Carbon starvation in the filamentous fungus Aspergillus niger

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Chapter 1

General introduction

The impact of filamentous fungi

Fungi are amongst the most simple eukaryotic organisms. Similar to other microorganisms, they play essential roles in decomposition of organic matter and nutrient cycling in nature. According to Whittakers early four-kingdom system (Whittaker, 1959; Hagen, 2012), they constitute their own taxonomic kingdom, the Mycetae, which is a very diverse group of microorganisms comprising generally known species including yeast, molds and mushroom forming fungi such as *Agaricus bisporus*, also called the champignon mushroom. In contrast to the majority of yeasts which are unicellular and grow by budding (e.g. *Saccharomyces cerevisiae* also known as baker's yeast) or fission (e.g. *Schizosaccharomyces pompe*), molds grow in a polar manner forming hyphae which are long tubular tip-growing cells. Due to this key morphological characteristic, molds are (more) frequently referred to as filamentous fungi.

Filamentous fungi have an immense impact on human society. *Penicillium camenberti* and *Aspergillus oryzae* have for example long been used for the production of camembert cheese and Japanese soy sauce (*shōyu*), respectively. According to legend, the production of the latter dates back to 1254 (Murooka *et al.*, 2008) and Japanese cuisine cannot be imagined without it. β-lactam antibiotics are another famous example for the significant impact of filamentous fungi on human society. In 1928 the physician Alexander Fleming, who worked with *Staphylococus* bacteria, accidently discovered that a mold contaminating his culture plates secreted an agent with antibacterial activity. The mold was identified as *Penicillium notatum* and the antibacterial compound was named Penicillin (Kardos *et al.*, 2011). Driven by increasing demands for anti-infective drugs during World War II, the development of industrial production technology, process and strain improvements are outstanding examples of industrial fungal biotechnology. In 1945, the annual world production of penicillin G amounted to 5 tons, this had increased to more than 12,000 tons in 1982 and reached 33,000 tons by 1995 (Beek *et al.*, 1984; Elander, 2003). However, industrial fungal biotechnology is not limited to pharmaceuticals. Most filamentous fungi used in industrial biotechnology are efficient saprophytes (e.g. *Trichoderma reesei* or *Aspergillus niger*) meaning that they naturally have high secretion capacities for various hydrolytic enzymes required to extracellularly decompose dead organic matter. These high secretion capacities together with their versatile primary and secondary metabolisms make filamentous fungi outstanding production hosts for a variety of commercial products including primary metabolites (e.g. organic acids and vitamins), secondary metabo-

lites (e.g. antibiotics and bioactive compounds such as alkaloids), natural secreted hydrolases (e.g. cellulases, pectinases and proteases) and, since the development of efficient molecular genetic tools for filamentous fungi, heterologous proteins (e.g. Interleukin 6 and manganese peroxidase) (Punt *et al.*, 2002). Thus, industrial sectors applying products from filamentous fungal biotechnology are as diverse as the products themselves and include amongst others, health, food, beverages and feed.

Industrial biotechnology is continually increasing its effort to establish economic and sustainable production processes which can compete with alternative processes from the chemical industry. Of great importance are second generation feedstocks (substrates) such as lignocellulosic biomass waste products (e.g. sugar cane bagasse or wheat straw) that can be used as substrates in bioprocesses after hydrolytic pretreatments. Second generation feedstocks are cheaper than traditional first generation feedstocks (e.g. sugar canes or corn) and do not compete with food or feed supply. However, as lignocellulosic biomass hydrolyzates contain complex mixtures of sugars and inhibitory compounds, there can be considerable differences in the performance of industrial microorganisms. Very promising results were recently shown for *A. niger* (Rumbold *et al.*, 2009; Rumbold *et al.*, 2010), thus further emphasizing the importance of this filamentous fungus as a versatile industrial cell factory.

Apart from the above-mentioned positive aspects, there is also a downside to filamentous fungi as many species can negatively impact health and the economy. These include, for example, field infection with phytopathogenic species (e.g. rice blast fungus *Magnaporthe oryzae*) and post-harvest contamination of food and feedstocks with mycotoxin-producing fungi such as *Aspergillus flavus* (carcinogenic aflatoxins) which can result in substantial economic losses in the agricultural industry. Airborne fungal spores are ubiquitous and can be found both indoors and outdoors where they often exceed pollen concentrations by up to 1,000-fold and are considered important perpetrators of respiratory allergies and asthma. Most allergenic filamentous fungal species are asexual Ascomycetes as, for example, *Alternaria alternata* and *Aspergillus fumigatus* (Horner *et al.*, 1995). For the latter, it has been estimated that humans inhale several hundred conidia per day (Latge, 1999). These conidia are a life-threatening danger for immunocompromised patients (e.g. cancer, organ transplantation and HIV), as they can cause invasive pulmonary aspergillosis with mortality rates up to 50%, even when treated with antifungals (Fedorova *et al.*, 2008).

The black mold *Aspergillus niger* is a ubiquitous saprophytic filamentous fungus belonging to the *Aspergillus* section *Nigri*, which together with the section *Flavi*, accounts for the most common mycotoxigenic fungi contaminating agricultural goods including, amongst others, corn, peanuts, raisins and onions (Mogensen *et al.*, 2010). Nevertheless, *A. niger* is one of the major hosts in industrial biotechnology for the production of food ingredients (e.g. cit-

ric acid/E330 or gluconic acid/E574) (Ruijter *et al.*, 2002) and industrial enzymes (e.g. Lipases, pectinases and catalases) (Fogarty, 1994). *A. niger* has a long history of safe use and many of its products have acquired GRAS status, meaning that they are generally recognized as safe food ingredients by the American Food and Drug Administration (FDA) (Schuster *et al.*, 2002). Industrial-scale production of citric acid with *A. niger* dates back to 1923. Nowadays, citric acid is a bulk product which, due to its flavor, acidity and chelating property, is a common ingredient in food, beverages, cosmetics and pharmaceuticals with an annual world production of 900,000 tons in the year 2000 (Ruijter *et al.*, 2002).

Carbon starvation: Dynamics of the fungal mycelium

In contrast to most laboratory growth conditions, microorganisms experience dramatic changes and fluctuations of environmental factors in their natural habitats, including light conditions, temperature, moisture, pH, osmolarity, nutrient availability and competitors. Depending on the niche, diverse stress responses have evolved which are dedicated to survival and propagation. The favored commercial production process in fungal biotechnology is submerged cultivation in stirred tank reactors which can reach volumes of up to 300,000 liters (Elander, 2003). Many of these processes are operated as fed-batch cultures (Zustiak *et al.*, 2008) preventing catabolite repression by ensuring a nutrient-limited growth regime. However, substrate limitation towards the end of production processes in particular, can cause severe stress to the production host which subsequently can translate into reduced yields. The focus in the following will be on carbon starvation during submerged cultivation. Various aspects related to the complex physiological and morphological consequences for the fungus will be briefly introduced.

The fungal mycelium; a network of hyphal cells

The formation of a mold colony from a single spore is initiated by germination, which involves isotropic growth (swelling) and the establishment of polarity in the form of a germ tube. Apical elongation and branching of the germ tube leads to the formation of individual substrate exploring hyphae. As a result of further apical extension of hyphae, branching and hyphal fusion (anastomosis) an interconnected hyphal network evolves which is referred to as "fungal mycelium" (see Figure 1.1).

Hyphae of higher fungi (e.g. Ascomycetes and Basidiomycetes) are multicellular and multinucleated. They consist of several compartments (see Figure 1.2). These compartments are separated from each other by a so-called septum, a structure consisting of cell wall material (mainly polysaccharides) that is continuous with the lateral cell wall of the fungus. Com-

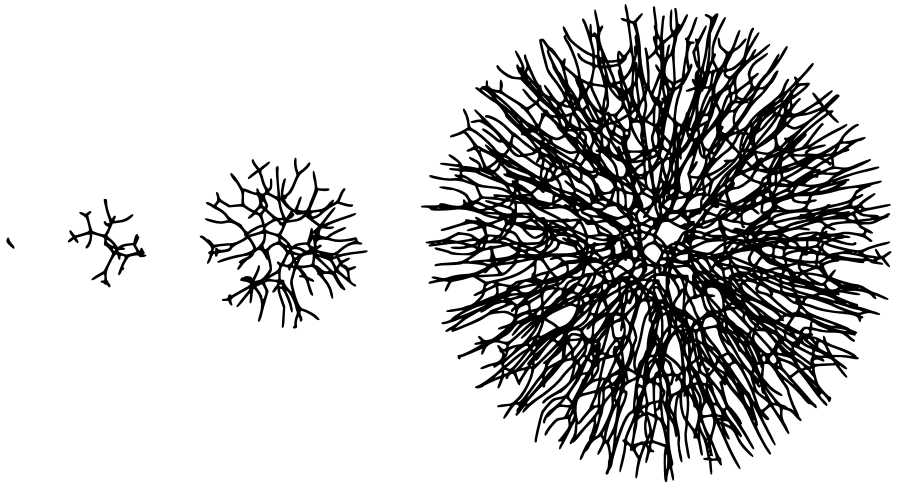

Figure 1.1 — Development of a filamentous fungal colony
Neighbor-Sensing model simulating colonial growth of a filamentous fungus on an isotropic substrate (Meškauskas *et al.,* 2004). A young germling undergoes branching forming a low density mycelial layer. Further branching and hyphal fusion (anastomosis) lead to the formation of a denser radially growing mycelial mat which forms a mycelial entity.

partments can communicate with each other via a septal pore. This pore in the septum is large enough to allow organelles (e.g. nuclei and mitochondria) and protein complexes (ribosomes) or proteins and metabolites to be transported from one compartment to the other. When required e.g. in response to hyphal damage of the tip cell or environmental conditions (*Schizophyllum commune*) (Peer *et al.*, 2009), filamentous fungi can close the septal pore by plugging it with a specialized organelle, which, in case of Ascomycetes, is called the Woronin body (Markham *et al.*, 1987). A recent study for *A. oryzae* (Bleichrodt, 2012) reported that 60% of the first three septa in substrate exploring hyphae were closed, independent of environmental conditions and the state of the other two septa. It was assumed that septal pore plugging in *A. oryzae* is a stochastic process, which consequently impedes cytoplasmic continuity and thus promotes hyphal heterogeneity. Indeed, monitoring glucoamylase gene expression, the authors demonstrated that hyphal heterogeneity was abolished in the Δ*hex*1 mutant, which does not form Woronin bodies and as a consequence cannot plug septal pores.

Autophagy

During surface growth on an isotropic substrate (e.g. agar plate cultures, a typical laboratory growth condition), colonies of filamentous fungi expand radially (see Figure 1.1). Hyphae at the periphery of the colony experience nutrient rich conditions, whereas the nutrient avail-

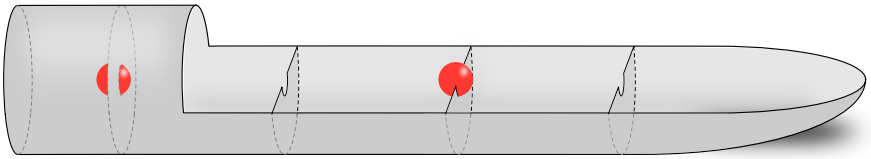

Figure 1.2 – Septated hypha
Schematic representation of the cell wall skeleton of a septated hypha. Hyphae are compartmentalized by septa, while compartments are connected via septal pores which allow intercompartmental trafficking of organelles, protein complexes, proteins and metabolites. Septal pores can be reversibly plugged to protect from cytoplasmic bleeding upon damage or to allow specification of compartments (e.g. commitment to conidiation.).

ability decreases towards the center of the colony, where nutrients eventually become depleted. Unlike colonies of unicellular microorganisms that form and expand by the production of daughter cells, colonies of filamentous fungi are able to grow beyond a nutrient depleted region, as for example, on a non-isotropic natural substrate, by providing hyphae at the periphery of the colony with resources from basal hyphal compartments. Interconnectivity of the mycelial network is essential for this foraging strategy and is a key-advantage of the filamentous lifestyle over unicellular microbes (Richie *et al.*, 2007).

Autophagy is a process that has been shown to be important for the maintenance of foraging hyphae in *A. fumigatus* (Richie *et al.*, 2007). Autophagy is a well conserved catabolic process constitutively active in eukaryotic cells. Together with proteasome-mediated degradation, autophagy is the major pathway for protein and organelle turnover being important for cellular homeostasis and maintenance (Reggiori *et al.*, 2002). A fine-tuned balance of biosynthetic (anabolic) and degradative (catabolic) pathways is essential to maintain cellular activity and allow adaptation to changing environmental conditions. There are two distinct mechanisms of autophagy, micro- and macroautophagy (Klionsky *et al.*, 1999). The first describes the invagination of cytoplasmic constituents directly at the vacuolar membrane, whereas the latter (from now on simply referred to as autophagy) involves engulfing cytoplasmic content in double-membrane vesicle cargos that finally fuse to the vacuole and become degraded (see Figure 1.3). Both non-specific bulk turnover of cytoplasm and organelle-specific forms of autophagy exist including pexophagy (degradation of peroxisomes), ribophagy (degradation of ribosomes), ERphagy (degradation of the endoplasmic riticulum) and mitophagy (degradation mitochondria) (Bernales *et al.*, 2007; Mao *et al.*, 2011). Although autophagy is known to be induced by nutrient starvation (carbon and nitrogen) and leads to recycling of building blocks from degraded cytoplasmic constituents, autophagy is not simply a recycling pathway as it is also clearly associated with cell death. Interestingly, autophagy has been shown to be both protective against and causative of cell death. It has been demonstrated in filamentous fungi,

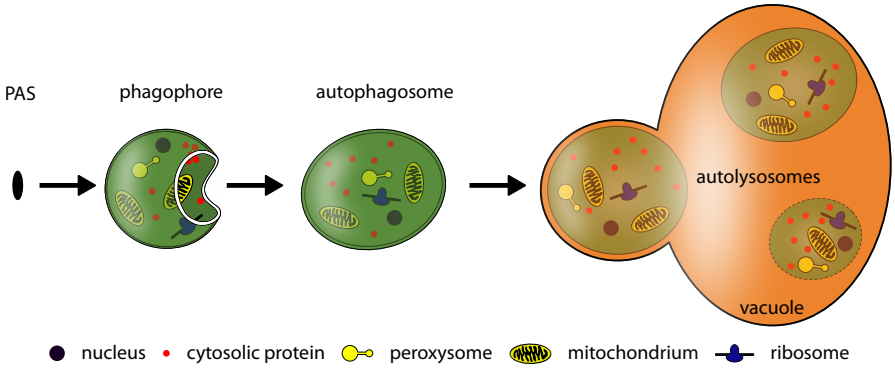

Figure 1.3 – Macroautophagy
Schematic representation of non-specific bulk macroautophagy. Macroautophagy is initiated by the formation of a pre-autophagosomal structure (PAS). Subsequently bulk cytoplasm becomes engulfed in double-membrane cargos that fuse to the vacuoles were they are finally degraded and building blocks are recycled.

for example, that autophagy protects against cell death during the heterokaryon incompatibility reaction (a mechanism of non-self recognition) in *Podospora anserina* (Pinan-Lucarré *et al.*, 2005) or during carbon starvation in *Ustilago maydes* (Nadal *et al.*, 2010). Contrary to this, autophagy induced cell death has been reported to be required for rice plant infection by *Magnaporthe grisea* (Veneault-Fourrey *et al.*, 2006). Shoji *et al.* (2011) suggest that autophagy in filamentous fungi might represent a final effort to endogenously recycle and translocate cytoplasmic resources to adjacent compartments prior to cell death. Shoji *et al.* (2010) even demonstrated autophagic degradation of whole nuclei for *A. oryzae* and suggested that nuclei might serve as storage for phosphorus and nitrogen in multinucleate organisms.

Programmed cell death

There is an immense body of literature on cell death but terms are frequently confused or inappropriately used (Lockshin *et al.*, 2004; Kroemer *et al.*, 2008). The increasing attention being paid to the field of cell death is explained by the fact that cell death plays a fundamental role in development, aging and serious human pathologies such as cancer and neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases. A brief, general introduction on programmed cell death (PCD) and a summary of a few important historic milestones followed by a more specific overview of fungal PCD are provided below.

The classical concept of PCD relates to the observation that fetal and larval structures regress during ontogenetic development of higher eukaryotes, a phenomenon that was possibly already known to Aristotle (384 BC – 322 BC) (Clarke *et al.*, 1996). However, it was not until the mid 19th century, when advancements in microscopy and histology allowed the observa-

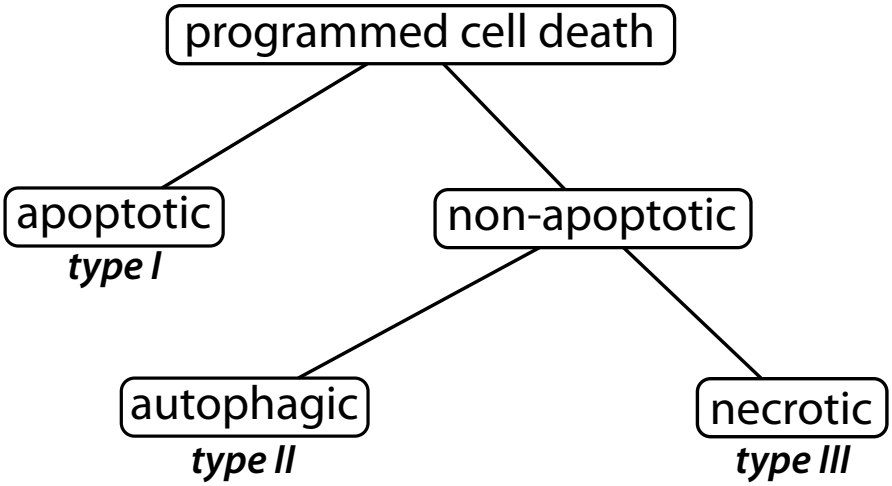

Figure 1.4 — Programmed cell death
Simplified flowchart showing the relationship of the three types of PCD: Type I (apoptosis), type II (autophagy) and type III (programmed necrosis or necroptosis).

tion of cells and the establishment of the cell theory by Schwann (1839) and Schleiden (1842), that cell death was first described and the concept of developmental cell death was initiated by Carl Vogt (Clarke *et al.*, 1996). In 1885, Walther Flemming was the first to suggest that cell death is caused rather by chemical changes within the cell than by mechanical disturbance. 80 years later, Lockshin *et al.* (1964) coined the term "programmed cell death" emphasizing the existence of a cell-intrinsic and genetically programmed developmental cell death. Around the same time, John Kerr histologically described a distinctive form of active necrosis, which he initially referred to as "shrinkage necrosis" and later coined it "apoptosis", a word that originates from the Greek word describing the falling-off of leaves (ἀπόπτωσισ) (Kerr *et al.*, 1972; Diamantis *et al.*, 2008).

In contrast to the traditional view which claims the existence of two principally distinct forms of cell death in higher eukaryotes, namely apoptosis as a programmed, cell-intrinsic suicide mechanism and necrosis as an unorganized, passive form of cell death, it has been suggested that cell death is generally organized and three types of PCD have been delineated (see Figure 1.4). Type I PCD refers to apoptosis, the traditional PCD, type II and type III PCD describe the non-apoptotic forms that are autophagy and programmed necrosis (also referred to as necroptosis (Galluzzi *et al.*, 2008)), respectively (Lockshin *et al.*, 2004). Subsequently, this clearly implies that the terms "apoptosis" and "programmed cell death" are not considered as synonyms (Kroemer *et al.*, 2008). It has to be noticed that in contrast to type I and type III PCD, which are clear cell death mechanisms, autophagy, as mentioned earlier, is also a normal

cell physiological process important for cellular homeostasis and maintenance. These different types of PCD can be distinguished by the presence or absence of certain sets of morphological and biochemical hallmarks which will not be outlined here. The interested reader is referred to a publication on cell death classification by Kroemer *et al.* (2008). Importantly, the authors emphasize that it is inappropriate to use specific biochemical assays as an exclusive means to define apoptosis, as this specific form of PCD can also occur without certain hallmarks such as internucleosomal DNA fragmentation. Interestingly Kroemer *et al.* (2008) say the following on autophagic cell death: "Although the expression 'autophagic cell death' is a linguistic invitation to believe that cell death is executed by autophagy, the term simply describes cell death with autophagy", thereby emphasizing that autophagy is simply associated with cell death. Indeed, depending on what is studied, autophagy has been demonstrated to be both causative of and protective against cell death.

Before proceeding with the description of PCD in fungi, it is worth noting that cell death in microbial and higher eukaryotes has substantially different consequences. For higher eukaryotes, ontogenetic, pathological or physiological cell death preserves the individual as an entity, whereas microbial cell death, especially in the case of unicellular eukaryotes such as *Saccharomyces cerevisiae*, has severe consequences, namely death of the individual organism. Although, of course, microbes act as communities and death of individuals does not lead to death of a microbial population. In multicellular filamentous fungi in particular, it is an interesting hypothesis that certain hyphal compartments undergo cell death for the benefit of the mycelial entity e.g. when confronted with life-threatening starvation conditions.

The best described form of PCD in fungi is type II. Interestingly, after the initial description of autophagy in mammalian cells in the 1960s, it was yeast as a genetically tractable model organism that considerably contributed to the understanding of the molecular mechanisms of autophagy in the 1990s (Yang *et al.*, 2010). The autophagic core machinery is highly conserved from yeast to man and more than 30 autophagy (*atg*) genes have been identified for *S. cerevisiae* and other fungi to date (Kanki *et al.*, 2011; Xie *et al.*, 2007; Meijer *et al.*, 2007). In contrast, only an ancestral core apoptotic machinery has been identified in fungi for type I PCD (Fedorova *et al.*, 2005) and the term apoptosis should be interpreted in a broader sense (Hamann *et al.*, 2008). For example, no orthologs of caspases, cystein-dependent aspartate-directed proteases, that play essential roles in mammalian apoptosis as initiator and effector enzymes, have been identified in fungi. Instead, it has been suggested that metacaspases are functional homologues of caspases in fungi (protozoa and plants). Metacaspases are cystein proteases that probably share the same ancestor as caspases (Hamann *et al.*, 2008). Similarly, orthologs of BCL-2-like proteins, which have pro- or anti-apoptotic properties, have not been identified in fungi, but their heterologous expression has been shown to affect apoptosis-like processes in yeast and

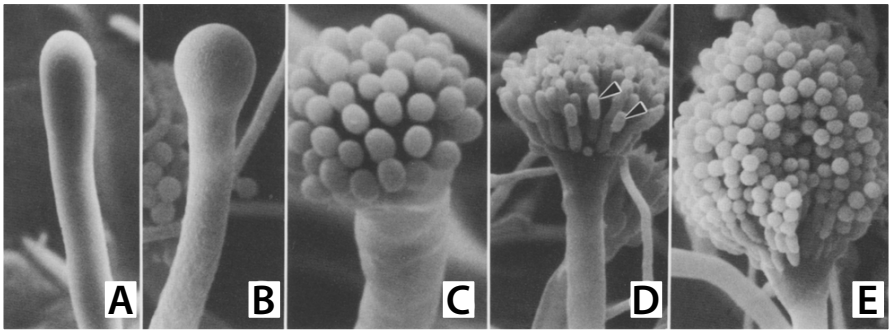

Figure 1.5 – Conidiophore development
Scanning electron micrographs showing different morphological stages of conidiophore development in *A. nidulans*: (A) Conidiophore stalk, (B) vesicle, (C) metulae, (D) phialides and (E) mature conidiophore with conidial chains. Adapted from Mims *et al.* (1988).

Colletotrichum gloeosporioides (Barhoom *et al.*, 2007; Owsianowski *et al.*, 2008). Cell death has been studied in fungi as valuable model organisms and in a variety of contexts of fungal biology including host defense mechanisms (e.g. oxidative burst and α-tomatin), sexual and asexual development, nutritional starvation, putative antagonistic mediators (e.g. antifungal proteins and farnesol), aging and heterokaryon incompatibility.

Carbon starvation, conidiation and autolysis in submerged cultures

A. niger belongs to the fungi imperfecti (Deuteromycota) as a sexual life cycle has never been observed for this species. Consequently, the only (known) reproductive mode is conidiation, the formation of asexual spores, so called conidia (see Figure 1.5E). Those conidia are airborne, highly melanized (dark pigmentation) and hydrophobic haploid cells which are more resistant to harsh conditions than vegetative cells and thereby improve the chance of successful proliferation of the fungus. Once a conidium meets the right conditions it can germinate and form a new mycelium, completing the life cycle (see Figure 1.1).

Asexual differentiation and the underlying spatio-temporal regulatory mechanisms leading to the formation of conidiophores, spore bearing structures consisting of multiple cell types (see Figure 1.5), were studied in detail in the model fungus *A. nidulans* (Adams *et al.*, 1998). Core genes involved in signal transduction and asexual development (see Figure 1.6) have also been identified in *A. niger* (Pel *et al.*, 2007), suggesting that the regulation of asexual development is conserved.

In the review by Adams *et al.* (1998) a model has been established describing the interactivity of important developmental regulators controlling vegetative growth and development in *A. nidulans* (see Figure 1.6). A central and early developmental regulator for asexual devel-

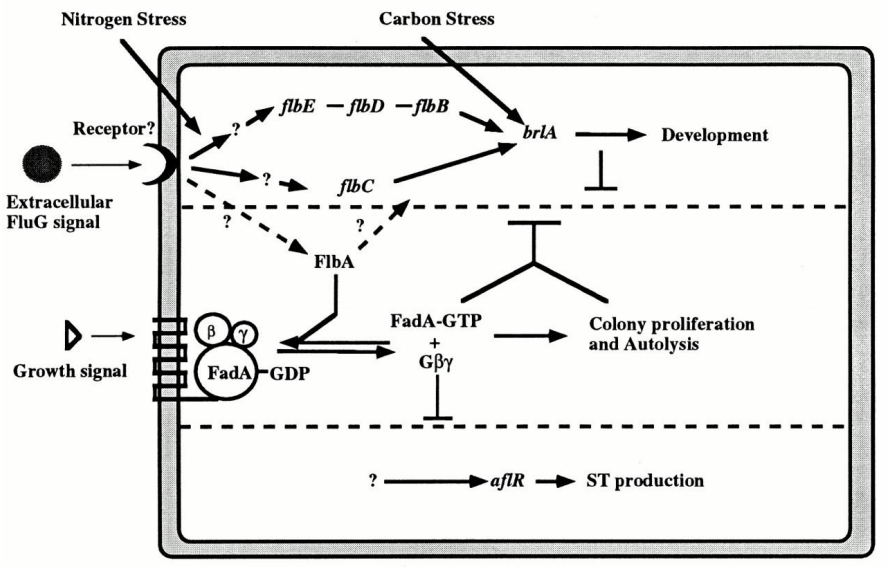

Figure 1.6 — Regulation of asexual development
Two antagonistic pathways were supposed to promote either vegetative growth or asexual development and secondary metabolism in *A. nidulans*. In its GTP-bound form, the Gα subunit FadA promotes vegetative growth and inhibits asexual development as well as secondary metabolism. Early developmental signals lead to the activation of FlbA, a regulator of G-protein signaling, which in turn enhances the FadA intrinsic GTPase activity and consequently leads to growth arrest, initiation of asexual development and activation secondary metabolism. Adapted from Adams *et al.* (1998)

opment is the transcriptional activator BrlA (bristle A), which acts downstream of the regulatory circuit consisting of five proteins FlbA-FlbE encoded by the so called fluffy genes *flbA* to *flbE*. BrlA induces its own transcription and that of downstream regulators including AbaA and WetA. Due to its autoregulation, BrlA mRNAs accumulate strongly during conidiation. Deletion of *brlA* completely blocks asexual differentiation at the stage after the formation of conidiophore stalks (see Figure 1.5A), giving mutant colonies a bristle-like appearance.

The transition from vegetative growth to asexual development is regulated by a heterotrimeric G-protein consisting of Gα, Gβ and Gγ subunits. In its active GTP-bound form, the Gα subunit FadA dissociates from Gβγ promoting colony proliferation and blocking asexual differentiation. FlbA, a regulator of G-protein signaling, is thought to enhance the intrinsic GTPase activity of FadA, whereby FadA becomes converted into its inactive GDP-bound form which associates with Gβγ forming the heterotrimeric Gαβγ complex. Consequently, vegetative growth is blocked and asexual development is initiated. Depending on upstream signals, FlbA is thus required for the initiation of conidiation by the inactivation of FadA. Similar to colonies of other mutants of the fluffy genes, FlbA mutant colonies have a fluffy a-conidial

appearance. However, in contrast to other fluffy mutants, the colony centers of FlbA mutants begin to disintegrate after about three days of growth resulting in collapsing of aerial hyphae and autolysis.

Although development and differentiation have mostly been studied at a substrate/air interphase, these processes also take place in submerged cultures under carbon or nitrogen starvation. Submerged cultivation of filamentous fungi can be accomplished by shaking liquid cultures or by cultivation in bioreactors. Aside from the industrial applicability, the latter has several advantages including high reproducibility, monitoring and controlling of physiological parameters. A number of recent studies for *A. niger* (Jørgensen *et al.*, 2009; Jørgensen *et al.*, 2010; Jørgensen *et al.*, 2011) have demonstrated the potential of bioreactor cultivation to dissect fungal physiology and development.

As described above, nutrient starvation in submerged cultures of filamentous fungi induces asexual development. In addition, complex physiological and morphological changes occur, leading to disintegration of the fungal biomass. These self-degradative processes are generally referred to as fungal autolysis and hallmarks are described to include biomass decline, hyphal fragmentation, emergence of empty hyphae, increase of extracellular hydrolase (proteases and glycosyl hydrolases) activities and extracellular ammonium (White *et al.*, 2002). Subsequently, cytological heterogeneity increases and it has been suggested that recycled resources fuel the maintenance of surviving compartments, conidiation and cryptic growth, a term used to describe re-growth under starvation conditions (Trinci *et al.*, 1969; Bainbridge *et al.*, 1971; McNeil *et al.*, 1998). As a consequence, yields of bioprocesses can be negatively affected e.g. by hydrolytic degradation of products, decreasing active biomass fractions or even problems during filtration (filter plugging by hyphal fragments) in downstream processing (White *et al.*, 2002; McNeil *et al.*, 1998). However, in some cases autolysis might even be desirable for the recovery of intracellular products.

Depending on the study, hydrolytic weakening of the fungal cell wall in aging nutrient deprived cultures of filamentous fungi has been reported to be either a key-characteristic or not evident. Excessive hyphal fragmentation has been reported, for example, for cultures of *P. chrysogenum* (McNeil *et al.*, 1998) and *A. nidulans* (Emri *et al.*, 2004), whereas another investigation with *A. nidulans* (Bainbridge *et al.*, 1971) stated that there were no cytological indications for lysis of cell walls. Studies with *Neurospora crassa* (Martinez *et al.*, 1969) and *A. niger* (Lahoz *et al.*, 1986) even assert that the stability of fungal cell walls in cultivations lasts up to 60 days.

The fungal cell wall is an essential component that determines the shape of the fungus, prevents it from lysis and protects it from the environment. It is rigid but dynamic and adapts its composition and molecular structure during spore germination, growth, development and in

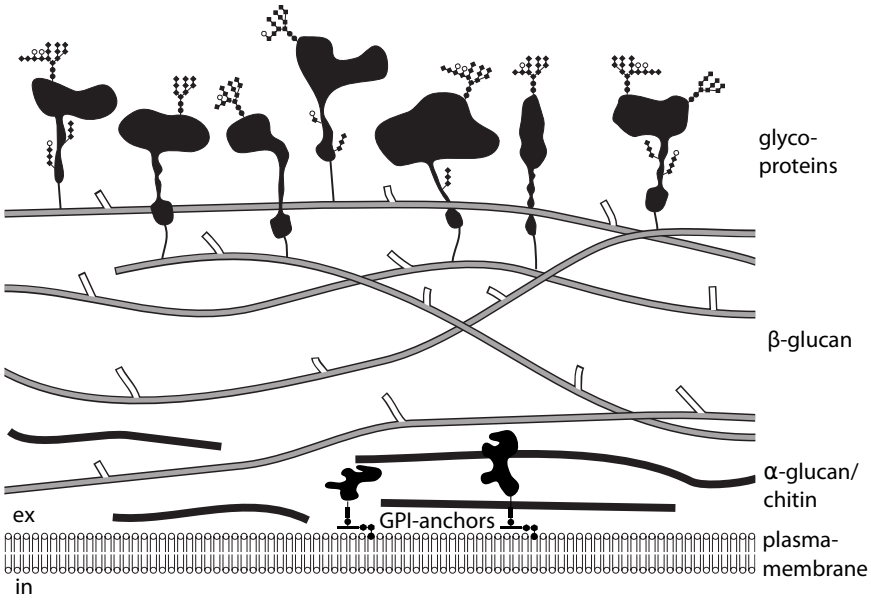

Figure 1.7 – The fungal cell wall
Schematic representation showing the organization of the different cell wall components: α-glucans, β-glucans, chitin and glycoproteins. Adapted from Grün (2003).

response to stress. The three major components of fungal cell walls are glycoproteins, glucan (mainly 1,3-β-glucan) and chitin (1,4-β-N-acetylglucosamine) polymers (see Figure 1.7). In general, there are considerable differences in the cell wall compositions between filamentous fungi and yeasts such as *S. cerevisiae* (Nobel *et al.*, 2000). Glucans and glycoproteins are the two major components of yeast cell walls, respectively, with 35-60% and 30-50% of the cell wall dry weight. Chitin is only a minor component accounting for 1.5-6% of yeast cell wall dry weight (Klis *et al.*, 2006). In comparison, the chitin content in the cell walls of filamentous fungi is up to 10-fold higher, amounting to 10-15% of the cell wall dry weight. Further differences in the cell wall composition of fungal species can be found in their glucan polymer compositions. For example, α-glucans are not present in the cell walls of *S. cerevisiae* and *Candida albicans*, whereas they are important cell wall polymers in many filamentous fungal species, including *A. niger* (R. A. Damveld *et al.*, 2005).

In particular, the role of chitinases in hydrolytic weakening of fungal cell walls during maintained nutrient deprived cultures of filamentous fungi has been studied in considerable detail. The most prominent autolytic chitinase is ChiB which has been identified as the major extracellular chitinase during the autolytic phase of carbon starved submerged cultures of *A. nidulans* (Pusztahelyi *et al.*, 2006). Deletion of *chiB* in *A. nidulans* has been shown to reduce

hyphal lysis in aging cultures (Yamazaki *et al.*, 2007). However, the role of *A. fumigatus* ChiB in cell wall weakening during carbon starvation is less clear. Although ChiB has been shown to be the major enzyme contributing to extracellular chitinolytic activity, its deletion did not affect the autolytic phenotype of *A. fumigatus* (Jaques, 2003). During autolysis, chitin is thought to be degraded in two successive steps involving degradation of chitin polymers by chitinases and subsequent degradation of chitin oligomers, mainly chitobiose, by enzymes having β-N-acetylglucosaminidase activities (Kim *et al.*, 2002). One such β-N-acetylglucosaminidase is NagA, which has been shown to be simultaneously early induced with ChiB upon carbon depletion in *A. nidulans* (Pusztahelyi *et al.*, 2006).

Based on the analysis of several developmental mutants of *A. nidulans* including FluG1, $\Delta brlA$, $\Delta flbA$-$\Delta flbD$ and $fadA^{G203A}$, Pósci and colleagues demonstrated that there is a relation between conidiation and autolysis and that both share common regulatory elements (Molnár *et al.*, 2004; Emri *et al.*, 2005b). For example the loss-of-function *fluG* mutant has been reported to have a non-autolytic phenotype with reduced biomass decline, low chitinase and protease activities as well as severely reduced hyphal fragmentation (Emri *et al.*, 2005b).

Outline of the thesis

This thesis aims at elucidating processes that are induced during carbon starvation in submerged cultures of the industrially important filamentous fungus *Aspergillus niger*. Carbon starvation has been achieved by prolonged cultivation in carbon-limited bioreactor batch cultures. During carbon starvation in aging batch cultures, carbon and energy for maintenance of surviving compartments, cryptic re-growth and asexual development are exclusively available by recycling of endogenous and exogenous resources. This is in contrast to cultivations with pulsed or continuous feeding regimes such as fed-batch or retentostat cultures, where requirements of carbon and energy are (partially) met by the fed substrate. The complex physiological changes during carbon-starved batch cultures were studied and described on a systems level covering physiology, morphology, transcriptomics and secretomics.

Chapter 1 provides an introduction to filamentous fungi, their importance as industrial production hosts and pathogens. Some characteristic traits of filamentous fungi contributing to their lifestyle are briefly introduced in general and/or with respect to nutrient (carbon) starvation.

Resources required for the analysis of omics data from *A. niger* were established and described in chapters 2 and 3. Chapter 2 focuses on transcriptomic data analysis which can generally be considered as a multi-step approach including primary quality assessment, normalization, background correction and condensation of raw data followed by computation of

differentially expressed or co-transcribed genes. Using public *A. niger* datasets, basic steps of transcriptomic data analysis with the open source statistical programming language R were described in a step-by-step tutorial and important theoretical background on the required statistics has been provided. In chapter 3, *A. nidulans* Gene Ontology (GO) annotation was mapped to all Aspergilli with published genome sequences and an online tool for GO enrichment analysis (FetGOat: http://www.broadinstitute.org/fetgoat/index.html) was implemented, thus establishing new resources that strongly facilitate omics data analysis.

Subsequently, in Chapter 4 the above tools for omics data analysis were applied in combination with physiological and morphological analyses to investigate carbon starvation in submerged batch cultures of *A. niger* on a systems level. This description provides a framework for detailed analysis of specific processes induced under carbon starvation conditions. Autophagy and conidiation were among the predominantly induced processes. Subsequently, in chapter 5 the role of autophagy during carbon starved batch cultivation was investigated by deletion of essential autophagy genes and phenotypic characterization during surface growth and submerged cultivation in carbon-limited batch cultures. Fluorescent microscopy and automated image analysis provide evidence that autophagy promotes survival by physiological adaptation to carbon starvation.