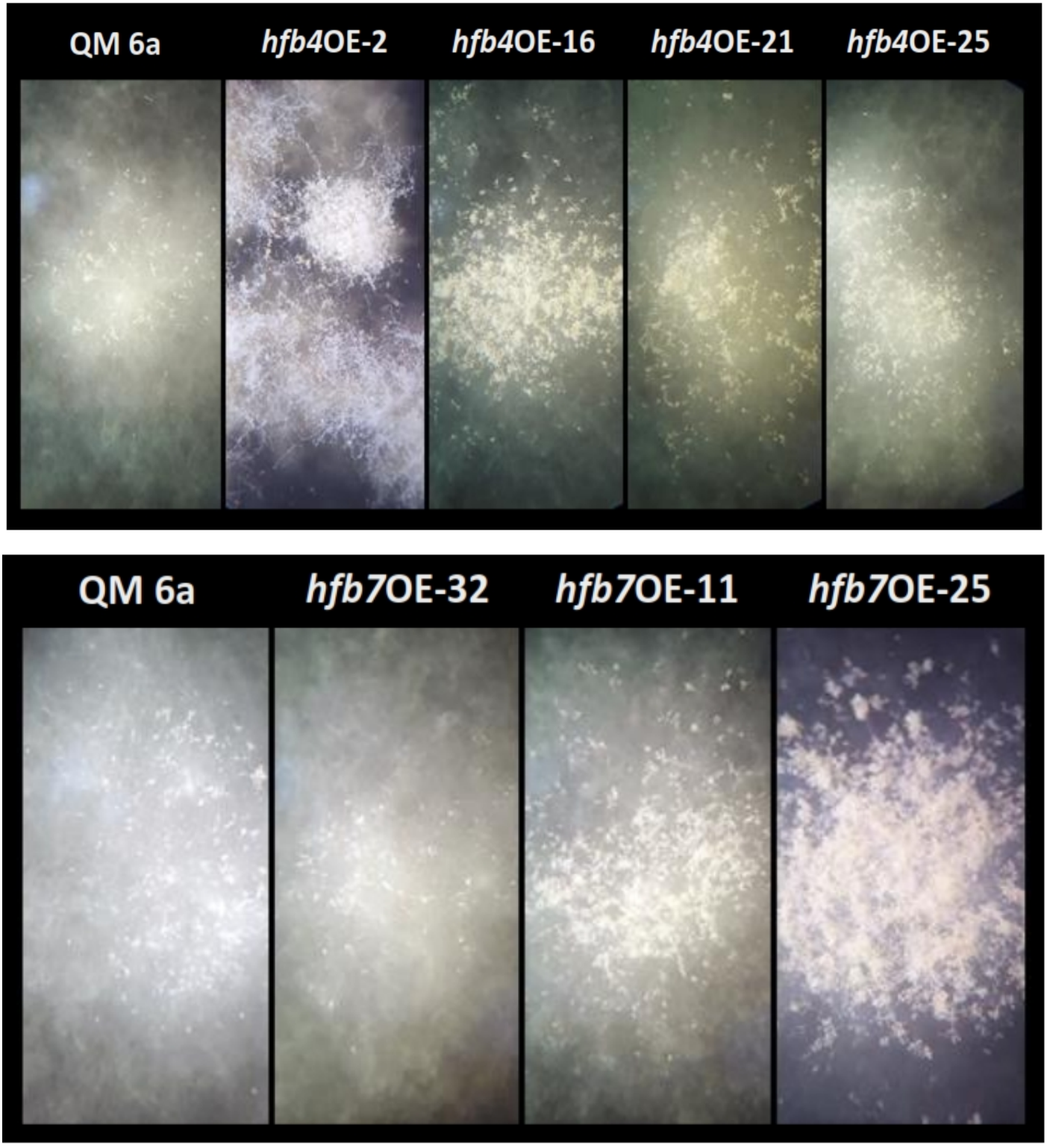


Figure 27. Pictures of mutant mycelia compared to QM 6a wild type after 1 week of incubation in liquid GSM medium on 24-well plates. Pictures taken through microscope to display mycelia variation.

4. Discussion

Considerable research is being focused on recombinant expression proteins in *Trichoderma reesei* due to the vast applications of the fungi as protein or metabolite cell factories (121, 172). The focus of the current study was to transform a *T. reesei* wild type QM 6a strain with a modified pUC19 plasmid containing a hydrophobin gene cassette in two instances: the former being a homologous transformation and the latter heterologous. In this manner HFB4 and HFB7 originating in *T. virens* were overexpressed in a *T. reesei* host.

4.1. Transformation Efficacy

The transformation was completed successfully generating a library of *hfb4*OE and *hfb7*OE mutants. During the development of overexpression systems, a few considerations must be made. The copy number represents the total amount of inserts of the vector, and is ultimately regulated by the degree that the organism is capable of overexpressing the protein of interest, within the limits of its secretion capacity and considering that the insertion must not prevent a normal fungal lifestyle (194). It is evident that the transformant *hfb4OE-25*, and likely *hfb7OE-11* were more capable of producing the target protein than *hfb4OE-16*. This could be due to inefficient integration of the target gene into the genome, or a higher copy number of the insert. In other situations, the availability of transcription factors may be the limiting factor for protein expression. The housekeeping gene *cdna1*, for example, is constitutively expressed. A too great number of *cdna1* promoters for the production of a heterologous protein would decrease the pool of transcription factors. Co-expressing transcription factors and/or auxiliary proteins has been explored in *T. reesei* to facilitate the expression of the target gene (195).

The transformation procedure used achieved a similar amount of transformants compared to previous studies using protoplasting or electroporating procedures (42, 106, 196, 197). These protocols will likely remain popular despite the emergence of CRISPR/Cas9 for still some time, due to their ease of use and reliable results in *T. reesei*. Other *Trichoderma* species that are usually harder to transform, modern techniques, once better implementation protocols are developed, will allow more flexibility when designing OE transformants with other genes suppressed.

4.2. Fermentation Assessment

Attention may further be directed to the medium composition. There is room for optimisation to promote the production of hydrophobins. The carbon source used also would have imparted a significant influence on the resulting hydrophobin concentration detected. Glycerol was chosen as the carbon source throughout the

fermentations, as it is a compound easily digested by *T. reesei* yet does not interfere with analytics in the same way as glucose, the latter which would interfere with subsequent analyses. Glycerol also is a good compromise between preventing catabolic repression (and therefore the biosynthesis of auxiliary proteins such as hydrophobins) and inducing cellulose degrading enzymes. Other studies have found lactose to be a good carbon source at instigating class II hydrophobin expression (61). The study also determined the optimal pH to be higher than that which was used in this study, although the trade-off between optimal organism pH and optimal product protein stability pH is a fine gradient. Steps in this direction will determine if the *T. reesei* overexpression mutants developed in the current study are economically sensible for hydrophobin production.

Alongside upstream improvement in the fermentation, the extreme surface activity of hydrophobin is a constant hindrance to purification and collection. During the fermentation, hydrophobins may be accumulating on many surfaces (Figure 28). Although it is assumed that majority hydrophobin yield will be in the supernatant, given the strong affinity of hydrophobins to hydrophilic and hydrophobic surfaces (50), it is possible that a significant portion of the yield is lost in this manner to glassware surfaces. To combat this, techniques such as pull-down assays may be developed - perhaps glass or PET beads could be used to seed bioreactors as a vector to collect hydrophobins.

During the shake flask fermentation, it became apparent that the OE mutants foamed considerably more than the wild type. This observation was later observed again during the bioreactor fermentation. It is well known that hydrophobins coalesce along air-liquid boundaries, thus it is no surprise that they should be present in foam (7, 198). As it is due to the presence of additional hydrophobins that foaming is more prevalent than in the wild type, a potential purification strategy involved collecting and condensing the foam. This system proved unsuitable for HFB4$_{virens}$ or HFB7$_{virens}$, however, as collected foam fractions did not result in visible bands on immunoblot membranes. The concentration of hydrophobins in the foam fraction has been reported as high as 502.0 mg/L (199). A possible reason why no band was detected in this study could be due to the fact that only 10 – 15 mL of foam was collected. In this situation, the antifoam prevented significant foam, and what foam existed could only be collected through a bottom drainage sampling mechanism. Therefore, the tested foam is likely not representative of the naturally produced foam at the top of the medium. Antifoaming was used twice throughout the fermentation on mutant *hfb*4OE-25, at a concentration of 1 mL antifoam to 1 L fermentation solution. This was an expected side effect of hydrophobin overexpression, explained by the high surface activity of hydrophobins. The mechanism is best demonstrated in beer gushing experiments of Lutterschmid *et al.* (200).

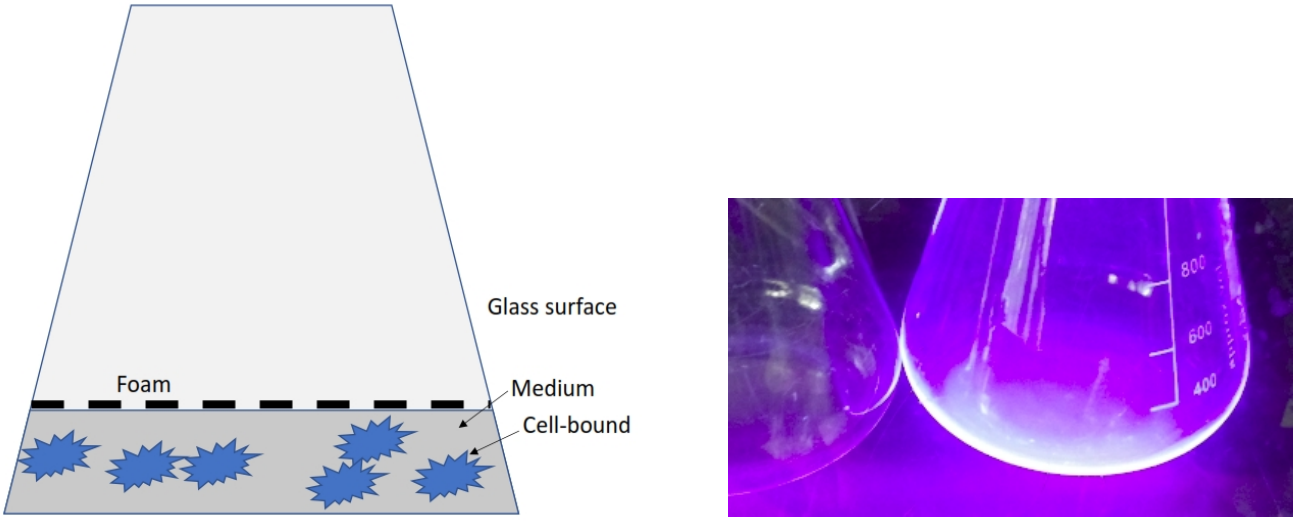


Figure 28. Sketch of a Erlenmeyer flask used in Shake Flask Fermentations, designating potential HFB fixation sites. Green represents the medium, blue represents mycelia, and the dotted line represents foaming (left). A shake flask fermentation containing mutants with a florescent hydrophobin tag showing significant binding of hydrophobin proteins to the glass post-removal of the medium. An empty Erlenmeyer flask adjacent provides a negative control (right).

The fermentation was terminated at 60 h due to extensive biomass growth by *hfb*4OE-25 preventing further sampling. The results of the biomass sampling indicate a significant increase in biomass at the very end of the fermentation. The medium became almost homogenously thick - in all mutants and the wild type. It was also determined that most of the hydrophobins were cell-bound, and not free flowing in the supernatant.

The bioreactor fermentation represents an attempt to create a scalable experiment for industrial application. The wild type and three mutants: the pair homologous transformation mutants *hfb*4OE-25 and *hfb*4OE-16, as well as the heterologous transformation mutants *hfb*7OE-11. It was predicted that the OE mutants would take on similar growth styles in the bioreactor as in the shake flask fermentation, i.e. *hfb4*OE-16 would grow slowly and have a cell density similar to the wild type, *hfb*4OE-25 would grow quickly and be a strong producer, and *hfb*7OE-11 would be similar, although not as pronounced, as *hfb*4OE-25.

4.3. Production Assessment

The samples were assessed across four measurements: biomass, total protein concentrations (BCA assay), proteins separated by size (SDS-PAGE) and HIS6 tag screening (Immunoblot). The BCA assay showed that the shake flask total protein concentrations were relatively high. The formation of mycelial pellets, especially after several days of fermentation, is an unavoidable consequence of the fermentation of filamentous fungi in submerged media. Regardless, care was taken to keep the fermentation as homogeneous as possible, so as to prevent large clumping and the implied cell starvation which commonly follows. The acidification of the shake

flasks over time, however, likely caused a greater number of cells to die than in the bioreactor. The additional cell debris may explain the discrepancy in the measured concentrations. Additionally, of course, the bioreactor fermentation must be optimised for the production of hydrophobins and the particular mutants.

The BCA assay indicated that, among the homologous overexpression group, *hfb*4OE-25, *hfb*4OE-28, *hfb*4OE-2 contained the most amount of protein within the supernatant at most time intervals. *Hfb*4OE-16, conversely, displayed a low protein concentration. SDS-PAGE results however showed a strong band at ca. 10 kDa, indicative of hydrophobins, but also other small molecular weight proteins. The western blot of the concentrated supernatant samples indicated that *hfb*4OE-25 and *hfb*4OE-16 both were capable of producing the desired homologously expressed HFB4. The pair represent two production extremes in terms of total protein production. It is not possible to quantitatively evaluate which mutant produces more of the desired protein using SDS-PAGE and BCA assay alone, thus both were chosen for the bioreactor fermentation. Among the heterologously overexpressed mutants, *hfb7*OE-11 demonstrated strong production potential as indicated by the BCA assay, SDS-PAGE analysis, and the western blot.

The SDS-PAGE analyses show that the total protein output of the bioreactor mutants was lower than the shake flask mutants. It is possible that some very high MW proteins interfered with the BCA assay. It may be, for example, that the expressed hydrophobins are aggregating, either as homogenous or heterogenous complexes with proteins in the sample. Otherwise, however, the trends seen during the shake flask fermentation were duplicated in the bioreactor fermentation. The western blots provided inconsistent results. Given sequencing validation for the success of the transformation and the detection of a hydrophobin band on the shake flask immunoblots, it is difficult to explain why *hfb4*OE-16 samples from the bioreactor fermentation do not show any band. As there is no efficient way to isolate hydrophobins from the medium, it is likely they were always lost during the purification procedure.

High product losses during the downstream processing of hydrophobins has been observed previously (201-204). The surface activity of the molecules enacts a heavy toll on the downstream processing, explaining the universally low protein yields of ca. 5-10% (22, 162, 201, 205). To date, the best hydrophobin expression achieved was *hfb1* from *T. reesei* in the native host using the *cbh1* promoter and the native terminator, reaching 0.7 g/L of purified protein (203). The gene *hfb2* was expressed in the native host and flanked by the *cbh1* promoter and terminator, however achieving a much lower yield at 0.240 g/L (204). It is difficult to compare the results from the current study to these in a direct manner, as a pure target protein extract was not achieved. Maximum protein concentrations in the bioreactor were near 1 g/L for the *hfb4* mutants and 1.6 g/L for the *hfb7* mutant, with shake

flask concentrations being higher at 2.5 g/L and 4 g/L, respectively. The purified protein, assuming a 10% yield and the bioreactor level concentrations, would be between 0.01 g/L and 0.016 g/L.

4.4. Future Directions

Hydrophobins, especially those of class II, often exist in solution as weakly associated oligomers (often dimers or tetramers) (206). Their affinity to liquid-air boundaries ensures that product recovery rates will depend heavily on the location the sample is taken from. Due to the apparatus set-up, sampling was always taken from the supernatant. Some solutions which may be applied in future research are suggested in the next section. In terms of ongoing research, there are two departure points from the current study: developing an efficient hydrophobin collection method for this mutant set-up, and comparing the produced protein of interest with its analogues within the native *T. virens* and other vectors.

Working with supernatant samples alone, the monomeric hydrophobin concentration is currently too low in *Trichoderma* to be of industrial interest and must be concentrated. One modern technique to achieve this in a dynamic, real-time system is CO_2-hydrophobin foam fraction extraction. Khalesi et al. (204) showed that CO_2 has a high affinity to the hydrophobic residues of the hydrophobin proteins, thus allowing them to be collected and concentrated simultaneously (207). Other methods proposed for class I hydrophobins use strong acids such as trifluoroacetic acid to monomerise the hydrophobins, thereby effectively concentrating them at the liquid-air boundary (208). Otherwise, post-fermentation scrubbing techniques may allow rapid processing of the supernatant. Aqueous two-phase purification systems (ATPS) allow efficient separation of water-soluble polymers and non-ionic detergents, as well as subsequent concentrating. The interaction of HFB2 and especially HFB1 from *T. reesei* with nonionic surfactants yields an efficient partitioning coefficient (203, 209). ATPS is probably one of the few effective techniques capable of efficiently purifying membrane proteins, and thus was an interesting choice to be applied to hydrophobins, however did not provide the separation efficacy needed for the industrial scale (210, 211). A type of ATPS called cloud point extraction (CPE) applies the ATPS partition principle to the system while in a cloud point equilibrium. The harsher conditions may perhaps be more efficient at keeping hydrophobins denatured during purification. If an efficient scrubbing technique is developed, it would suffice to simply remove a portion of the medium for hydrophobin collection, and replenish the reactor with fresh medium – a strategy commonly used in the production of many common products.

The second direction that may be taken from this study is the thorough characterisation of the OE proteins, and their subsequent comparison with their *T. virens* analogues. It would be interesting to see if the hydrophobins synthesised heterologously by *T. reesei* are identical to *T. virens*. Furthermore, it is important to understand how

hydrophobins produced in the native host differ from their analogous produced in yeast (i.e. *Pichia pastoris*) or in bacteria (*E. coli*), as recently characterized by Przylucka *et al.* (209). In the way, the capacity of alternative expression systems to efficiently produce hydrophobins may be assessed.

In terms of the experimental procedure, it is unlikely that the initial expression configuration is the most efficient possible. Further optimisation of the procedure necessitates testing out different expression cassettes (210, 211), promoters (212), fusion protein tags (213, 214), as well as reconfiguring the bioreactor set up. For example, HFB2 is often detected on 2D gels and SDS-PAGE gels at near or greater concentrations that the target protein. Replacing the *cdna1* promoter with that of *hfb2* or deletion of *hfb2* might knock out a persistent background protein while hijacking an obviously highly efficient promoter.

5. Conclusion

This study overexpressed the two hydrophobins (HFB4 and HFB7) originating from *T. virens* in a *T. reesei* host. The transformation was completed successfully. Mutants were characterised based on their growth rates and protein production in a shake flask and bioreactor fermentations. Selected mutants are described as well as their interest for future research, which should focus on further characterisation of the overexpressed protein and optimisation of the downstream processing to allow a better assessment of the production system for industrial purposes.

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7. Curriculum vitae

VICTOR P. LOBANOV

vplobanov@gmail.com

PERSONAL DATA:

Date of Birth: 10.09.1992

Place of Birth: Seattle, WA, USA

Nationality: American, Canadian

Family status: unmarried

EDUCATION:

TU Wien (Vienna, Austria)	Jan. 2016 – Mar. 2018
Master in Technical Chemistry: Biotechnology and Bioanalytics	
University of Calgary (Calgary, Canada)	Sep. 2012 – Nov. 2016
B.Sc., Natural Sciences (Concentrations: Chemistry, Statistics)	

RESEARCH EXPERIENCE

Research Group Microbiology and Applied Genomics	Oct. 2015 – Mar. 2018
Technical University of Vienna (Vienna, Austria)	
Master Student; Advisor: Dr. Irina Druzhinina	
Projects:	
• Hydrophobin overexpression, production, and characterisation in *Trichoderma* spp.	
Clinical Neurosciences, Section Physical Medicine & Rehabilitation	Sep. 2012 – Dec. 2015
Foothills Medical Centre (Calgary, Canada)	
Research Assistant; Advisor: Dr. Chester Ho	
Projects:	
• Care and Needs Survey write-up and internal publication	
• Champion Nursing Program survey	
• Telehealth patient satisfaction assessment	
• Ulcer frequency analysis	

PROFESSIONAL EXPERIENCE

Research Assistant, Foothills Medical Centre (Calgary, Canada)	Sep. 2012 – Dec. 2015
Language Tutor in German, Russian (Calgary, Canada)	Sep. 2014 – Aug. 2015
Professional Ski Patroller, Winsport (Calgary, Canada)	Sep. 2011 – May 2012
Volunteer, Canadian Blood Service (Calgary, Canada)	Sep. 2012 – Mar. 2014
Volunteer Ski Patroller, Snoqualmie Summit Central, USA (Snoqualmie, USA)	Sep. 2010 – May 2011

SCIENTIFIC PUBLICATIONS

Przylucka A, **Lobanov VP,** Druzhinina IS. A Daedalian Network of Hydrophobin Production Strategies: why terminal applications influence method selection. Appl. Microbiol. Biotechnol. *In submission to AMB*.

Przylucka A, Akcapinar GB, Bonazza K, Mello-de-Sousa TM, Mach-Aigner AR, **Lobanov VP,** Grothe H, Kubicek CP, Reimhult E, Druzhinina IS. "Comparative Physical-Chemical Analysis of Hydrophobins Produced in Escherichia coli and Pichia pastoris." Colloids and Surfaces B: Biointerfaces, 2017, ISSN 0927-7765.

Morris TT, Keir JL, Boshart SJ, **Lobanov VP**, Ruhland AM, Bahl N, Gailer J. "Mobilization of Cd from human serum albumin by small molecular weight thiols." J Chromatogr B Analyt Technol Biomed Life Sci. 2014 May; 1(958):16-21.

CONFERENCE PROCEEDINGS

Lobanov VP. " Learning from Fungi: New Applications from Surface-active Proteins". Poster and Talk at the Austrian Days of Chemistry. 2017 September.

Lobanov VP, Cai F, Przylucka A, Sidarenka L, Shen Q, Druzhinina IS. "Improvement of Hydrophobin Production in *Trichoderma."* Poster presented at the Vienna young Scientists Symposium. 2017 June.

Lobanov VP, McKenzie N, Ho C. "Financial Security as a Motivational Facilitator for Rehabilitative Care." Poster presented at the Academy of Spinal Cord Injury Professionals Conference. 2015 September.

Lobanov VP, McKenzie N, Ho C. "Determining the Current State of Caregiver Burden for the SCI Population in Southern Alberta". Poster presented at the International Spinal Cord Society Conference. 2015 May.

LANGUAGES

Language	**Proficiency (CEFR scale)**
English	C2 (greatest proficiency)
Russian	C1
French	C1
German	C1
Dutch	B1

8. Supplementary Materials

8.1. Vector Sequences

Legend:

ATACCTGTCCGCCTTTCTC: Primer sequence

AAGCAATC = cdna1P

CCCAAGTGC = *hfb4* or 7 gene

TCGCCGCTCTTC = *Tcbh* terminator sequence

ATCGAT = CLA 1 restriction site

GTCGAC = SAL 1 restriction site

AAGTGT = following segment pUC cloning vector

>HFB4

GAGGGAGTGAGAAACTGAAGAGAAGGAAAGCTTCGAAGGTTCGATAAGGGATGATTTGCATGGC
GGGCGACAGGATGCGATGGCTCGTTGGGATACATAATGCTTGGGTTGGAAGCGATTCCAGGTCG
TCTTTTTTTGGTTCATCATCACAGCATCAACAAGCAACGATACAAGCAATCCACTGAGGATTAC
CTCTCAACTCAACCACTTTCCAAACCATCTCAACTCCCTAAGATTCTTTCAGTGTATTATCACT
AGGATTTTTCCCAAGCCGGCTTCAAAACACACAGATAAACCACCAACTCTACAACCAAAGACTT
TTTGATCAATCCAACAACTTCTCTCATCGATATGCAGTTCTCAGCCATCATTGCCCTCTTTGCC
TCTCTGGCTATTGCCGCGCCCGCTGGGGAGACGACTACTCACGAGCTCGCTGCTCGTGACGGCC
CTTGCTCTTCTGGTGTTACTAACAACGTCCCCAAGTGCTGTGGTACTGGTGTCCTCGACCTCCT
CTACCTGGACTGCAAGACTCGTAAGTCGTTGAAAGTGGCCTCTTATCAAAAGATCATGTTCTAA
CACATCTCCAGCCACTCAAGCCACCTCCGTTCTCAACCCCTTGAGTGCTGTCTGCGGCCGTGTT
GGTCTCCAGGCCAAGTGCTGCACCGCCGGTATCGTAAGTATTCTCAGCTGCATCATCCTCCATT
ATGAATTGTATTAACGAGTTTTCAGGGCTCTGTCGGTGTTTTGTGCCAGGATGCTCTTCCCGAG
CATCATCACCATCACCACTAAGTCGAC
AGCTCCGTGGCGAAAGCCTGACGCACCGGTAGATTCTTGGTGAGCCCGTATCATGACGGCGGCG
GGAGCTACATGGCCCCGGGTGATTTATTTTTTTTGTATCTACTTCTGACCCTTTTCAAATATAC
GGTCAACTCATCTTTCACTGGAGATGCGGCCTGCTTGGTATTGCGATGTTGTCAGCTTGGCAAA
TTGTGGCTTTCGAAAACACAAAACGATTCCTTAGTAGCCATGCATTTTAAGATAACGGAATAGA
AGAAAGAGGAAATTAAAAAAAAAAAAAAAACAAACATCCCGTTCATAACCCGTAGAATCGCCGC

TCTTCGTGTATCCCAGTACCACGGCAAAGGTATTTCATGATCGTTCAATGTTGATATTGTTCCC
GCCAGTATGGCTCCACCCCCATCTCCGCGAATCTCCTCTTCTCGAACGCGGTAGTGGCGCGCCA
ATTGGTAATGACCCATAGGGAGGTCGAC
CTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC
GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT
CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAA
AGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTAT
AAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
TACCGGATACCTGTCCGCCTTTCTC

>HFB7

GAGGGAGTGAGAAACTGAAGAGAAGGAAAGCTTCGAAGGTTCGATAAGGGATGATTTGCATGGC
GGGCGACAGGATGCGATGGCTCGTTGGGATACATAATGCTTGGGTTGGAAGCGATTCCAGGTCG
TCTTTTTTTGGTTCATCATCACAGCATCAACAAGCAACGATACAAGCAATCCACTGAGGATTAC
CTCTCAACTCAACCACTTTCCAAACCATCTCAACTCCCTAAGATTCTTTCAGTGTATTATCACT
AGGATTTTTCCCAAGCCGGCTTCAAAACACACAGATAAACCACCAACTCTACAACCAAAGACTT
TTTGATCAATCCAACAACTTCTCTCATCGATATGCAGTTCTTCGCCGTCGCCGCTCTCTTCGTC
GCCAGCGCCTTGGCCGCCCCTGCTGGCGATGAAGGTTGCCCAGGTGGTATCCTTCACAACGTCC
CGCTGTGCTGCGCTACCAATGTGCTCGGTGTCGCTACCCTCGACTGTGGCACTCGTAAGTCTCC
TTCTTCAATCTACCTTTCAGCAACTCCCACATGGAGCATTTCACTGAGCAAATACATCGCAGCT
ACTGTGCCCGTCCTCACTCCTGCCCAGTTCCAGAGCCACTGTGCTGGCAAGGGCAAGCAGCCTG
TCTGCTGCAGCATCCCCGCTGTAAGTATCCCCTCCAAATGATATTCAACAATATTGAATTGTGA
GATTCTGATGCTCATTTCTTTCTATCAAGGCTGGCCTGGGACTCCTTTGCGAGAAGCCCGTCGG
AACCCAGCATCATCACCATCACCACTAGGTCGAC
AGCTCCGTGGCGAAAGCCTGACGCACCGGTAGATTCTTGGTGAGCCCGTATCATGACGGCGGCG
GGAGCTACATGGCCCCGGGTGATTTATTTTTTTTGTATCTACTTCTGACCCTTTTCAAATATAC
GGTCAACTCATCTTTCACTGGAGATGCGGCCTGCTTGGTATTGCGATGTTGTCAGCTTGGCAAA

TTGTGGCTTTCGAAAACACAAAACGATTCCTTAGTAGCCATGCATTTTAAGATAACGGAATAGA
AGAAAGAGGAAATTAAAAAAAAAAAAAAAACAAACATCCCGTTCATAACCCGTAGAATCGCCGC
TCTTCGTGTATCCCAGTACCACGGCAAAGGTATTTCATGATCGTTCAATGTTGATATTGTTCCC
GCCAGTATGGCTCCACCCCCATCTCCGCGAATCTCCTCTTCTCGAACGCGGTAGTGGCGCGCCA
ATTGGTAATGACCCATAGGGAGGTCGAC
CTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG
CCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC
GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT
CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAA
AGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTAT
AAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
TACCGGATACCTGTCCGCCTTTCTC

8.2.CLUSTAL 2.1 Multiple Sequence Alignment

```
                          hfb4 virens reesei comp.txt
CLUSTAL 2.1 multiple sequence alignment

T. reesei       GCGCCTGCTCCGCCGCCGCCGCCGTCATCGAACCCCCCATCCACCCCGTCGCCGCCCCGA
T. virens       ------------------------------------------------------------A
                                                                             *

T. reesei       GATGCGCCCCGGCCACCGCCGCCAGCGGCGTCTGCGTGCCCGTCGCCAGGTGCGCCTGGA
T. virens       TGCAGTTCTCAGCCATCATTGCCCTCTTTGCCTCTCTGGCTATTGCCGCGCCCGCTGGGG
                      * * **** *   ***  *   * **   ** *  * ***  *  ***  **

T. reesei       AG-TAGTTGCTCCGGCCCTGCCCGCCCACGCCCAGGATTCCCGGCGTCGACGTCCCCAGA
T. virens       AGACGACTACTCACGAGCTCGCTGCTCGTGAC-GGCCCTTGCTCTTCTGGTGTTACTAAC
                **     * ***  *  **  * ** *  * *  *   *  *      *  **  * *

T. reesei       AACGCCCGCCCGAGGTCGTGCCCGCTGAGGTTCATGCCGGGGCTCATGCTCATCGTGTGC
T. virens       AACGTC--CCCAAG--------TGCTGTGGT----ACTGGTGTCCTCGACC-TCCTCTAC
                **** *  *** **         **** ***     * ** *  *  *  * ** * * *

T. reesei       ATGTTGGGCGTGCTGGCGTTCATGTGCGAGCCCAAGCCGAGCCCCATCCCC-GTCCCTGT
T. virens       ---CTGGAC-TGCAAG-ACTCGTAAGTCGTTGAAAGTGGCCTCTTATCAAAAGATCATGT
                   *** * ***  *   ** *  *       ***  *   *  ***    *  * ***

T. reesei       TCCCATCCCCATCCCCATCCCCAGGCCACCGCCCATGTTCATGCTCATCTCGCTGCGCAG
T. virens       TCTAACACATCTCCAGCCACTCAAGCCACC-------TCCGTTCTCAACCCCTTGAGTGC
                **  *  *   ***     * ** ******       * * * **** * *  ** *

T. reesei       CTCCCGCAGCCCGTTTTCCCTCCTCCTCGCCTCCATCATCGCCGCCGCCAGCCGTTCCTT
T. virens       TGTCTGCGGCCGTGTTGGTCTCCAGGCCAAGTGCTGCACCGCCGGTA----TCGTAAGTA
                  * ** ***   **   ****    *   * *  ** *****        ***   *

T. reesei       TTCCCTGTCCCTGTCCCTGTCCCTGTCCCTATTCCTATCCCTCTCCGCAAAGCTCTTGTC
T. virens       TTCTCAG---------CTGCATCATCCTCCATTATGAATTGTATTAACGAG---------
                *** * *         ***   *   * * ***   *    * *   * *

T. reesei       ATCTCCACCGCTCTCCCCGCCCTCCTCATCACCCCCATCCTCATCATCATCATCATCATC
T. virens       -TTTTCAGGGCTCTGTCGGTGTTTTGTGCCAGGATGCTCTTCCCGAGCATCATCACCATC
                 * * **  *****  * *   *      **      ** **   * ******** ****

T. reesei       ATCATCATCATCATCCTGCTCGTAGAAATCCGGCAGAGGGTCCGTAATGTCCTTCAACTC
T. virens       ACCACTAA-----------------------------------------------------
                * **  *
```

8.3. Protoplasting Media

Constituent	Amount per L Solution
1.2 M Sorbitol	43.72g
0.1 M KH_2PO_4 (pH 5.6)	2.72 g

Table 9. Solution A to maintain the osmotic and electrolytic equilibrium of the protoplasting cells following cell-wall removal via lysing enzymes.

Constituent	Amount per L Solution
25 % PEG 6000	25.0 g
50 mM $CaCl_2$	0.735 g
10 mM Tris-HCl (pH 7.5)	1.576 g

Table 10. Solution B to facilitate plasmid uptake by increasing nuclear permeability.

8.4. SDS-PAGE Composition and Buffer Solutions

Composition of the SDS-PAGE medium:

Constituent	Amount per L solution	
	Seperating Gel	Stacking Gel
dH_2O	1.35 ml	1.40 ml
Buffer	1.41 mL*	0.70 mL**
30% Acrylamide	0.42 mL	7.5 mL
10 % SDS	56 µL	28.5 µL
TEMED	3.38 µL	5.625 µL
10% APS	56 µL	18.75 µL

*Table 11. Constituents are added in the ordered specified from top down. * Separating Gel buffer consists of Tris – HCl buffer, 1.5M, pH 8.8. ** Stacking Gel buffer consists of Tris – HCl buffer, 0.5 M, pH 6.8.*

Constituent	Amount per L Solution
TRIS	90.75 g
SDS	2.0 g

Table 12. Separating Gel: 1.5 M Tris – HCl buffer (pH 8.8).

Constituent	Amount per L Solution
TRIS	30.25 g
SDS	2.0 g

Table 13. Stacking Gel: 0.5 M Tris – HCl buffer (pH 6.8).

Constituent	Amount per L Solution
TRIS	90.75 g
SDS	10.0 g
Glycine	144.0

Table 14. 10x Running Buffer (pH 8.3). Running buffer constituents for SD-PAGE. Solution diluted to 1x before use.

Constituent	Amount per 30 mL Solution
0.5 M Tris – HCl	3.75 mL
10% SDS	6.0 mL
50% Glycerol	15.0 mL
1% Bromophenol Blue	0.3 mL
ß-mercaptoethanol	50 µL to 950 µL sample buffer

Table 15. SDS-PAGE sample buffer composition 2x (Laemmli).

8.5. Western Blot Buffer Solutions

Constituent	Amount per L solution
MeOH	200 mL
190 mM glycine	14.26 g
25 mM TRIS	3.03 g

Table 16. Constituents for transfer buffer. Solution must be diluted to 1x before use.

The gel composition detailed above for SDS-PAGE corresponds to that used in the first electrophoresis of the western blot assay.

8.6. Commercial Kits

Name	**Supplier**	**Origin**
In Fusion® HD cloning Kit	Clontech	Mountain View, California
PureYield™ Plasmid Midiprep System	Promega	Bavaria,Germany
QIAprep Spin *Miniprep Kit*	Quiagen	Madison,USA
QIAquick Gel Extraction Kit	Quiagen	Madison,USA
RNeasy Plant Mini Kit	Quiagen	Madison,USA

8.7. Equipment List

Name	**Supplier**
Analytical balance	Sysmatec
Centrifuge	Eppendorf
Centrifuge 3 -18k	Sigma – Aldrich
Gel electrophoresis power unit	BIO RAD
Gel imaging system	BIO RAD
Incubators 25˚C,28˚C, 37˚C	Heraus
Mini – PROTEAN Tetra cell	BIO RAD
PCR Thermocycler	BIORAD
Pipette tips (1 mL)	Eppendorf
Pipette tips (10 µl)	Eppendorf
Sorvall RC 6 plus Superspeed Centrifuge	Thermo scientific
Spectrophotometer Nanodrop ND - 1000	Peqlab
Thermomixer compact	Biometra
UV Spectrophotometer	Thermo scientific