Deletion of Hydrophobin Genes in *Aspergillus niger*

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Layman's summary

Industries use microorganisms to make small compounds. These small compounds can have all kinds of functions. For example, they can be used for preservation of food products. In the industrial production line, a lot of factors can influence the productivity of the microorganism. That is why environmental conditions like temperature and oxygen content need to be set precisely and monitored closely. Biotechnology can help in researching ways to improve this production process. These researchers try to understand the microorganism in question by studying its growth and development. Then, they think of biotechnological ways for this microorganism to make more of the desired compound. In this research, the fungus *Aspergillus niger* is studied.

In nature, *A. niger* lives on decaying materials and is therefore very good at breaking down organic matter. It does so by secreting acids and enzymes. Humans have discovered a way to harvest these acids and enzymes for their own use. For example, a certain enzyme called 'glucoamylase' makes soluble sugars from longer chains of sugars. Therefore, this enzyme is very useful for making sweet beverages. Currently, *A. niger* has been used as a cell factory for industrial fermentation for decades. It is grown in large fermentation tanks called bioreactors. These bioreactors contain soluble nutrients and are shaken to mix them in the solution. Here, *A. niger* colonies clump together to form so called 'pellets' that are visible to the naked eye. These are spherical structures that are composed fungal colonies that have clumped together.

In earlier studies it was shown that these clumps were formed due to an aggregation process. The fungal spores bump into each other in the liquid shaken culture and then stick together. After that they grow 'hyphae' which are long branching tubular cells that make up a mycelium. These bump into even more spores that stick together, creating a large unit. Studies on this subject in species of the same genus showed that the hydrophobin genes may be important. These proteins are secreted by fungi to facilitate *A. niger* in interactions between surfaces that repel and attract water. Removal of these hydrophobin genes resulted in changes in size, biomass and other properties of the pellets in the liquid shaken cultures. Therefore, the hydrophobin genes in *A. niger* were studied for the same properties. Strains were made in which each of the eight hydrophobin genes were removed. These were compared to the strain without any hydrophobins removed. No differences were seen in the properties tested. This means that not one hydrophobin gene on its own is responsible for these properties. Instead, the other hydrophobin genes probably take over its role. A strain where all eight genes are knocked out would be interesting to study in the future.

Abstract

Aspergillus niger is a saprophytic fungus that is widely used for the industrial production of enzymes and small molecules that find applications in food, feed, pharmaceutical and cosmetic industries. Optimization of industrial conditions requires understanding of the pellet formation process in liquid shaken culture. In this study, cell wall proteins called hydrophobins are studied regarding their role in pellet formation. Single knockouts were made of each of the eight hydrophobins of *Aspergillus niger* as well a triple knockout. Genes were removed using pairs of cas9 plasmids that were designed with sgRNA that targets regions upstream and downstream of the gene to be deleted. The knockout strains were assessed on solid and liquid shaken cultures for growth, spore dispersal and pellet formation. No major differences were found between the wildtype and the deletion strains for all properties. Functional redundancy is regarded as the most likely explanation for this outcome. Future studies should focus on creating a strain with a knockout of all eight hydrophobin genes to assess their function.

1 General introduction

In ecosystems, fungi are regarded as important decomposers of organic materials. They return nutrients back to the soil to be recycled for new life. Unfortunately, the ability to degrade organic materials often manifests itself too early as fungi are widely regarded as prominent spoilers of food and as invasive pathogenic agents. It is less known that fungal interactions also lead to beneficial forms of symbiosis. For example, mycorrhiza have been an integral part of worldwide ecosystems for hundred millions years. Additionally, fungi are essential in brewing, food preparation, medicine and other industries. Fungi also intrigue scientists in their biological adaptations and inspire artists in design and philosophy.

The genus Aspergillus contains 492 species that display large genetic diversity (Yu et al., 2021). Well-known species in this genus are the model organism *Aspergillus nidulans,* the food producer *Aspergillus oryzae*, the human pathogen *Aspergillus fumigatus* and the cell factory *Aspergillus niger* (Cairns et al., 2018). Spores of this genus disperse via air currents and germinate into mycelia on a solid or liquid substrate (Bennet et al., 2010). Aspergilli are very adept at consuming starches, (hemi-)celluloses, pectins and other sugar polymers (Bennet et al., 2010). Aspergilli also provide food for many soil organisms while secreting acids and enzymes for digestion. *Aspergillus niger* is typified by its characteristic black spores that bud from conidiophores. This fungus has been found all over the world and can be isolated from locations like air, soil, agricultural products like plants, foods and textiles (Yu et al, 2021). Beside their ubiquitous spores, the success of *A. niger* in both nature and industry is attributed to their thermo- and acid tolerance. The fungus can grow in a temperature range of 6-47°C and a pH-range of 1.5 to 9.8 (Lima et al., 2019).

As a food contaminant, *A. niger* can be found on fruits, vegetables, grains and dairy products (Hayden & Maude, 2007; Gougouli & Koutsoumanis, 2017; Garnier et al., 2017). Sometimes the fungus is referred to as ‘black mold’ or ‘black rot’ for the powdery black spores that it leaves behind. Spoilage of food by *A. niger* is a problem for producers as well as consumers as it can negatively affect color, odor, taste and nutritional value of the product (Pitt & Hocking, 2009). Additional care should be taken *as A. niger* is also a producer of mycotoxins like fumonisin and ochratoxin (Nielsen et al., 2009). A. niger rarely causes adverse health effects in comparison to other species from its genus. The United States Food and Drugs Administration (FDA) gave the products of this fungus the status of GRAS (Generally Regarded As Safe) and *A. niger* has been used for decades in the food and beverage industry. In addition, the lowest biosafety level in laboratory environments apply for *A. niger.*

After food scientist James Currie showed that A. niger was a very efficient producer of citric acid in sugar media, Phizer began fermentation of citric acid on an industrial scale and production quickly outweighed extraction from citrus fruits (Cairs et al., 2018). Nowadays, industrial fermentation of citric acid is a multibillion-dollar industry (Pau et al., 2015). It is a very common ingredient in food, pharmaceuticals and cosmetics. Besides citric acid, *A. niger* also produces other organic acids like gluconic acid, malic acid and itaconic acid (Cairns et al., 2021). After the success of citric acid, researchers discovered and optimized other metabolites from *A.niger* with glucoamylase being one of the most important enzymes (Cairns et al., 2018). This enzyme catalyzes hydrolysis of starch to yield soluble sugars. It is therefore used in the food and beverage industries but also in the fermentation of bioethanol (Jòzef, 2007) (Kumar & Satyanarayana, 2009). Other important proteins produced by *A. niger* are enzymes like mannanases, lipases, peptidases, asparaginases and pectinases (Petersen et al., 2006; van den Brink et al.,2006; Mhetras et al., 2009; Ahmed et al., 2016.)

Researching *A. niger* and its growth and development can give us insight in how to control and optimize industrial conditions as well as find new possibilities of applications. In this research, cell wall proteins called hydrophobins will be studied to find out the effect of their deletion on the morphology of the fungus.

2 Growth and development of A. niger

2.1 Life cycle and development

A. niger has an asexual reproduction cycle in which conidiophores produce haploid spores called conidia. These conidia disperse and when they germinate, hyphae are formed that grow by apical extension. The hyphae branch to form a vegetative network of mycelium that in turn form conidiophores, hereby closing the asexual life cycle of the fungus. The conidiophores form specialized thick hyphae. These so-called stalks swell at their tip to form a vesicle. Metulae and phialides bud from the surface of this vesicle and the phialides undergo mitotic division to produce a string of conidia (Mims et al., 1988; Adams et al., 1998). When conidia mature, they separate from the conidiophores and disperse into the environment. When exposed to favorable conditions, the conidia break dormancy and start to germinate. During germination there is a period of spherical growth. In this swelling period, the spores take up water and increase metabolic activity. Then, germ tubes emerge that extend and branch in an apically polarized manner. Colonies vary in diameter between (sub-)millimeter (micro-colonies) to centimeter (macro-colonies) scale depending on the substrate (Krijgsheld et al., 2013). Hyphae extend at the edge of the colony to colonize new substrate. This exploring region of the mycelium is composed of the youngest hyphae. At the hyphal tips, enzymes like glucoamylase are secreted that digest polymers into smaller products (Wösten et al., 1991; Vinck et al., 2005). Just behind the exploring zone, hyphae branch thoroughly and thereby fill up the substrate increasing the surface area for nutrient uptake. In the substrate surrounding the centre of the colony, nutrients have depleted. Sporulation is often initiated in this region. It should be noted that differentiation also occurs within zones of the colony and even within a single hypha (Wösten et al., 2013; Tegelaar et al., 2020ab).

2.2 Pellet aggregation

A. niger is grown bioreactors in the industry, enabling control of culture conditions like temperature and oxygen transfer. In such bioreactors, *A. niger* forms pellets or can grow dispersed (Papagianni, 2004). These morphologies develop due to aggregation of spores and germlings or the absence of these processes, respectively. Grimm et al. (2004) showed that medium composition, mechanical forces, strain characteristics and inoculum impact aggregation. Dispersed mycelium is considered more productive when compared to pelleted morphology but the latter exhibits lower viscosity reducing problems in oxygen transfer (Riley et al., 2000). Grimm et al. (2004) distinguish a two-step aggregation process in *A. niger.* In the first step, resting conidia aggregate, which is affected by the pH of the medium (Grimm et al., 2005). This is followed by aggregation of germlings. This step depends on the pH of the medium as well as on fluid dynamic conditions like agitation and aeration. These aggregation processes result in the formation of pellets. These micro-colonies also disintegrate due to separation forces like shear stress (Grimm et al., 2004; Tegelaar et al., 2000c).

2.3 Cell wall composition

Saprophytic fungi like *A. niger* grow under varying environmental conditions like osmolality, temperature and pH. The cell wall is often the first to be exposed to these stressors and therefore has evolved adaptations in structure, protection and interaction with its surroundings. The cell wall of hyphae is very similar to the cell wall of conidia. Both consist of cell wall proteins and polysaccharides like chitin. However, the outer cell wall of the hypha is composed of galactosaminogalactan, while layers of melanin and rodlets make up this layer in the case of spores. In *A. fumigatus*, the melanin and rodlet layers are involved in hiding cell wall compounds to prevent immunological reactions in the host. During conidia swelling, the cell wall changes. The melanin and rodlet layer disappear and polysaccharide chains as well as other cell wall proteins become exposed. Galactosaminogalactan in Aspergillus fumigatus plays a role in fungal growth and has anti-inflammatory properties to prevent host immune reaction (Gresnigt et al, 2014) (Robinet et al, 2014).

2.4 Hydrophobins

Hydrophobins are small proteins secreted by filamentous fungi. They are for instance involved in lowering surface tension, facilitating escape of hyphae into the air, spore dispersal and attachment to a host (Wösten, 2001). Hydrophobins have low amino acid homology but are characterized by the presence of eight cysteine residues that form four disulphide bonds (Wösten et al., 1999). Hydrophobins are divided in class I and class II based on hydropathy patterns and physical properties. However, for the hydrophobins of Aspergilli this division cannot clearly be made as the majority are considered an

intermediate form (Jensen et al., 2010). Both class I and class II hydrophobins have been found among Aspergilli and both types have even been found within the single species *Aspergillus terreus* (Jensen et al., 2010). Hydrophobins self-assemble at the interface between hydrophobic and hydrophilic surfaces into an amphipathic membrane, often called the rodlet layer in conidiospores (Wösten, 2001). The disulphide bridges keep monomers soluble in water and prevent precocious self-assembly. During self-assembly, the hydrophobins undergo conformational changes resulting in increased β-sheets content and resembling amyloid fibrils. In *A. nidulans*, the influence of the hydrophobins DewA and RodA on the aggregation of submerged conidiospores showed that absence of either of these hydrophobins decreased the amount of biomass of pellets, increased the average size of the pellets and decreased the pH values (Dynesen & Nielsen, 2003). In *A. fumigatus*, RodA is one of the seven hydrophobins (RodA-RodG) and deletion of the corresponding gene led to the absence of the rodlet layer as well as lower sporulation and lower hydrophobicity (Valsecchi et al., 2017).

2.5 The knowledge gap

Surface properties of conidia are a predominant factor in the physical behavior of the spores in submerged culture, especially in the first aggregation step (Grimm et al., 2005). Adhesion of spores and/or hyphae results in a three-dimensional structure known as pellets. Glycoproteins, hydrophobins, carbohydrates and lipids have already been identified as important molecular surface properties (Cole et al., 1979). Notably, Gerin et al. (1993) attribute spore aggregation to salt bridging between polysaccharides and not to hydrophobicity or electrical properties of spores. Ryoo and Choi (1999) reported that pellet formation in A. niger is influenced by an interaction between the cell wall surface and the medium. Important influences were electrostatic effects like pH-value. The surface charge of most microbial cells in submerged culture depends on the ionization of surface-associated basic and/or acidic functional groups (Wargenau et al., 2013). Aggregation is easier when the surface potential is around zero. More positive or negative groups result in repulsion. At lower pH, the positive contribution from basic groups is more present. At higher pH, the negative charge from acidic groups is more present (Wargenau et al., 2013). According to Dynesen & Nielsen (2003), both electrical charge and hydrophobicity effect pellet formation. In this study, the goal is to better understand the role of hydrophobins in pellet aggregation in *A. niger*. This goal will be pursued by performing a knockout experiment in *A. niger*.

3 Materials and methods

3.1 Strain and culture conditions

Escherichia coli NEB10 and its derivatives were cultured at 37 °C on lysogeny broth agar (LB) either or not containing 1.5% agar. Ampicillin (100 mg/L) was used as a selection marker. Hydrophobin deletions were made in strain *A. niger* MA612.27, which is a derivative of strain N400 (a.k.a CBS120.49) that contains a *kusA* deletion. *A. niger* was incubated in a box in an incubator at 30 °C. Solid culture medium consisted of potato dextrose agarose (39 g/L). Hygromycin B (1,5 ml/L) was added as a selection marker. For harvesting, conidia produced on a Petri dish were taken up in 5 mL saline tween solution (0,8% NaCl and 0,005% Tween-80) and filtered through a cottonwool syringe to remove mycelium. Spores were counted with a Bürker Türk Counting Chamber. Spores were stored at 4 °C for a maximum of 1 month.

3.2 Construction of plasmids

Plasmid strain pMT12.5 includes tracrRNA under control of an *A. niger* proline tRNA promotor and terminator, a cas9 protein, GFP under control of a pseudomonas sp. merR operon promotor and an ampicillin and a hygromycinB resistance cassette. The coding sequence of GFP in pMT12.5 can be replaced by a sgRNA using BtsI. GFP thereby can be used to screen for transformants that have received a plasmid with an sgRNA. The pMT12.5 plasmid was cloned with an sgRNA sequence using golden gate and the restriction enzyme.

3.2.1 Designing sgRNA

The amino acid sequence of each hydrophobin gene in *A. niger* was retrieved from aspgd.org and FungiDB.org. Sequence were blasted on mycocosm.jgi.doe.gov against the genome of *A. niger* strain NRRL3 which is parental to N400. The best match of the blast was chosen and its nucleotide sequence, along with the sequence upstream and downstream, was visualized using the program ApE (v.3.0.9). The website chopchop.cbu.uib.no returned the best cutting sites when provided with the upstream and downstream sequence of the gene. Primers were chosen based on GC-percentage (between 40-60%), a lack of multiple complementarities and the shortest distance to the hydrophobin gene. The PAM site was replaced by two overhanging nucleotides that function as sticky ends. The primer for the forward strand ends with GT and the reverse primers ends with GA. (see supplementary Table 2 for the nucleotide sequences). The sgRNA pairs were annealed with a RAMP program in a thermal cycler. The oligos (5 µM in 100 µL) were denatured at 95 °C for 3 min, then slowly cooled to room temperature (rt) for 30 min with a speed of 0,03-0,04 °C/s. Annealed oligos were stored at -20 °C.

3.2.2 Assembling plasmid

Plasmid pMT12.5 was isolated from *Escherichia coli* using a DNA isolation kit (Machery-Nagel). Success of extraction was confirmed by agarose gel electrophoresis showing a band of approximately 16 kb. By combining BtsI-v2 (NEB), linearized pMT12.5 and the annealed single guide RNA's, 16 different recombinant DNA molecules were formed. The fragments were introduced in pMT12.5 with Golden Gate assembly. The sticky ends of the annealed sgRNA's are compatible with this restriction site. Additionally, ATP (10 µM), rCutsmart buffer (NEB) and T4 ligase (NEB) were added, and the solution was put in a thermal cycler for 30 repetitions at 37 °C of 5 min, followed by 16 °C for 5 min and then 37 °C for 5 min.

3.2.3 Competent cells

E. coli strain NEB10 was grown in a 1000 mL flask containing 250 mL SOB (5 g/L yeast extract, 20 g/L tryptone, 0.584 g NaCl, 0.186 g KCl, 2.4 g MgSO4). The culture was propagated at rt at 250 rpm until an optical density of 0.3. The solution was centrifuged (3000 rpm, 4 °C) for 10 min. The supernatant was discarded and the remaining cells were resuspended in 300 µL CCMB80 buffer (10 mM KOAc pH 7.0, 80 mM $CaCl_2.2H_2O$, 20 mM $MnCl_2.4H_2O$, 10 mM $MgCl_2.6H_2O$, 10% glycerol, pH6.4). This buffer was added to 80 mL and the solution was incubated for 20 min on ice. This step was repeated but the cells were then resuspended in 10 mL buffer. 50 µL of suspension was then mixed with 200 µL of SOC for measuring of OD. Buffer was added until an OD of 1.0-1.5 after which the competent cells were snap frozen in liquid nitrogen in aliquots of 50 µL in 1.5 mL tubes and stored at -80 °C.

3.2.4 Transformation of NEB10

For transformation, 3 µL pDNA was added to thawed competent cells. After incubation on ice for 30 min, cells were heat shocked by putting the suspension in a water bath of 42 °C for 30 s and then returned to ice for 5 min. 900 µL SOC was added and the cells were regenerated at 37 °C, 200 rpm for 60 min. 100 µL was inoculated onto prewarmed LB+Amp plates overnight. Only successful transformants should grow on the selection plates. However, it is possible not all plasmids were properly assembled during golden gate if a competent cell took up a backbone plasmid. Therefore, fluorescence microscopy was used to distinguish successful transformants without fluorescence from colonies with fluorescence and therefore backbone plasmids. To this end, a flueorescence Leica MZ16 stereomicroscope was used with a Leica GFP2 filter and a mercury lamp. Colonies were selected and the plasmid DNA was extracted with a Midiprep using a DNA isolation kit (Machery-Nagel). The extracted pDNA was sent for sequencing to Macrogen Europe, Amsterdam, The Netherlands.

3.3 Construction of *A. niger* knockout strains

3.3.1 Protoplasting

For protoplasting, 100 mL CM (MM with 0.5% yeast extract and 0.2% enzymatically hydrolysed casein) with 1% glucose was inoculated with 10^7 N400 spores in a 500 mL Erlenmeyer. After 16 h incubation, the spores were harvested using a Buchner funnel and filter paper. One gram filtered mycelium was mixed with 400 mg *Trichoderma harzianum* enzymes (received from Dr. L.G. Lugones), 50 mg chitinase, 50 mg BSA and 15 mL NMC (0,7 M NaCl, 0,2 M $CaCl_2$, 10 mM MES, pH 5,6). Cells were incubated at 30 °C, 200 rpm until 10-50 protoplasts were visible at 400x magnification using a Bürker Türk counting chamber (approximately 4h). Then 15 mL STC (1,33 Sorbitol, 10 mM Tris-Cl, 50 mM $CaCl_2$) was mixed and the solution was filtered through a cottonwool syringe to remove mycelial hyphae. After washing with STC the protoplasts were counted and diluted to a concentration of 10^7 protoplasts /ml. Aliquots of 600 µL were snap frozen and stored at -80 °C.

3.3.2 Transformation of N400

The transformed cells were cultured on special transformation medium composed of two layers. The bottom layer (MMS) was composed of minimal medium (MM), sucrose (0,95 M), and agar (1,2%). The top layer was composed of MM (de Vries et al, 2004), sucrose (0,95%) and agar (0,6%). Hygromycin was added to both layers. One square plate had approximately 30 mL MMS and 15mL MMST with the DNA solution on top. Repair sequences were added as a *kusA* deletion strain of N400 was used. These repair sequences provide a template for homologous recombination and were therefore complimentary to the 45 base pairs before the upstream cut and 45 base pairs after the downstream cut (see supplementary Table 3 for the sequences). The protoplasts (200 µL) were mixed with 1 µL upstream cutting plasmid, 1 µL downstream cutting plasmid, 10 µL repair template and 10 µL STC. 1 mL PEG6000 (25% PEG6000, 50 mM $CaCl_2$, 10 mM Tris-Cl, pH 7.5) was added drop by drop, followed by incubation for 5 min at rt. Finally, 2 mL STC and the MMST with hygromycin were added before pouring the solution onto the MMS plates. As a control, repair DNA was omitted. The transformation plates were incubated for 3 or 4 days before colonies became visible.

3.3.3 Candidate confirmation

Transformants were transferred to a new selection plate followed by two purification rounds in the absence of antibiotic. To extract the genomic DNA, the spores were cultured in CM with 1% glucose overnight. After centrifugation and removal of supernatant, the mycelium was turned to powder by adding magnetic beads, dropping the tubes in liquid nitrogen, and using a Tissuelyzer. For further breakdown, 500 µL lysing solution (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl and 1% sodium dodecyl sulfate) was added and the mixture was incubated for 10 min at rt. 150 µL potassium acetate buffer (5 M potassium acetate, 11,5 mL glacial acetic acid) was added and after two times transfer of supernatant, the DNA was precipitated by using 300 µL 70% ethanol. The solution was centrifuged and dried at 60 °C. DNA was resuspended in 50 µL milliQ and stored at 4 °C. PCR was performed on the extract to confirm deletion of the hydrophobin gene(s). Primers were chosen using as selection criteria: GC% in between 40% and 60%, annealing temperature between the two primers did not differ more than 5 °C, there were little multiple complementarities, and the sequence was close to the coding sequence of the gene. For PCR, Taq One polymerase, Taq One buffer and dNTP's were used alongside the template and the primers (see supplementary Table 4 for the sequences and the details regarding annealing temperature and the band length of a successful deletion). Annealing temperature was calculated using https://tmcalculator.neb.com/#!/main.

3.4 Phenotyping

3.4.1 Solid culture

Mycelium growth of the knockout strains and sporulation after 3 and 5 days was assessed on solid medium in triplo. Petri dishes had a diameter of 9 cm and were filled with 10 mL medium. To assess colony growth, plates were inoculated in the middle of the plate with 2 10^7 spores. Pictures of the Petri dishes were taken for three consecutive days and taken from a set height and on a dark background. The area of mycelium growth was analyzed using the program ImageJ (1.53k). To assess sporulation, plates were inoculated by spreading the spores over the whole plate with a spreader. Spores were harvested after 3 and after 5 days. Concentration of spores was determined by constructing a trendline of absorbance values from a spectrophotometer and this was related to a dilution series of which the spore number was counted.

3.4.2 Liquid culture

In liquid shaking cultures, pellets were compared for morphology, area and biomass. Moreover, pH-value and protein composition of the culture medium were determined. 10^7 Spores of the wildtype as well as the knockout strains were inoculated in 250 mL Erlenmeyer flasks. These flasks were filled with 50 mL transformation medium (MM with yeast extract (5 g/L), casamino acids (2 g/L), and xylose (25 mM)). After incubation at 30 °C and 200 rpm for 24 h the pellets were transferred to 50 mL MM in 250 mL Erlenmeyer flasks and incubated for another 24 h at the same settings. Samples with pellets were taken from each flask. Pictures were taken from a set height on a dark background. These images were also used for calculation of pellet area using ImageJ (1.53k). For segmentation, the image was transformed to 8-bit greyscale and separated by intensity values. After testing which values only segmentate the desired pellets, an intensity threshold of 175-255 was applied to all images. The parameters used when performing particle analysis are a minimum area size of 0.01 cm^2 to infinity and a circularity of 0.30 to 1.00. The mean area of each pellet and strain was calculated and then compared to the wildtype. Afterwards the pellets were harvested on filter paper, dried overnight, and weighed to measure biomass. Beforehand, the filter papers were dried at 60 °C overnight and then weighed so their biomass could be subtracted.

4 Results

Plate morphology can be seen in Figure 1. No obvious difference in growth of the mycelium can be seen for any of the knockout colonies in comparison to the wildtype strain. The stains seem to grow and develop normally with no delay or complications at day 1, 2 or 3. After the third day, spores started to disperse over the plate and accurate comparison was not feasible. Table 2 shows the area of the mycelium for all strains. The area was calculated based on the pictures from Table 1. For day 1, the knockout strains deviated between -0,010 cm^2 to +0,340 cm^2 from the wildtype. For day 2, the knockout strains deviated between -0,170 cm^2 to +1,520 cm^2 from the wildtype while these numbers were -0,930 cm^2 and +2,710 cm^2 at day 3. The triple knockout strain showed the largest deviation.

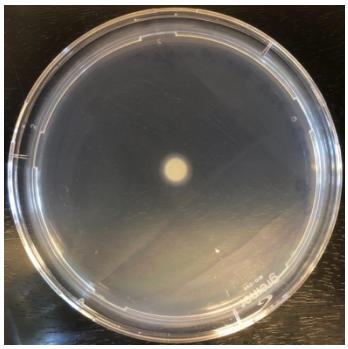
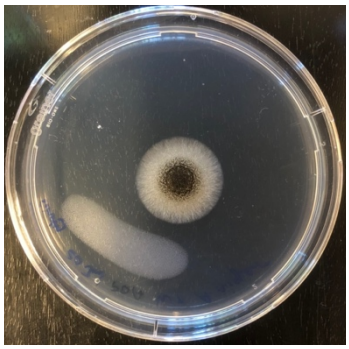
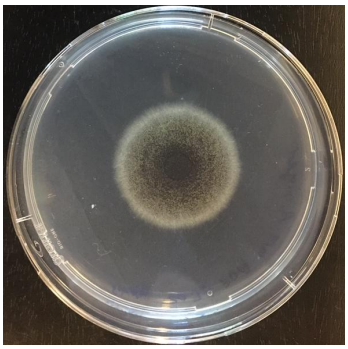
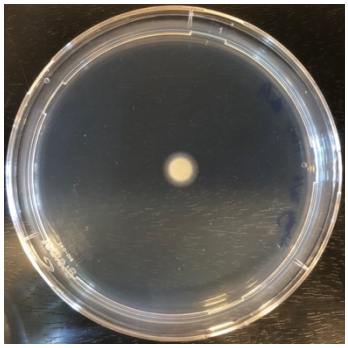
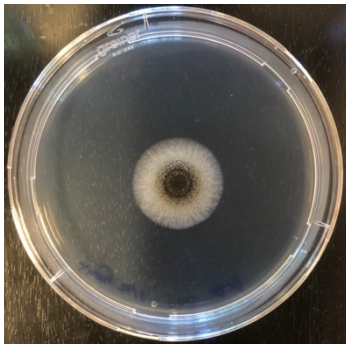
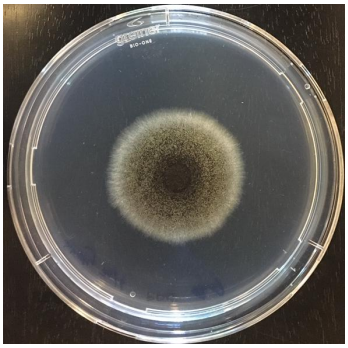
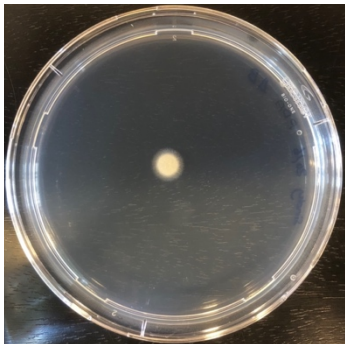
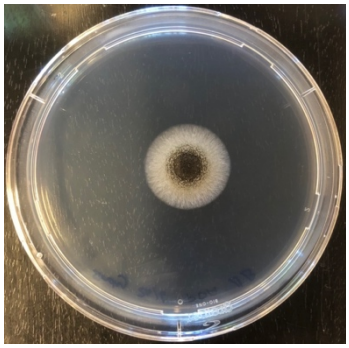
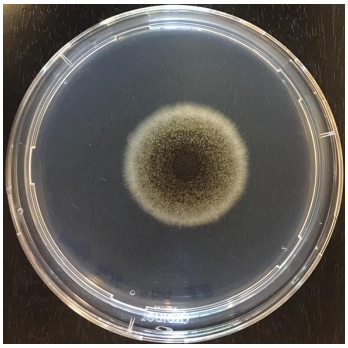
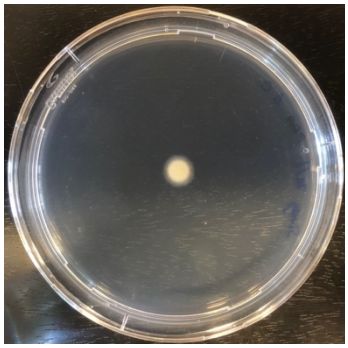
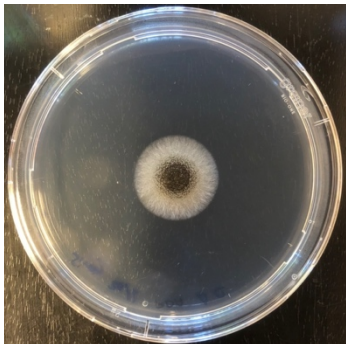
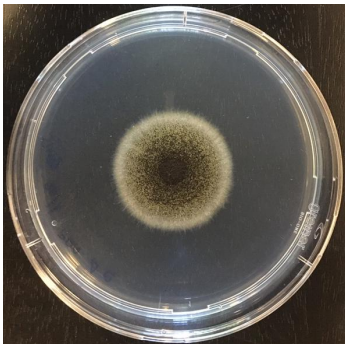
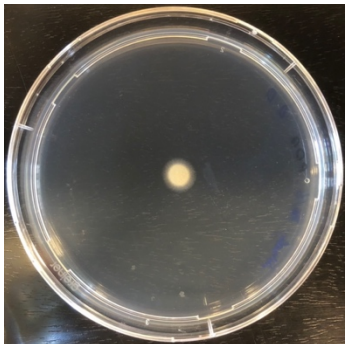
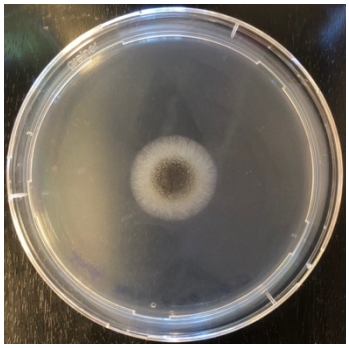
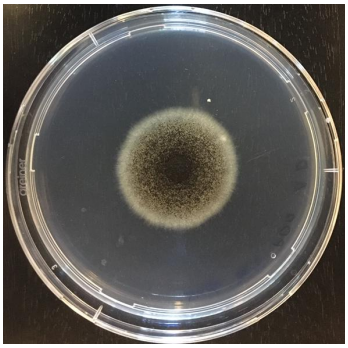
Table 1 Area of Aspergillus niger 3-day-old mycelium on plate.

Strain	Day 1 (cm2)	Deviation from WT	Day 2 (cm2)	Deviation from WT	Day 3 (cm2)	Deviation from WT
Wildtype	0,610	n.a.	4,120	n.a.	9,360	n.a.
ΔHypA	0,810	0,200	4,960	0,840	10,440	1,080
ΔHypB	0,650	0,040	4,310	0,190	9,320	-0,040
ΔHypC	0,750	0,140	4,540	0,420	9,000	-0,360
ΔHypD	0,720	0,110	4,420	0,300	9,050	-0,310
ΔHypE	0,630	0,020	4,280	0,160	9,660	0,300
ΔHypF	0,730	0,120	3,950	-0,170	8,430	-0,930
ΔHypG	0,600	-0,010	4,440	0,320	9,000	-0,360
ΔHypH	0,750	0,140	4,310	0,190	9,060	-0,300
ΔHyp Triple	0,950	0,340	5,640	1,520	12,070	2,710

All strains had formed about 10^7 spores at day 3 and 5. For day 3, the knockout strains deviate between -4,74 10^4 to +9,35 10^6 from the wildtype. For day 5, the knockout strains deviate between -6,63 10^5 to +8,48 10^6 from the wildtype. ΔHypD had the largest negative deviation in spores in comparison to the other strains.

Table 3 Spore count on Day 3 and 5. For details, see supplementary Table 6.

Strain	Mean spores Day 3	Deviation from WT	Mean spores Day 5	Deviation from WT
WT	1,17E+07	n.a.	2,15E+07	n.a.
ΔHypA	1,34E+07	1,70E+06	2,18E+07	3,31E+05
ΔHypB	1,51E+07	3,31E+06	2,57E+07	4,21E+06
ΔHypC	1,38E+07	2,04E+06	2,04E+07	-1,09E+06
ΔHypD	1,17E+07	-4,74E+04	2,08E+07	-6,63E+05
ΔHypE	1,61E+07	4,36E+06	3,00E+07	8,48E+06
ΔHypF	1,25E+07	8,05E+05	2,50E+07	3,55E+06
ΔHypG	1,42E+07	2,46E+06	2,41E+07	2,60E+06
ΔHypH	1,33E+07	1,56E+06	2,36E+07	2,08E+06
ΔHyp Triple	2,11E+07	9,35E+06	2,49E+07	3,43E+06

Strain	Day 1	Day 2	Day 3
WT			
ΔHypA			
ΔHypB			
ΔHypC			
ΔHypD			

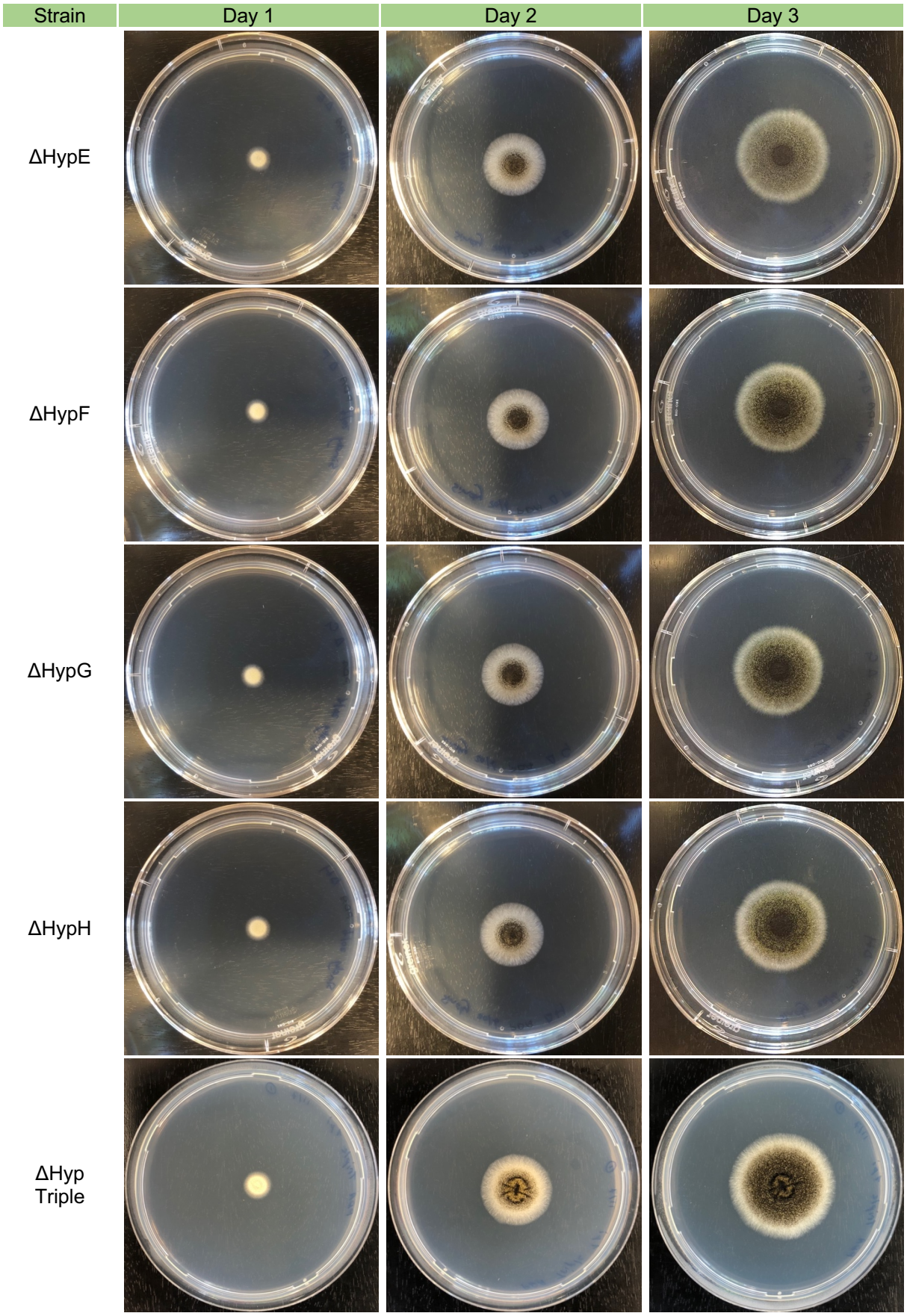


Figure 1 Morphology of A. niger wildtype and knockout strains. First three days after inoculation at the center of the plate.

In Figure 2, pellets can be seen which are taken from the flasks from the liquid shaken cultures. Morphologically, the different strains had quite similar pellets. ΔHypF showed more dispersed pellet formation in one sample. However, this phenotype could not be reproduced by re-culturing. The area of the pellets (Table 5) was calculated based on the pictures from Table 4. The knockout strains deviate between -0,022 to +0,027 cm^2 from the wildtype.

Table 5 Area of pellets of Aspergillus niger wildtype and knockout strains from liquid shaken cultures. For details, see supplementary Table 7.

Strain	Mean Area cm^2	Deviation from WT
Wildtype	0,112	n.a.
ΔHypA	0,131	0,019
ΔHypB	0,127	0,015
ΔHypC	0,108	-0,004
ΔHypD	0,130	0,019
ΔHypE	0,139	0,027
ΔHypF	0,117	0,005
ΔHypG	0,117	0,005
ΔHypH	0,114	0,002
ΔHyp Triple	0,090	-0,022

Biomass of the pellets of the knockout strains deviated between -35 mg and +5 mg from the wildtype which had a biomass of 71 mg in 50 mL (Table 6). The pH-value of the culture medium of the wildtype was 2.8 (Table 7). The knockout strains deviated between -0,06 and +0,23 from the wildtype.

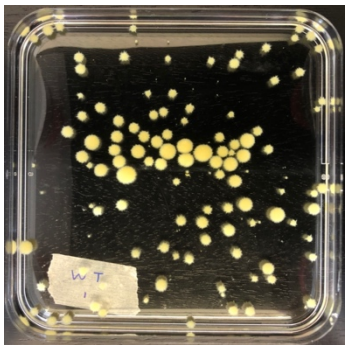
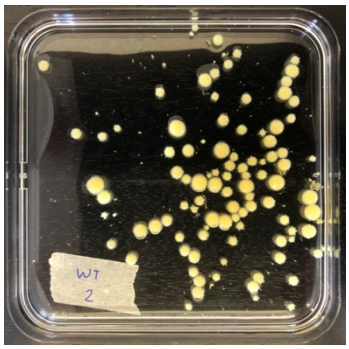
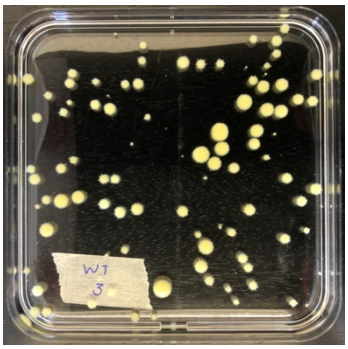
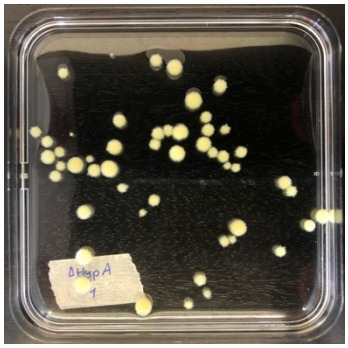
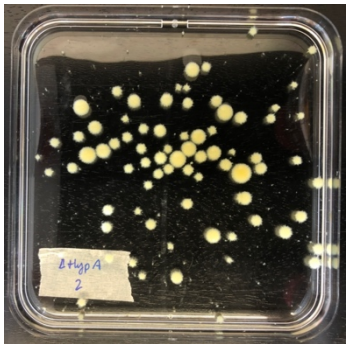
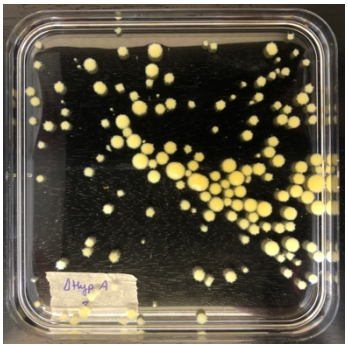
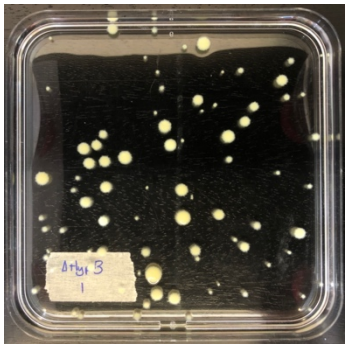
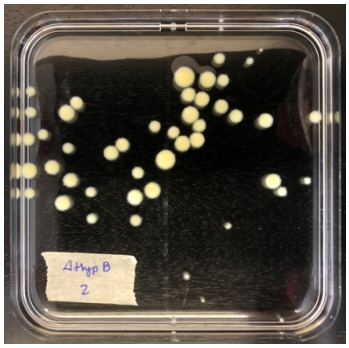
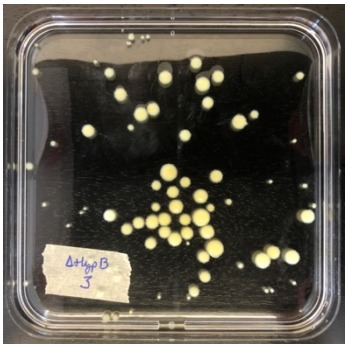
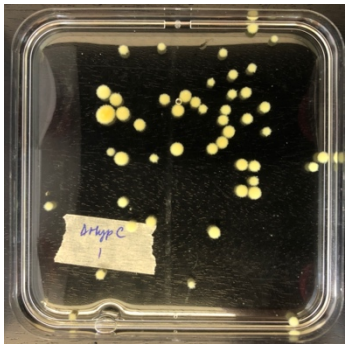
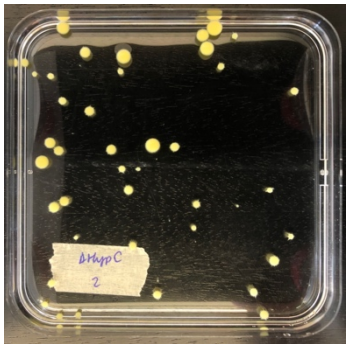
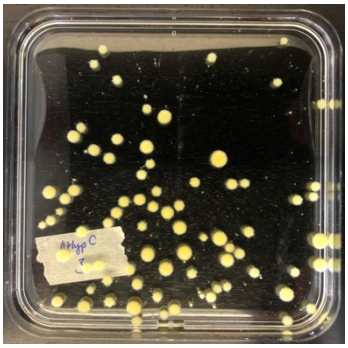
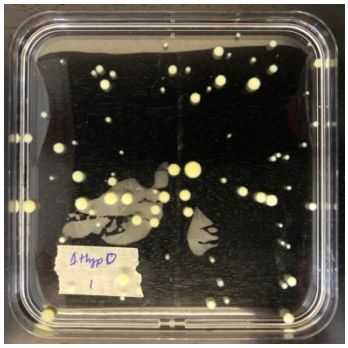
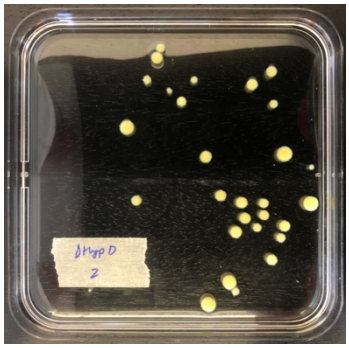
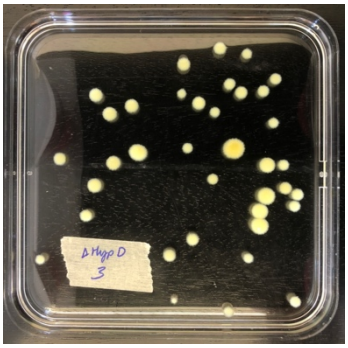
Table 6 Biomass of pellets of Aspergillus niger wildtype and knockout strains. For details, see supplementary Table 8.

Strain	Mean Biomass (mg)	Deviation from WT (mg)
Wildtype	71	n.a.
ΔHypA	73	2
ΔHypB	60	-11
ΔHypC	49	-22
ΔHypD	50	-21
ΔHypE	36	-35
ΔHypF	63	-8
ΔHypG	55	-16
ΔHypH	76	5
ΔHyp Triple	61	-10

Table 7 Pellet supernatant pH-value of wildtype and hydrophobin knockout strains of A. niger. For details, see supplementary Table 9.

Strain	Mean pH-value	Deviance from WT
Wildtype	2,80	n.a.
ΔHypA	2,74	-0,06
ΔHypB	2,81	0,00

ΔHypC	2,98	0,18
ΔHypD	2,92	0,12
ΔHypE	3,04	0,23
ΔHypF	2,92	0,12
ΔHypG	2,96	0,16
ΔHypH	2,95	0,15
ΔHyp Triple	3,02	0,22

Strain	Sample 1	Sample 2	Sample 3
Wildtype			
ΔHypA			
ΔHypB			
ΔHypC			
ΔHypD			

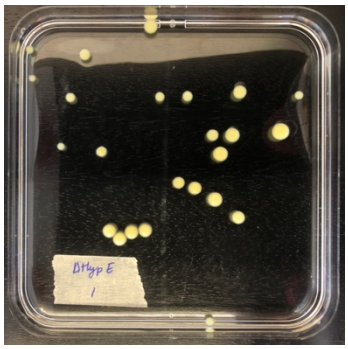
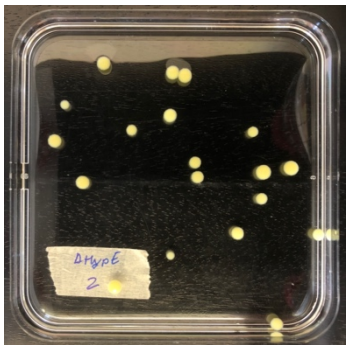
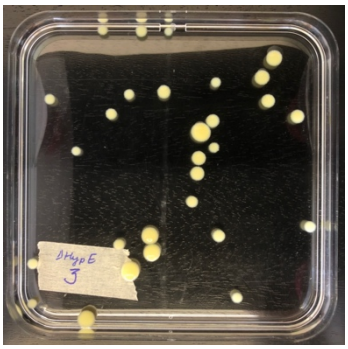
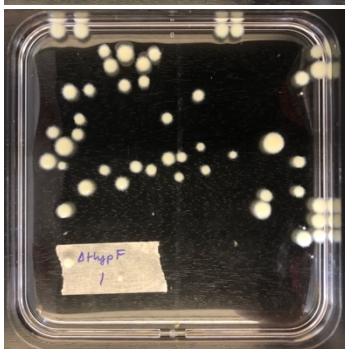
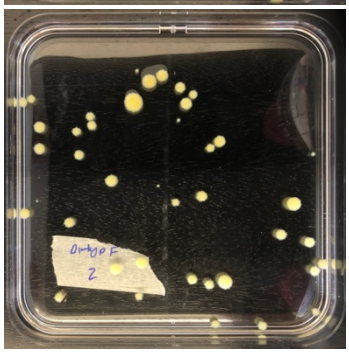
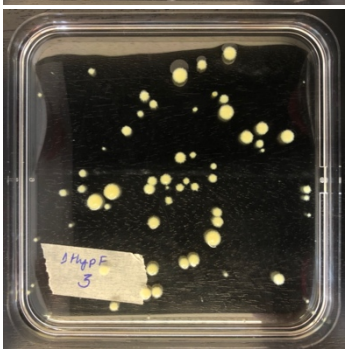
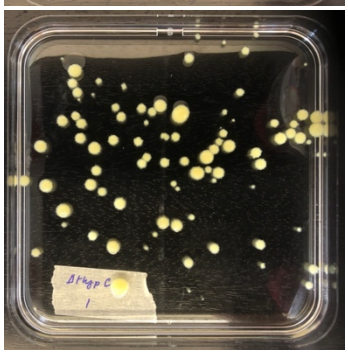
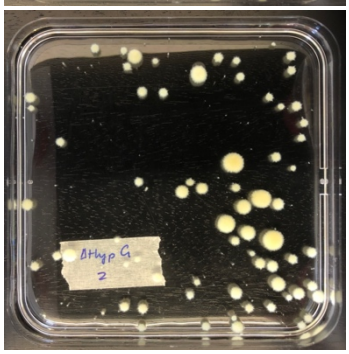
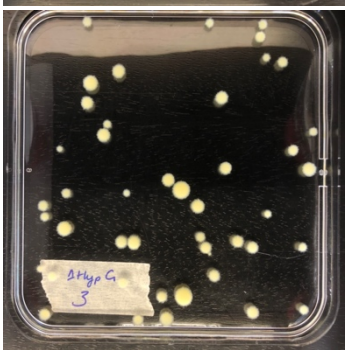
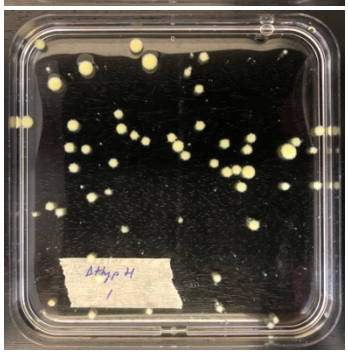
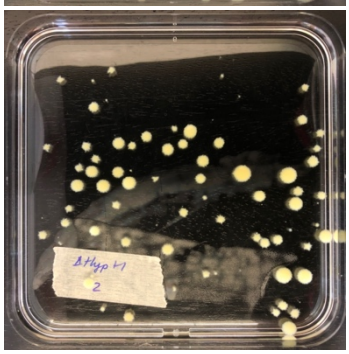
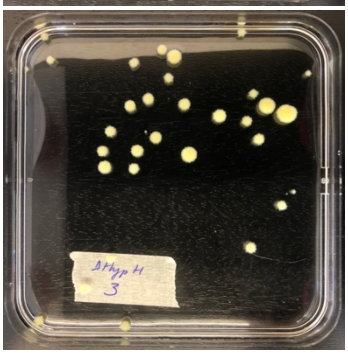
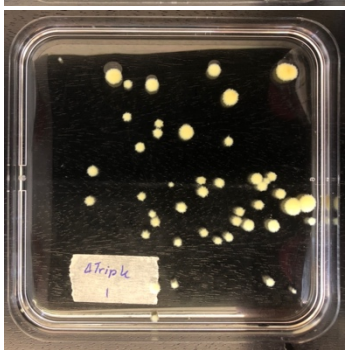
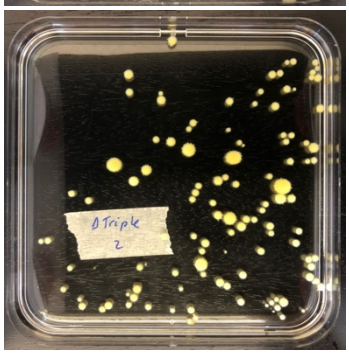
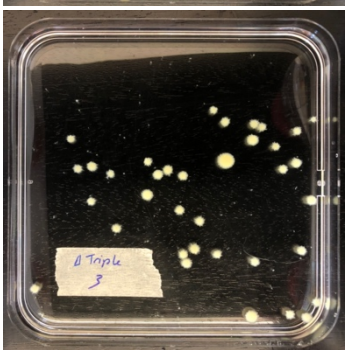
Strain	Sample 1	Sample 2	Sample 3
ΔHypE			
ΔHypF			
ΔHypG			
ΔHypH			
ΔHyp Triple			

Figure 2 Pellets from Aspergillus niger wildtype and knockout strains from liquid shaken culture.

5 Discussion

This study attempted to better understand the role of hydrophobins in pellet formation of *A. niger.* In the introduction, the importance of cell wall proteins in this process was already elucidated. However, I showed that single hydrophobin A-H deletions did not affect growth and spore and pellet formation. Even more so, the triple knockout did not show any affect in growth, spore and pellet formation either. Together, data indicate that the role of hydrophobins in these processes is not yet clear.

An explanation for the absence of an effect on fungal morphology could be that the hydrophobin genes are functionally redundant. Functional redundancy is a good possibility as hydrophobins have shown to be essential in other fungi. As mentioned in the introduction, the absence of DewA and RodA in A. nidulans impacted biomass, pellet size and pH-values. This indicates that the hydrophobins of *A. niger* are different from DewA and RodA in *A. nidulans.* To test whether the hydrophobins of *A. niger* are functionally redundant, a multiple gene knockout was made. Deletion of multiple genes in one transformation still proves difficult and time consuming. Multiple attempts of doing an eight gene knockout in one transformation have failed. Possibly, the presence of at least one hydrophobin gene is essential for viability of *A. niger*. Hydrophobins are necessary for facilitating escape into the air. If there are no hydrophobins, then surface tension cannot be lowered and hyphae could not break through the substrate. However, this conclusion cannot be drawn as the construction of an 8-gene knockout failed. It could be that too many cutting plasmids and repairs were offered at the same time and that this reduced repair of the chromosomes. For a multiple gene knockout, only three genes (HypB, hypC and hypE) were able to be deleted in a single strain. The triple knockout was made using a ΔHypC strain and then deleting HypB and HypE in one transformation. This strain also did not show any obvious differences from the wildtype. This could mean that the other hydrophobin genes could make up for the absence of hydrophobin B, C and E. Future deletion of other combinations or all hydrophobin genes in *A. niger* could show a different phenotype. It would be interesting to find out whether each hydrophobin gene could replace one another or to know that deletion of all hydrophobin genes of HypA-H has no effect at all.

Hydrophobins are assumed to influence the first aggregation step in pellet formation. Besides hydrophobins, the melanin layer is also involved in pellet formation of *Aspergillus niger*. Inactivation of the *olvA* gene led to the disappearance of the rodlet layer as well as a change in zeta potential (Veluw et al., 2013). The melanin layer is involved in the first aggregation step and both the rodlet layer and melanin layer are removed during germination. The melanin surface coat is rich in acidic carboxyl groups that make the surface negatively charged. The gene ALB1 is essential in melanin biosynthesis (Priegnitsz et al., 2012). In a deletion study in *A. niger*, the ΔALB1 mutant had a more positive surface charge and depending on the pH of the surrounding medium, the strain with surface charge closest to 0 showed more aggregates after 3 hours (Priegnitsz et al., 2012). However, both wildtype and mutant had similar aggregates at later time points. This indicates that germ tube and hyphal adherence can compensate for deficiencies in the initial aggregation step (Priegnitz et al., 2012) and contradicts the two-step aggregation model (Priegnitz et al., 2012). In this study, phenotypes of the pellets of the knockout strains had similar aggregates as the wildtype. This supports the idea that the second aggregation step is most crucial for pellet formation. In Aspergillus fumigatus, cell wall α-1,3-glucans are involved in aggregation of germinating conidia as α-1-3-glucanase was found to prevent conidial aggregation (Fontaine et al., 2010). Here I propose that the impact of first aggregation step is negligible in pellet formation. Instead, the phenomenon of coagulation of ungerminated conidiophores depends on the α-1,3-glucans and the interaction between the pH-value of the medium and the surface charge from the cell wall components. Certain hydrophobins may influence the surface charge if they have acidic carboxyl groups for example. Functional analysis of hydrophobins would be more successful on solid media.

In conclusion, single deletion of the hydrophobin genes HypA-H have no obvious effect on normal growth, spore dispersal and pellet formation. Functional redundancy was tested by making a multiple gene knockout of HypB, HypC and HypE but this strain also did not show a different phenotype. Future studies should make a knockout of all hydrophobin genes or with other combinations to understand its role in *Aspergillus niger.*

6 Acknowledgements

In this year I have learned a lot and accomplished a lot too. This would not have been possible without the people that guided and supported me. Therefore, I would like to thank my teachers: Han Wösten, Jun Lyu and the Aspergillus group for their confidence and their patience. I would like to thank Paco Olmedo, Jurian Wijnheijmer, Wieke Teertstra and all other labpartners in the microbiology group that helped me in my work and brightened my day. Lastly, I would like to thank my friends and family for always supporting me in what I do.

7 Literature

Adams, T. H., Wieser, J. K., & Yu, J. H. (1998). Asexual sporulation in Aspergillus nidulans. *Microbiology and molecular biology reviews*, *62*(1), 35-54.

Ahmed, I., Zia, M. A., Hussain, M. A., Akram, Z., Naveed, M. T., & Nowrouzi, A. (2016). Bioprocessing of citrus waste peel for induced pectinase production by Aspergillus niger; its purification and characterization. *Journal of radiation research and applied sciences*, *9*(2), 148-154.

Beauvais, A., Fontaine, T., Aimanianda, V., & Latgé, J. P. (2014). Aspergillus cell wall and biofilm. Mycopathologia, 178(5), 371-377.

Bennet, J.W. (2010) *Aspergillus: Molecular Biology and Genomics.* Poole, UK: Caister Academic Press.

Cairns, T. C., Barthel, L., & Meyer, V. (2021). Something old, something new: challenges and developments in Aspergillus niger biotechnology. *Essays in Biochemistry*, *65*(2), 213-224.

Cairns, T. C., Nai, C., & Meyer, V. (2018). How a fungus shapes biotechnology: 100 years of Aspergillus niger research. *Fungal biology and biotechnology*, *5*(1), 1-14.

Cole, G. T., Sekiya, T., Kasai, R., Yokoyama, T., & Nozawa, Y. (1979). Surface ultrastructure and chemical composition of the cell walls of conidial fungi. *Experimental Mycology*, *3*(2), 132-156.

Currie, J. N. (1917). The citric acid fermentation of Aspergillus niger. *J Biol Chem*, *31*(1), 15-37.

Dynesen, J., & Nielsen, J. (2003). Surface hydrophobicity of Aspergillus nidulans conidiospores and its role in pellet formation. *Biotechnology progress*, *19*(3), 1049-1052.

Ellena, V., Sauer, M., & Steiger, M. G. (2020). The fungal sexual revolution continues: discovery of sexual development in members of the genus Aspergillus and its consequences. *Fungal Biology and Biotechnology*, *7*(1), 1-7.

Fontaine, T., Beauvais, A., Loussert, C., Thevenard, B., Fulgsang, C. C., Ohno, N., ... & Latgé, J. P. (2010). Cell wall α1-3glucans induce the aggregation of germinating conidia of Aspergillus fumigatus. *Fungal Genetics and Biology*, *47*(8), 707-712.

Garcia-Rubio, R., de Oliveira, H. C., Rivera, J., & Trevijano-Contador, N. (2020). The fungal cell wall: Candida, Cryptococcus, and Aspergillus species. Frontiers in microbiology, 10, 2993.

Garnier, L., Valence, F., & Mounier, J. (2017). Diversity and control of spoilage fungi in dairy products: An update. *Microorganisms*, *5*(3), 42.

Gerin, P. A., Dufrêne, Y., Bellon-Fontaine, M. N., Asther, M., & Rouxhet, P. G. (1993). Surface properties of the conidiospores of Phanerochaete chrysosporium and their relevance to pellet formation. *Journal of bacteriology*, *175*(16), 5135-5144.

Gougouli, M., & Koutsoumanis, K. P. (2017). Risk assessment of fungal spoilage: A case study of Aspergillus niger on yogurt. *Food microbiology*, *65*, 264-273.

Gravelat, F. N., Beauvais, A., Liu, H., Lee, M. J., Snarr, B. D., Chen, D., ... & Sheppard, D. C. (2013). Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. PLoS pathogens, 9(8), e1003575.

Gresnigt, M. S., Bozza, S., Becker, K. L., Joosten, L. A., Abdollahi-Roodsaz, S., van der Berg, W. B., ... & van de Veerdonk, F. L. (2014). A polysaccharide virulence factor from Aspergillus fumigatus elicits anti-inflammatory effects through induction of Interleukin-1 receptor antagonist. *PLoS pathogens*, *10*(3), e1003936.

Grimm, L. H., Kelly, S., Hengstler, J., Göbel, A., Krull, R., & Hempel, D. C. (2004). Kinetic studies on the aggregation of Aspergillus niger conidia. *Biotechnology and bioengineering*, *87*(2), 213-218.

Grimm, L. H., Kelly, S., Völkerding, I. I., Krull, R., & Hempel, D. C. (2005). Influence of mechanical stress and surface interaction on the aggregation of Aspergillus niger conidia. *Biotechnology and bioengineering*, *92*(7), 879-888.

Grünbacher, A., Throm, T., Seidel, C., Gutt, B., Röhrig, J., Strunk, T., ... & Fischer, R. (2014). Six hydrophobins are involved in hydrophobin rodlet formation in Aspergillus nidulans and contribute to hydrophobicity of the spore surface. *PloS one*, *9*(4), e94546.

Hayden, N. J., & Maude, R. B. (1992). The role of seed-borne Aspergillus niger in transmission of black mould of onion. *Plant Pathology*, *41*(5), 573-581.

Hou, L., Liu, L., Zhang, H., Zhang, L., Zhang, L., Zhang, J., ... & Wang, D. (2018). Functional analysis of the mitochondrial alternative oxidase gene (aox1) from Aspergillus niger CGMCC 10142 and its effects on citric acid production. *Applied microbiology and biotechnology*, *102*(18), 7981-7995.

Jensen, B. G., Andersen, M. R., Pedersen, M. H., Frisvad, J. C., & Søndergaard, I. (2010). Hydrophobins from Aspergillus species cannot be clearly divided into two classes. *BMC Research Notes*, *3*(1), 1-6.

Jòzef, S. (2007). The use of starch processing enzymes in the food industry. In *Industrial Enzymes* (pp. 19-34). Springer, Dordrecht.

Krijgsheld, P., Bleichrodt, R., van Veluw, G. J., Wang, F., Müller, W. H., Dijksterhuis, J., & Wösten, H. A. (2013). Development in Aspergillus. *Studies in mycology*, *74*(1), 1–29. https://doi.org/10.3114/sim0006

Kumar, P., & Satyanarayana, T. (2009). Microbial glucoamylases: characteristics and applications. *Critical reviews in biotechnology*, *29*(3), 225-255.

Lima, M. A. S., Oliveira, M. D. C. F. D., Pimenta, A. T., & Uchôa, P. K. (2019). Aspergillus niger: A hundred years of contribution to the natural products chemistry. *Journal of the Brazilian Chemical Society*, *30*, 2029-2059.

Lin, P. J., Grimm, L. H., Wulkow, M., Hempel, D. C., & Krull, R. (2008). Population balance modeling of the conidial aggregation of Aspergillus niger. Biotechnology and bioengineering, 99(2), 341-350.

Marín-Navarro, J., & Polaina, J. (2011). Glucoamylases: structural and biotechnological aspects. *Applied microbiology and biotechnology*, *89*(5), 1267-1273.

Mhetras, N. C., Bastawde, K. B., & Gokhale, D. V. (2009). Purification and characterization of acidic lipase from Aspergillus niger NCIM 1207. *Bioresource technology*, *100*(3), 1486–1490. https://doi.org/10.1016/j.biortech.2008.08.016

Mims, C. W., Richardson, E. A., & Timberlake, W. E. (1988). Ultrastructural analysis of conidiophore development in the fungusAspergillus nidulans using freeze-substitution. *Protoplasma*, *144*(2), 132-141.

Ni, M., Gao, N., Kwon, N. J., Shin, K. S., & Yu, J. H. (2010). Regulation of Aspergillus conidiation. *Cellular and molecular biology of filamentous fungi*, 557-576.

Nielsen, K. F., Mogensen, J. M., Johansen, M., Larsen, T. O., & Frisvad, J. C. (2009). Review of secondary metabolites and mycotoxins from the Aspergillus niger group. *Analytical and bioanalytical chemistry*, *395*(5), 1225-1242.

Papagianni, M. (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology advances*, *22*(3), 189-259.

Pel, H. J., de Winde, J. H., Archer, D. B., Dyer, P. S., Hofmann, G., Schaap, P. J., ... & Stam, H. (2007). Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. *Nature biotechnology*, *25*(2), 221-231.

Petersen, S. G., Rahbek-Nielsen, H., Hellmuth, K., & Harboe, M. (2006). Increased production of chymosin by glycosylation. *Journal of biotechnology*, *125*(2), 304-310.

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage* (Vol. 519, p. 388). New York: Springer.

Priegnitz, B. E., Wargenau, A., Brandt, U., Rohde, M., Dietrich, S., Kwade, A., ... & Fleißner, A. (2012). The role of initial spore adhesion in pellet and biofilm formation in Aspergillus niger. *Fungal genetics and biology*, *49*(1), 30-38.

Riley, G. L., Tucker, K. G., Paul, G. C., & Thomas, C. R. (2000). Effect of biomass concentration and mycelial morphology on fermentation broth rheology. *Biotechnology and bioengineering*, *68*(2), 160-172.

Robinet, P., Baychelier, F., Fontaine, T., Picard, C., Debré, P., Vieillard, V., ... & Elbim, C. (2014). A polysaccharide virulence factor of a human fungal pathogen induces neutrophil apoptosis via NK cells. *The Journal of Immunology*, *192*(11), 5332-5342.

Ryoo, D., & Choi, C. S. (1999). Surface thermodynamics of pellet formation in Aspergillus niger. *Biotechnology letters*, *21*(2), 97-100.

Show, P. L., Oladele, K. O., Siew, Q. Y., Aziz Zakry, F. A., Lan, J. C. W., & Ling, T. C. (2015). Overview of citric acid production from Aspergillus niger. *Frontiers in life science*, *8*(3), 271-283.

Schuster, E., Dunn-Coleman, N., Frisvad, J. C., & Van Dijck, P. W. (2002). On the safety of Aspergillus niger–a review. *Applied microbiology and biotechnology*, *59*(4), 426-435.

Tegelaar M, Aerts D, Teertstra WR, Wösten HAB. Spatial induction of genes encoding secreted proteins in micro-colonies of Aspergillus niger. Sci Rep. 2020 Jan 30;10(1):1536. doi: 10.1038/s41598-020-58535-0. PMID: 32001779; PMCID: PMC6992626.

Tegelaar M, van der Lans GPA, Wösten HAB. Apical but not sub-apical hyphal compartments are self-sustaining in growth. Antonie Van Leeuwenhoek. 2020 May;113(5):697-706. doi: 10.1007/s10482-020-01383-9. Epub 2020 Jan 9. PMID: 31919791; PMCID: PMC7138781

Tegelaar M, Bleichrodt RJ, Nitsche B, Ram AFJ, Wösten HAB. Subpopulations of hyphae secrete proteins or resist heat stress in Aspergillus oryzae colonies. Environ Microbiol. 2020 Jan;22(1):447-455. doi: 10.1111/1462-2920.14863. Epub 2019 Nov 24. PMID: 31736205; PMCID: PMC6972715.

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latge, J. P., & Paris, S. (1994). Rodletless mutants of Aspergillus fumigatus. *Infection and immunity*, *62*(10), 4380-4388.

Vala, A. K., Sachaniya, B., Dudhagara, D., Panseriya, H. Z., Gosai, H., Rawal, R., & Dave, B. P. (2018). Characterization of L-asparaginase from marine-derived Aspergillus niger AKV-MKBU, its antiproliferative activity and bench scale production using industrial waste. *International journal of biological macromolecules*, *108*, 41-46.

Valsecchi, I., Dupres, V., Stephen-Victor, E., Guijarro, J. I., Gibbons, J., Beau, R., ... & Beauvais, A. (2017). Role of hydrophobins in Aspergillus fumigatus. Journal of fungi, 4(1), 2.

Veiter, L., Rajamanickam, V., & Herwig, C. (2018). The filamentous fungal pellet—relationship between morphology and productivity. *Applied microbiology and biotechnology*, *102*(7), 2997-3006.

Van Veluw, G. J., Teertstra, W. R., De Bekker, C., Vinck, A., Van Beek, N., Muller, W. H., ... & Wösten, H. A. B. (2013). Heterogeneity in liquid shaken cultures of Aspergillus niger inoculated with melanised conidia or conidia of pigmentation mutants. Studies in mycology, 74(1), 47-57.

Vinck, A., Terlou, M., Pestman, W. R., Martens, E. P., Ram, A. F., van den Hondel, C. A., & Wösten, H. A. (2005). Hyphal differentiation in the exploring mycelium of Aspergillus niger. Molecular microbiology, 58(3), 693-699.

de Vries, R.P., Burgers, K., van de Vondervoort, P.J., Frisvad, J.C., Samson, R.A., Visser, J. 2004. A new black Aspergillus species, A. vadensis, is a promising host for homologous and heterologous protein production. Appl Environ Microbiol, 70, 3954-3959.

Ward, O. P., Qin, W. M., Dhanjoon, J., Ye, J., & Singh, A. J. A. I. A. M. (2005). Physiology and biotechnology of Aspergillus. *Advances in applied microbiology*, *58*, 1-75.

Wargenau, A., Kampen, I., & Kwade, A. (2013). Linking aggregation of Aspergillus niger spores to surface electrostatics: a theoretical approach. *Biointerphases*, *8*(1), 7.

Wösten, H. A., Moukha, S. M., Sietsma, J. H., & Wessels, J. G. (1991). Localization of growth and secretion of proteins in Aspergillus niger. *Microbiology*, *137*(8), 2017-2023.

Wösten HA, van Veluw GJ, de Bekker C, Krijgsheld P. Heterogeneity in the mycelium: implications for the use of fungi as cell factories. Biotechnol Lett. 2013 Aug;35(8):1155-64. doi: 10.1007/s10529-013-1210-x. Epub 2013 Apr 17. PMID: 23592308.

Wösten, H. A. (2001). Hydrophobins: multipurpose proteins. *Annual review of microbiology*, *55*, 625-646.

Wösten, H. A., van Wetter, M. A., Lugones, L. G., van der Mei, H. C., Busscher, H. J., & Wessels, J. G. (1999). How a fungus escapes the water to grow into the air. *Current Biology*, *9*(2), 85-88.

Yu, R., Liu, J., Wang, Y., Wang, H., & Zhang, H. (2021). Aspergillus niger as a Secondary Metabolite Factory. *Frontiers in chemistry, 9*, 701022.

Zhang, J., & Zhang, J. (2016). The filamentous fungal pellet and forces driving its formation. *Critical reviews in biotechnology*, *36*(6), 1066-1077.

van Zyl, P. J., Moodley, V., Rose, S. H., Roth, R. L., & van Zyl, W. H. (2009). Production of the Aspergillus aculeatus endo-1,4-beta-mannanase in A. niger. *Journal of industrial microbiology & biotechnology*, *36*(4), 611–617. https://doi.org/10.1007/s10295-009-0551-x