Chitin in the fungal cell wall: Towards valorization of spent biomass of Aspergillus niger

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CHAPTER 1

General Introduction

Filamentous fungi

Filamentous fungi are commonly found in all sorts of places on earth. Most people have a negative association with these fungi, causing black spots in poorly ventilated bathrooms or as food spoilers; found as green patches on old bread or as white fluffy mold in a jar of pesto that is past the due date. Despite this negative association, filamentous fungi have been used by humans for beneficial purposes. Universal examples are the fermentation of certain cheeses and dried sausages, but they are also used to produce soy sauce and antibiotics (e.g. penicillin). The term "filamentous" applies to these fungi because they grow as hair-like filaments that give them a fluffy appearance. The correct scientific name for these hair-like filaments is hyphae. These hyphae (singular = hypha) contain cells enveloped by a cell wall that extends laterally through cell division and also branches out sideways, similar to a branch from a tree. Hyphal extension and branching together are colloquially referred to as vegetative growth. During vegetative growth, compartmentalization occurs within the hyphae through the formation of walls to separate cells. However, these separating walls (septa, singular = septum) are porous which means that the cells stay physically connected to one another to form a large interconnected network, known as a mycelium. The mycelial lifestyle is very different from yeast-like unicellular fungi, where growth occurs through separation of mother and daughter cells into individual entities. In mushroom-forming fungi, these mycelial networks can grow to an impressive size and form fairy rings (Dutch: heksenkring) that can be characterized by a circle of mushrooms (fruiting bodies). Most filamentous fungi do not form such fruiting bodies and produce either sexual or asexual spores as a means of proliferation (Cole, 1996). Asexual reproduction is commonly found among filamentous fungi and has the upside of not having to invest in finding a mating partner for the production of (sexual) spores. Instead, such as in the case of the filamentous fungus *Aspergillus niger*, asexual spores are formed as genetically identical (clonal) cells from a single vegetative mycelium. To produce these asexual spores, aerial hyphae are formed that extend from the vegetative mycelium below. Then, *A. niger* forms vesicles on top of aerial hyphae that sprout asexual spore chains (Figure 1). The shapes and forms of which these asexual reproduction structures are formed vary greatly between different filamentous fungi and so do the number of spores produced.

Spores are quite easily distributed when a spore-chain is physically disturbed, for example by a breeze of wind or by the touch of an animal, allowing colonization far away from the parental mycelium. Because spores are hardy survival structures, they can remain dormant for quite some

time before they germinate. A spore will germinate upon finding favorable conditions (such as in that pesto jar), forming an initial germ tube that will be the first-formed hyphae (germling). From here on out, germlings grow into a vegetative mycelium that starts the lifecycle all over again. What these favorable conditions are, depends on the type of fungus and its preferred environment. These environments can be rougly categorized into either dead material (saprophytic) or living material (parasitic). Hence, fungi are very efficient in colonizing many different places on earth.

Fungi are in many ways very similar to animal and plant cells, all belonging to the domain of Eukaryotes (true nuclei). In addition to nuclei, eukaryotic cells also harbor other compartments such as endoplasmic reticula, Golgi apparvatus and mitochondria, that can be found in all kingdoms of

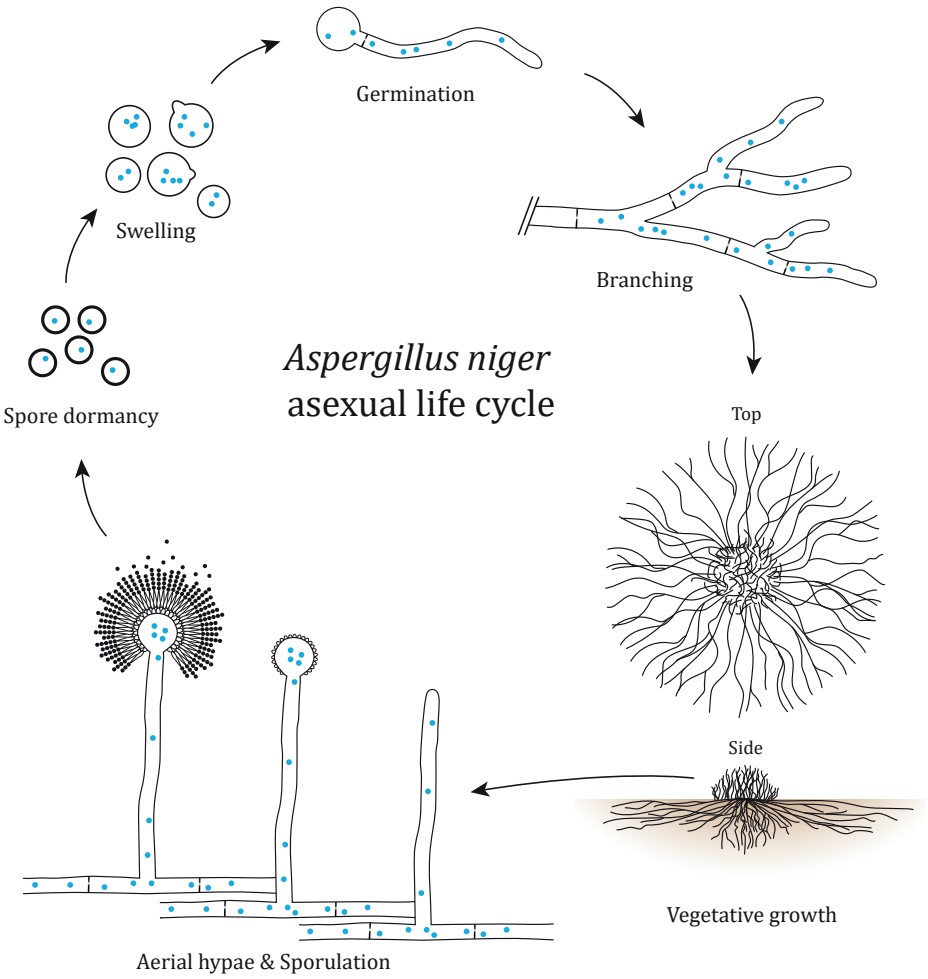

Figure 1. ***Aspergillus niger*** **asexual life cycle.**

Eukaryotes (Animalia, Fungi and Planta). When looking into more detail of what sets apart fungi from animals, we find that fungi possess a unique feature compared to animal cells: the cell wall. This cell wall dictates the shape of fungi and protects them from the outside world. Even though plants also have cell walls, fungal cell walls are very different in their compositional (polymeric) make-up. This difference in composition is one of the characteristics what makes filamentous fungi unique. This unique feature of the fungi is of interest for fundamental studies, but is also relevant for clinical/agricultural sectors to develop anti-fungal compounds (Garcia-Rubio et al., 2020), as infectious colonization of fungi is found in both kingdoms of Animalia and Planta. As mentioned above, the cellular similarity of fungi to both human and plant cells makes it difficult to specifically inhibit growth of fungi with antibiotic drugs, without affecting the host organism. An antibiotic drug that kills fungal cells due to an interaction with one of its cellular mechanisms is also likely to damage or kill the cells of animals, humans or plants crops. The cellular similarity between the kingdoms of Animalia, Planta and Fungi signifies why fungal research is important in the pursuit of finding and designing anti-fungal medication.

Besides infections and their potential danger, fungi have been (and will continue to be) used for the benefit of humanity. Common examples are cheese, beer, wine and fermented soy products, such as soy sauce. Less commonly known, but of high importance to (food)industry, is the use of filamentous fungi to produce different types of enzymes, antibiotics and organic acids. Organic acids, such as those that provide building blocks for other chemicals (Aurich et al., 2012) or those used as natural food flavor enhancers, acidifiers, stabilizers or preservatives (Magnuson and Lasure, 2004). A highly familiar example of citric acid application is in sugar-substituted Cola beverages. These either contain citric acid (light) or trisodium citric acid (zero), both for preservation and flavor and are also known as E330 and E331 food additives, respectively. Annual citric acid production quantities range up to 900,000 metric tons and is by far the largest organic acid made through fermentation, primarily by *A. niger* (Karaffa and Kubicek, 2003). Another organic acid, itaconic acid, is naturally produced by *Aspergillus terreus* (Bentley and Thiessen, 1957), and has its primary application in the polymer industry as a co-monomer, but is additionally used to manufacture coatings, adhesives and synthetic fibers (Magnuson and Lasure, 2004). However, *A. terreus* produces itaconic acid in less than economically feasible quantities (Weastra, 2011). Genetic engineering of *A. niger* has successfully led to production of itaconic acid to respectable titers for industrial production (Hossain et al., 2016). Both the production of plant cell wall degrading enzymes (e.g. to hydrolyze plant biomass to produce sugars which can be converted to bio-ethanol by yeasts) and production of organic acids of *A. niger* are two major exploits used by biotechnological industries (Cairns et al., 2018). Yet, another organic acid, lactic acid, is often used as a food preservative, flavor enhancer and acid regulator and can also be used as a precursor for the production of a biodegradable plastic: Poly Lactic Acid (PLA). The majority of lactic acid is produced by bacteria of the Genera *Lactobacillus* and *Bacillus*, but also fungi are capable of the production of this industrially promising organic acid. One of these is *Rhizopus oryzae*, a fungus traditionally used for the fermentation of soy to create tempeh (Magnuson and Lasure, 2004).

Next to large scale organic acid production, the production of enzymes can also be facilitated by fungi, including recombinant and genetically engineered, optimized enzymes. In 2016, annual world sales of enzymes by filamentous fungi are estimated at ca. 4.7 billion euros, which is expected to double in the next 10 years (Meyer et al., 2016). Both the production of enzymes and the use of cell factories for the biosynthesis of other products require large scale fermentations of micro-organisms, such as bacteria, yeasts, fungi. The best suited organism depends on each different enzyme, but in the case of filamentous fungi, *Aspergillus spp., Penicillium spp., Fusarium spp.* and *Trichoderma spp*. are most commonly used.

Aspergillus niger

The filamentous fungus *Aspergillus niger* is a haploid, saprophytic member of the phylum Ascomycota and is frequently used for industrial purposes as described above. The Genus "*Aspergillus*" was coined by its first discoverer in 1729, Pier Antonio Micheli, who found resemblance between the spore-bearing structure of the fungus and an "aspergillum", an object used by Roman Catholic clergy to sprinkle holy water (Bennett, 2010). The species name "*niger*" owes its name to the black color of the heavily melanized spores, providing protection from harmful UV-radiation. In our daily lives, the most likely place to encounter *A. niger* is in the form of black conidia on the outside of onions (Gherbawy et al., 2015).

A. niger has been reported to produce only asexual spores, but may yet have a cryptic sexual cycle (Dyer and O'Gorman, 2012) as it is also able to produce sclerotia (Frisvad et al., 2014; Jørgensen et al., 2020). Sclerotia are a type of macrostructure of aggregating hyphae that resemble the shape of psychedelic sclerotial "magic truffles" that one can buy in Dutch smartshops. These very hardy sclerotial structures are commonly formed by many fungal species and come in various shapes and sizes. In case of *A. niger*, this is the size of a sesame seed. It has been postulated that sclerotia formation has an evolutionary advantage as a way to survive adverse environmental conditions such freezing, drought, nutrient depletion or microbial attack (Smith et al., 2015). In addition to enhancing survivability in hostile environments, sclerotia formation is considered to be part of the sexual reproduction process in certain filamentous fungi (Smith et al., 2015). For example, in *Aspergillus flavus*, a known opportunistic pathogen and common spoiler of peanuts and peanut butter (Adeyeye, 2016), the sclerotia have been shown to also act as reproductive structures that contain ascospores (Horn et al., 2009). In studies of *A. niger*'s sclerotia (Frisvad et al., 2014; Jørgensen et al., 2020), the correct genetic requirements to induce sexual spore formation (two opposite mating types) have not been met. This means that the existence of a true sexual cycle of *A. niger* remains elusive, for now.

Because the existence of a sexual cycle can be very useful for genetic investigation of *A. niger*, a workaround technique has been developed—which is also used in the research described in this thesis (**Chapter 4** and **Chapter 5**)—known as the parasexual cycle (Pontecorvo et al., 1953). A schematic representation of how this process works is shown in Figure 2. The parasexual cycle

is a technique to redistribute/shuffle the chromosomes from both parents without true meiosis. Crucially, this means that there is no meiotic cross-over, only sparce mitotic cross-over events. In essence, two auxotrophic haploid *A. niger* strains are coerced to fuse (anastomosis) and then undergo cytoplasmic mixing (plasmogamy) based on non-complementary auxotrophic deficiencies (Figure 2A and B). Post-fusion a mycelium with two types of haploid nuclei (heterokaryon) will form, shown in Figure 2C. On low chance occurrence, nuclei in this heterokaryon can undergo karyogamy (nuclear fusion), resulting in a heterozygous diploid. In addition to the auxotrophic markers, the use of visually distinguishable color markers (specific complementation groups of in melanin synthesis, Jørgensen et al., 2011) may be added to visually track karyogamy in time.

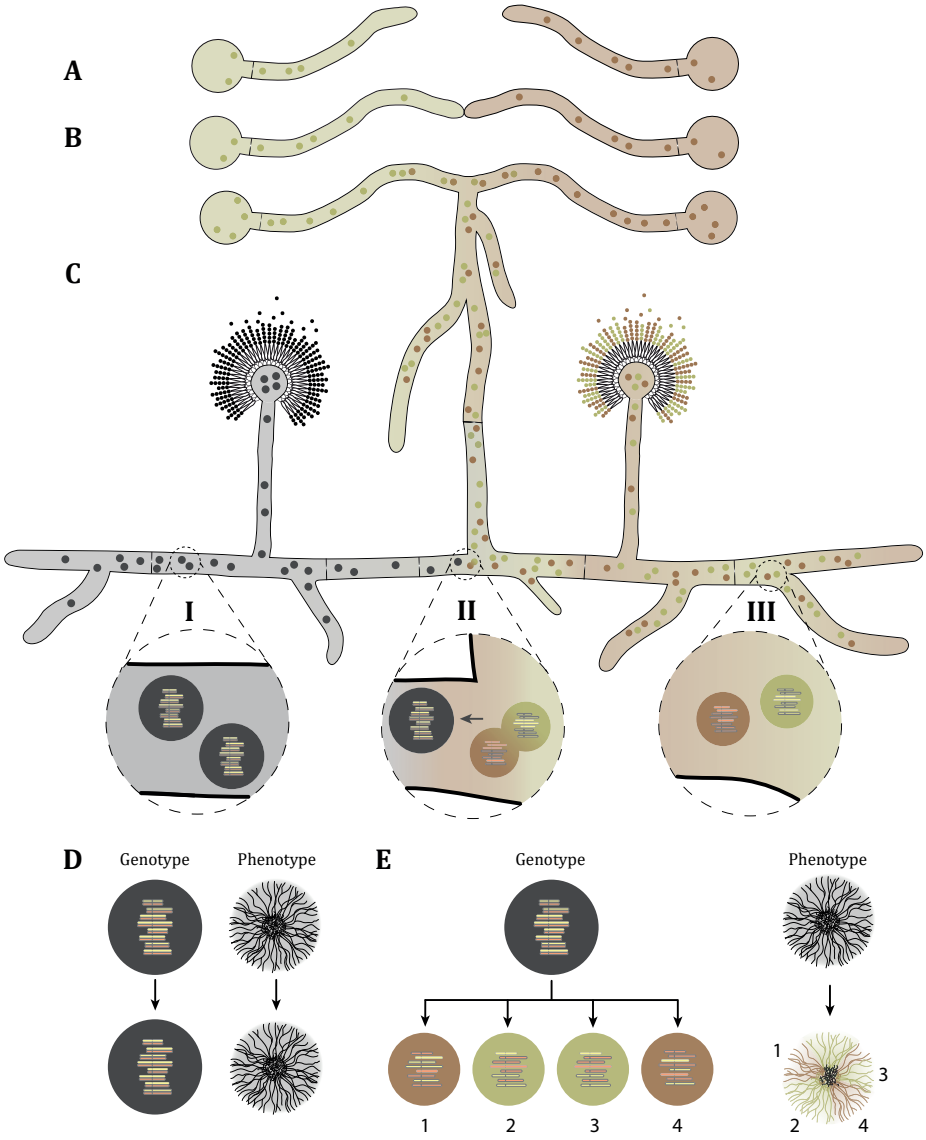

Figure 2. The parasexual cycle of *Aspergillus niger*. (A) Two complementary, haploid auxotrophic strains carrying different spore color mutations, *ΔbrnA* or *ΔolvA*, (Jørgensen et al., 2011b). (B) Initiation of directed hypal fusion, called anastomosis. Complementary auxotrophic strains are coerced to undergo plasmogamy (the fusion of cytoplasm) in absence of nutrients required for either of the two parental strains (C) Following plasmogamy, a heterokaryotic mycelium is formed that contains nuclei from both strains. On rare-chance occurrence, a heterozygous diploid (I) is formed through karyogamy, the fusion of nuclei from both parental strains (II). A heterozygous diploid forms black spores due to complementation of both parental spore color mutations. Without karyogamy, a heterokaryotic mycelium (III) produces the two parental type uninuclear, haploid spores, unable to germinate in the absence of the required nutrient supplementation. (D) The heterozygous diploid has a uniform spore color phenotype. (E) Benomyl induced haploidization of a heterozygous diploid. Benomyl destabilizes chromosome segregation that causes random distribution of chromosomes from either parent into haploid segregants with all different spore color and nutrient phenotypes.

Haploid heterokaryons produce spores of either genotype resulting in different spore colors, based on the fact that spores are generally uninuclear—or at least have clonal nuclei—whereas a heterozygous diploid will have black spores. A diploid is stable (Figure 2D), but can be artificially haplodized into haploid segregants upon addition of benomyl (Arentshorst and Ram, 2018). Benomyl destabilizes chromosome segregation, causing random distribution of chromosomes that result in all different color marker and nutrient phenotypes (Figure 2E).

A. niger is a saprobe, meaning that it thrives on decaying organic matter. As such, it secretes enzymes into its environment to degrade complex plant (cell wall) polysaccharides maily to release either dimeric or monomeric sugars. As described previously, *A. niger* is able to convert these sugars into large amounts of organic acid. These organic acids can then be consumed at later stage after sugar depletion. The conversion of sugar into acid(s)—thereby acidifying the surroundings—has been postulated to act as evolutionary benefit (Andersen et al., 2009), keeping other microbes at bay that would otherwise try to snatch the plant-cell-wall-released sugars from *A. niger*. Contrarily to the natural soil habitat of *A. niger*, industrial profiteering of *A. niger*'s secreted compounds is attained from submerged cultivation in large bioreactors. These bioreactors range in volume from a few hundred to up to more than hundred thousand liters. These (unnatural) liquid growth conditions cause *A. niger* to either grow dispersed, as clumps (germlings sticking together) or even as larger aggregates: pellets (P. Krijgsheld et al., 2013). In these submerged growth conditions, *A. niger* is able to produce large quantities of aforementioned products, while also converting much of the energy into acquisition of fungal biomass. When the secreted products (enzymes or organic acids) have been obtained, the remaining mycelial biomass is a left-over product. Currently, this mycelium is typically used as a low-cost fertilizer for agricultural crop plant production or as cattle feed (NRC, 1983). However, these disposal routes are a significant cost factor in the biotechnological production process, and clearly under-exploit this potentially precious material.

The potential of cell walls in mycelial waste

As the number of industrial scale fungal fermentation increases, so does the accumulation of fungal biomass waste. Most of the dry weight of the mycelial biomass is comprised of polymers from

fungal cells (50-80%) that may be of high value when extracted (Isaza-Pérez et al., 2020). These cell wall polysaccharides are mainly made up of differently linked monomers of either glucose, (*N*-acetyl-)glucosamine, galactose or mannose (Free, 2013; Gow et al., 2017; Ruiz-Herrera and Ortiz-Castellanos, 2019). Glucose residues can be found in either an α-linked or β-linked orientation, such as α-1,3-glucan, mixed α-1,3/1-4-glucans or β-1,3-glucan, β-1,6-glucan and mixed β-1,3/1,4 or β-1,3/1,6 glucan variations, and comprise the major fraction of the fungal cell wall. The second most abundant polymer of fungal cell wall is chitin. Chitin is also found in the exoskeletons of crustaceans (shell fish), insect cuticles and squid gladius/pen (the hard, internal body part that you may find on the beach). As such, chitin also happens to be second-most abundant world-wide, after cellulose (present in the cell walls of plants (Kaur and Dhillon, 2014). Comprised of β-1,4-linked *N*-acetylglucosamine monomers (GlcNAc), chitin naturally occurs as either one of three different crystalline, polymorphic forms: α-chitin, β-chitin or γ-chitin (Rudall and Kenchington, 1973). Fungal cell walls only harbor α-chitin which consists of anti-parallel GlcNAc chains that form the strongest type of intrachain hydrogen bonds of all chitin forms. A small percentage of the cell wall contains de-acetylated chitin (i.e. lack the (*N*-linked) acetyl group on the glucosamine monomer which liberates the amino group NH_2). The definitions of chitin and chitosan are based on the degree of acetylation (DA); any given β-1,4-(*N*-acetyl) glucosamine polymer that contains a DA ≥ 50%, is termed chitin, whereas if the DA < 50%, the term is chitosan (Muzzarelli, 1973; Pillai et al., 2009).

Variation in DA and degrees of polymerization (DP), molecular weight and pattern of charge distribution of chitin/chitosan determine the active properties and solubility of chitosan, giving rise to a wide range of applications in biomedical, pharmaceutical and agricultural sectors (El Gueddari et al., 2014; Rinaudo, 2006). Many (potential) applications have recently been reviewed and include promotion of wound healing, anti-microbial activity, but also anti-tumor, anti-inflammatory, immuno-stimulating, anti-diabetic, anti-Alzheimer's and anti-HIV activity, just to name a few (Naveed et al., 2019). In addition, chitosan can be used to inhibit microbial growth and extend the shelf-life of fresh produce as edible coatings, either by itself or combined with other natural (anti-microbial) biomaterials (Yousuf et al., 2018). Furthermore, chitosan has been shown to be an environmentally-friendly alternative for pesticides in disease control due to antimicrobial activity (Orzali et al., 2017). A broad spectrum of antimicrobial activity of chitosan has been described for both phytopathogenic fungi and bacteria, such as the fungi *Botrytis cinerea,* infectious to grapevine and strawberry (Feliziani et al., 2015; Reglinski et al., 2010), and *Rhizoctonia solani*, infectious to multiple crops, e.g. potatoes, sugar beet, cucumber and rice (Liu et al., 2012), but also bacteria *Agrobacterium tumefaciens* (crown gall disease – formation of tumors) and *Erwinia carotovora* (both multi-crop pathogens) (Badawy et al., 2014). Application of chitosan-mediated antimicrobial defense is often achieved by either amending soil or by coating of plant seeds, both treatments which have been shown in inhibit *Fusarium* infections (Bell et al., 1998; Lafontaine and Benhamou, 1996; Orzali et al., 2014). Aside from anti-microbial activity, chitosan addition to healthy plants enhanced seedling growth, total biomass production, crop yields (Corsi et al., 2015; Kowalski et al., 2006; Lizárraga-Paulín et al., 2013; Utsunomiya et al., 1998; Ziani et al.,

2010), and have also been found to protect plants against abiotic factors such as drought (Zeng and Luo, 2012). The effects of chitosan on plant protection are more extensively reviewed in Hidangmayum et al., 2019. The main mode of action in which chitosan achieves these feats is through electrostatic interactions with other molecules—through their protonated amino (NH_3^+) groups—causing plasma membrane damage, chitosan-DNA/RNA interactions, metal chelation, but also elicit plant defense responses through receptor binding, as well as affecting complex signal transduction networks (Xing et al., 2015). The almost innumerable range of applications that have been shown to positively use chitosan or substitute current plant treatments, signify the likely increase in demand for chitin and chitosan in the future. The large left-over fungal mycelial biomass production that currently being discarded could thus provide a means to close the gap in the market.

Exploitation of fungal chitin – challenges and perspectives

Currently, large scale commercial production of chitosans proceeds almost exclusively from shrimp and crab shell wastes through hot alkali extractions (Aranaz et al., 2009; Roberts, 1992). The chitosan obtained by such treatments shows inconsistent levels of de-acetylation, high molecular weight and protein contamination, which results in variable physico-chemical characteristics (Knorr & Klein, 1986; No and Meyers, 1997). Additional problems associated with these sources concern varying qualities due to seasonal variations, and spoilage during storage and transport (Aranaz et al., 2009). Also, animal origin *per se* poses a problem for certain markets, either due to consumer preferences such as in cosmetics, or due to regulatory issues concerning possible contaminations with allergens or viruses, particularly in the biomedical and pharmaceutical markets (Dhillon et al., 2012). Finally, the limited regional supply of raw material does not allow truly large-scale chitosan production from these sources, and traceability of the material back to the original source is also difficult in most cases.

A. niger provides a valuable source for chitin/chitosan not only because of its large scale production quantities, but also because of its already relatively high (~16%) extractable chitin/chitosan content (Isaza-Pérez et al., 2020). Extraction of chitin/chitosan from fungal cell walls is not too difficult, but necessitates rather harsh alkali extraction conditions (as mentioned above). High molarity alkali exposure can lead to uncontrolled partial de-polymerization and partial de-acetylation (Dhillon et al., 2012; Klis et al., 2007). This translates to low chitosan yields that are of poor quality in terms of polymer size and purity (Dhillon et al., 2012; Maghsoodi et al., 2009). Alkali extraction has only been scaled up to the extent that fungal chitosan is available in limited quantities on the market today (Dhillon et al., 2013). An alternative, but less cost-efficient strategy, involves enzymatic degradation of the cell wall, which can lead up to a 3-fold increase in chitosan yield along with increased polymer size (Cai et al., 2006).

Next to cultivation conditions and extraction methods, we hypothesized that the covalent linkages between chitin and glucans present in the fungal cell walls (Arroyo et al., 2016) may hamper

extraction and may cause impurity in fractionation. In *S. cerevisiae*, this covalent chitin/glucan linkage—the nature of which is not entirely understood in filamentous fungi—has been shown to be removed upon deletion of the genes encoding specific cross-linking enzymes. The genes encoding these cross-linking enzymes were named after their hypersensitivity to Congo Red (Crh), a cell wall disturbing compound, that signified the importance of these enzymes in cell wall integrity (Rodríguez-Peña et al., 2000b). Later, it was shown that removal of the Crh enzymes completely separated the chitin from the glucan fraction in the cell wall (Cabib, 2009; Cabib et al., 2007). As such, cell wall chitin from glucan separation may provide a valuable method of enhancing chitin extraction with both higher quantatiy and purity.

In order to use mycelial biomass waste as an economically feasible source of chitin/chitosan that can compete with the current chitosan production from shrimp waste, two major issues need to be addressed: (i) The overall chitin content in fungal cell walls should be boosted to increase yields. (ii) To enhance the extraction of chitin from the fungal cell walls, removal of covalent linkages between chitin and glucan is required. The work in this thesis addresses both of these topics by using a forward genetics screens to identify high chitin producing fungal strains and through complete removal of the seven *crh* genes encoding chitin-glucan crosslinking enzymes in a single *A. niger* strain.

Outline of the research presented in this thesis

Aspergillus niger is an important industrial producer of organic acids and enzymes producing large amounts of spent fungal biomass. In the European Research Area Industrial Biotechnology (ERA-IB) funded project, we effectively aimed to improve the composition of post-fermentation fungal biomass for extraction of the value-added product chitosan as a derivative of cell wall chitin (FunChi). The chapters of this thesis following this introductory chapter (**Chapter 1**), will discuss the identification of genes that are important for chitin deposition in the cell wall of *A. niger*, and also specifically discuss the genes that facilitate chitin cross-linking to the cell wall.

To enable mutant strain construction, **Chapter 2** describes the development of a very efficient and recyclable, marker free CRISPR/Cas9-based gene-editing system for *A. niger*. Using this system, we generated a complete seven-fold knockout of a cell wall chitin cross-linking enzyme family. By doing so, we aimed to understand the effect of removing Crh enzymes that covalently link chitin to the cell wall on cell wall integrity as a proxy for chitin extractability. The effects on morphology, cell wall composition and transcriptomic response in the seven-fold deletion mutant are discussed in **Chapter 3**. The results show that removal of the *crh* gene family does not affect cell wall composition, and the experiments hint towards an important relation between chitin and α-glucan to compensate for *crh* loss. In addition, we identified and characterized *A. niger* mutant strains with increased cell wall chitin deposition by exploiting the cell wall chitin compensatory response of the cell wall integrity pathway. Using the cell wall stress mutant library (Damveld et al., 2008), we screened mutants for increased cell wall chitin deposition. Two selected mutants

were characterized using classical genetics (parasexual crossing) combined with genome sequencing. Identified SNPs were carefully re-created in a wild type strain using CRISPR/Cas9 gene editing to confirm single gene involvement (discussed in **Chapter 4** and **Chapter 5**, respectively). These genes are a transcriptional repressor of cell wall chitin (*cwcA*) and Rab GTPase dissociation inhibitor (*gdiA*). Next to the cell wall mutant library, an industrially favorable hyperbranching *A. niger* mutant was selected and tested for increased chitin deposition. The mutant strain in question, *ΔkexB*, was chosen for its visibly thicker and shorter hyperbranched hyphae, resulting in a low-viscosity fermentation phenotype, a favorable trait in large scale fermentations. Effects of this gene deletion are discussed in relation to its morphology, transcriptome and cell wall chitin phenotype in **Chapter 6**. The results showed an increase in cell wall chitin content; however, this was not related to its hyperbranching phenotype. An overall discussion of all findings relating to the FunChi project, and an outlook to the future, are presented in **Chapter 7**.

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