General Introduction

1. The importance of filamentous fungi in product formation

The fungal kingdom is estimated to exist of over 1.5 million species among which only a small number of species have been described in detail (Hawksworth 2001). There is a huge diversity among fungi, both in their morphology as well as in the products they can produce. Morphologically, the fungal kingdom includes unicellular yeast cells as well as multi-cellular hyphal cells that form a mycelium. Hyphal cells grow exclusively at the tip of the hyphae in a highly polarized way (Momany 2002). Many filamentous fungi are saprophytes meaning that they feed on dead organic material. It is, therefore, necessary for them to secrete large amounts of extra-cellular hydrolytic enzymes to decompose these often complex organic materials in nature.

For centuries, filamentous fungi have been used in many traditional food processes such as cheese-making, sake (rice wine) and soy sauce production. Nowadays, filamentous fungi are also used as cell factories in biotechnology to produce a wide variety of products such as enzymes, primary and secondary metabolites including organic acids and antibiotics. Filamentous fungi have been explored as expression hosts for protein production of both fungal and non-fungal origin (Punt et al. 2002; Su et al. 2012). So the industrial application areas utilizing filamentous fungi are very diverse including food and beverage, pulp and paper, feed and pharmaceuticals (Fleissner and Dersch 2010; Meyer 2008; Saloheimo and Pakula 2012).

Relatively few species have been developed into a commercially exploited filamentous fungal expression system. The most frequently used industrial fungi include *Trichoderma reesei, Aspergillus oryzae*, *A. niger* and *Myceliophthora thermophila* (previously known as *Chrysoporium lucknowense* C1 (Berka et al. 2011; Verdoes et al. 2007; Visser et al. 2011)). *T. reesei* has mainly been used for the production of cellulases and other plant cell wall-degrading enzymes (Saloheimo and Pakula 2012; Schuster and Schmoll 2010). Fermented foods including soy sauce and sake are produced by *A. oryzae* (Machida et al. 2008) and citric acid and amylases that are used in the beverage and food industry respectively are mostly produced by *A. niger* (Pel et al. 2007). *M. thermophila* is a recently described fungal expression system for neutral cellulases and heterologous protein production (Berka et al. 2011; Visser et al. 2011). Recently, increased attention has been paid to second generation feedstocks using wheat straw or sugar cane bagasse. Compared to first generation feedstocks, these are cheaper more environmentally friendly and less competitive in

relation to food supply (Kumar et al. 2008; Zheng et al. 2012). Filamentous fungi have been shown as predominant hosts for the enzymes that are used for the saccharification process (Gusakov 2011; Sims et al. 2010) as well as for the direct microbial conversion from crude feedstock into bioproducts (Beeson et al. 2011; Lin et al. 2010; Rumbold et al. 2009; van den Brink et al. 2013). *A. niger* has also been shown as a potential and promising host for those processes (Delmas et al. 2012; Rumbold et al. 2010; Rumbold et al. 2009).

2. Factors affecting the productivity

A successful commercialization of fungal enzymes in relation to lignocellulose degradation or the production of metabolites is only possible when these proteins or metabolites are efficiently produced. Productivity is affected by many factors such as culture conditions, morphology and the fungal host strain. In the next sections, these different factors are discussed in more detail.

2.1. Culture conditions

Different culture conditions including the composition of culture medium, pH, temperature and the type of cultivation affect product formation. The carbon source is not only important as energy source, but also an important factor that decides which product is to be formed. For example, when starch or maltose is used as a carbon source, amylolytic enzymes including glucoamylase (GlaA) are highly induced and secreted, whereas xylose induces the synthesis of (hemi)cellulolytic enzymes (de Oliveira et al. 2011). Galacturonic acid, the main component of pectin induces the expression of pectinolytic enzymes (Martens-Uzunova and Schaap 2009). In addition, the carbon source affects the productivity. It has been shown that the production rate of extracellular proteins is three times higher on maltose than xylose (Jørgensen et al. 2009). Besides the carbon source of the medium, the pH, temperature and agitation speed of culture medium can affect the productivity. Withers et al. found that the GlaA productivity was not affected in the pH range 3.5-5.5 and culture temperature 30 and 37^{o}C, but significantly reduced at pH 6.5 and at 25^{o}C (Withers et al. 1998). In addition, the effect of pH on extracellular protease activity in relation to the heterologous protein (GFP) production was investigated using P*gpdA*-GlaA-GFP in which about ten times higher GFP was yielded at pH 6 compared to pH 3 because of lower protease activity (O'Doonnell et al. 2001).

The cultivation method such as solid-state or submerged cultivation can influence the protein production (Gamarra et al. 2010; Oda et al. 2006; te Biesebeke et al. 2006). Using the same substrate (wheat bran), te Biesebeke et al. found important differences in the

secretome between submerged and solid state conditions. Whereas the enzyme α-amylase was identified under both conditions, arabinosidase and xylanase were abundantly produced in submerged cultures, while a chitinase and two new proteins that were thought to be involved in filamentous fungal-specific functions were produced in solid state cultures in *A. oryzae* (te Biesebeke et al. 2006). The culture condition in which the fungus grows can also affect the product formation. For example, Jørgensen et al. reported that transcript levels of genes encoding secondary metabolites such as potential polyketide synthase (PKS), nonribosomal peptide synthetase (NRPS) and small cysteine-rich proteins (e.g. hydrophobines) were enhanced at near-zero growth conditions. The expression of extracellular hydrolases (e.g. GlaA) was strongly downregulated under these conditions (Jørgensen et al. 2010).

2.2. Fungal morphology

The morphology of filamentous fungi has a strong influence on their production properties (Grimm et al. 2005; McIntyre et al. 2001; Papagianni 2004). In submerged cultures, filamentous fungi grow either as freely dispersed mycelium including loosely intertwined hyphae (dispersed mycelium), as clumps or as pellets (Grimm et al. 2005; Riley et al. 2000). The preferred morphology cannot be generalized because it varies depending on the product. In *A. niger* pelleted forms are preferred for citric acid production while dispersed filaments of *A. niger* are favored for enzyme production (Braun and Vecht-Lifshitz 1991; Gibbs et al. 2000; Papagianni 2004). In addition, the growth-form can strongly affect mixing and mass transfer properties. Dispersed long hyphae cause high viscosity of culture broth and these long hyphae are sensitive to shear forces in a bioreactor. Grown as pellets, the mycelia is less viscous but is confronted with difficulties of nutrient and oxygen access inside the pellet (Gibbs et al. 2000; Grimm et al. 2005). Thus, the preferred fungal macromorphology would consist of loosely grown mycelia with short filaments derived from an optimum branching frequency to ensure the high productivity as well as better nutrient and oxygen supply for the cells (Grimm et al. 2005; Kaup et al. 2008; Papagianni 2007). Several studies identified multiple factors affecting fungal morphology which include agitation, medium composition, temperature and the way of inoculating cultures (Amanullah et al. 2002; Papagianni 2004; Wucherpfennig et al. 2010). In addition, a recent study showed that the age of conidia also influenced the adhesion properties thereby leading to different diameters of pellets (Colin et al. 2013). Addition of talc, alumina, or titanate microparticles was used to manipulate different morphology states to increase product formation in *A. niger* and other filamentous microorganisms (Driouch et al. 2012; Driouch et al. 2010; Kaup et al. 2008).

It is generally accepted that protein secretion occurs mainly at the hyphal apex as demonstrated by several studies e.g. localization of glucoamylase or α-amylase at hyphal tips (Gordon et al. 2000; Muller et al. 2002; Wösten et al. 1991). Some studies suggested a positive correlation between the amount of hyphal branches and protein secretion yields (Amanullah et al. 2002; Muller et al. 2002; te Biesebeke et al. 2005; Wongwicharn et al. 1999), while other reports demonstrated no correlation (Bocking et al. 1999; McIntyre et al. 2001). Our recent study showed that simply making more hyphal tips did not result in an increase of a protein production level using a hyperbranching mutant (Kwon et al. 2013b). Therefore, it is still a matter of debate whether a hyperbranching strain could improve protein production yields. Recently, it has been shown that the secretion also takes place at the septa using GFP fused α-amylase (AmyB-GFP) in *A. oryzae* (Hayakawa et al. 2011).

2.3. The fungal host strain

Filamentous fungi are main sources for the production of many commercial enzymes and organic compounds. Products of each fungal host strain can overlap but also be distinct (Table 1). *M. thermophila*, *T. reesei*, *A. oryzae* and *A. niger* are very efficient for the production of proteins. On one hand, several enzymes such as glucose oxidase, protease and phytase are produced from both *A. oryzae* and *A. niger*, or xylanase is produced from *T. reesei* and *A. niger*. Plant-polysaccharide-degrading enzymes such as cellobiohydrolases are produced from *T. reesei* and *M. thermophila* (Visser et al. 2011). On the other hand, certain products are only specifically produced from certain fungi: itaconic acid from *A. terreus*, kojic acid from *A. oryzae*, citric acid mainly from *A. niger* (Table 1). Here we focus on *A. niger* and why and/or how this fungus possesses high protein secretion capacity.

3. The protein secretion capacity of *A. niger*

A. niger is well known for its tremendous capacity to secrete proteins into the culture medium and is used as a cell factory not only for native products e.g. glucoamylase (GlaA), but also for heterologous protein production (Meyer 2008; Meyer et al. 2011b; Pel et al. 2007). The complete genome sequence comparison of several fungi including *A. nidulans* and *S. cerevisiae* revealed that the number of genes related to protein secretion do not correlate with the secretion capacity of the species (Pel et al., 2007). It still remains unknown what makes *A. niger* a good protein secretor. In the following section, a few potential mechanisms are described which could contribute to the high secretion capacity of *A. niger*.

3.1. Uncoupling of growth and secretion

Growth and secretion in fungi are considered to be tightly linked processes. The high capacity of protein secretion of *A. niger*, however, cannot be explained by a more rapid growth of *A. niger* in comparison to e.g. *S. cerevisiae* or *A. nidulans* which secret much lower levels of proteins. Therefore, it is possible that *A. niger* has developed mechanisms in which growth and high levels of protein secretion can be uncoupled. One possible mechanism could be the existence of parallel secretory pathways that independently deliver proteins for secretion (e.g. GlaA) and proteins related to growth (e.g. plasma membrane proteins and cell wall synthesizing enzymes) to the cell surface. Several studies including studies in fungi, plants and mammalian cells show that different populations of Golgi derived vesicles exist. In *S. cerevisiae* two populations of vesicles have been described: one is characterized by the presence of an enzyme involved in cell wall biosynthesis (Bgl1p) and the major plasma membrane ATPase (Pma1p) and the other population contains secreted enzymes, invertase and acid phosphatase (Harsay and Bretscher 1995). In *Yarrowia lipolytica*, mutants have been identified that are specifically affected in the export of plasma membrane and cell wall associated proteins but not in the secretion of extracellular proteins (Titorenko et al. 1997). Also in plants, it has been demonstrated that secretory proteins and cell wall synthase complexes move to different secretory pathways (Leucci et al. 2007).

Table 1. Selected examples of industrially important acids and enzymes produced by filamentous fungi.

Compound	Organism	Main application areas
Acids		
Citric acid	*A. niger*	Food and beverage
Itaconic acid	*A. terreus*	Polymer
Kojic acid	*A. oryzae*	Food
Enzymes		
α-Amylase	*A. niger, A. oryzae*	Starch processing and food
Cellulase	*Trichoderma viride, T. reesei, M. thermophila*	Textile, pulp and paper
Cellobiohydrolase	*T. viride, T. reesei, M. thermophila*	Textile, pulp and paper
Glucoamylase	*A. phoenicis, Rhizopus delemar*	Starch processing
Glucose oxidase	*A. niger, A. oryzae*	Textile and Biosensor
Lipases	*A. niger, A. oryzae*	Food and detergent
Pectin lyase	*T. reesei*	Food
Proteases	*A. niger, A. oryzae, R. delemar*	Food and detergent
Phytase	*A. niger, A. oryzae*	Food
Xylanases	*T. reesei, T. konignii, A. niger, M. thermophila*	Textile, pulp, paper and bakery

Table is adapted from (Meyer 2008) with a small modification in which *M. thermophila* was included (Ustinov et al. 2008; Visser et al. 2011).

A study in the filamentous fungus *T. reesei* revealed the possible presence of more than one pathway for exocytosis based on spatial segregation of different SNARE complexes in the fungal tip cell (Valkonen et al. 2007). More recently, a distinct secretory route that occurs behind the apex independently of the Spitzenkörper was demonstrated in *Neurospora crassa* (Fajardo-Somera et al. 2013). There a GFP-tagged plasma membrane (PM) H+-translocating ATPase (PMA-1) localized at the subapical PM (>120 μm), but not at the tip or in the Spitzenkörper was described. In addition, fluorescence recovery after photobleaching (FRAP) analysis suggested that PMA-1 was incorporated directly into the PM indicating an alternative secretion pathway in filamentous fungi (Fajardo-Somera et al. 2013). Also in *A. niger*, the existence of parallel secretion pathways has been suggested based on the phenotype of the *srgA* mutant. SrgA is the ortholog of the Sec4 protein, a Rab GTPase essential for exocytosis and cell viability in *S. cerevisiae*. In *A. niger,* deletion of *srgA* is not lethal, but the extracellular protein production on glucose was severely reduced while the production on maltodextrin was only marginally reduced (Punt et al. 2001). These results might be explained by postulating the existence of the two different secretion pathways: a constitutive pathway, highly dependent on SrgA and an additional, inducible pathway that is less dependent on the function of SrgA. We postulate that these parallel secretion pathways are present in all fungi and are used by *A. niger.* Possibly also other high secretors are using parallel secretion pathways to be able to increase secretion capacity of extracellular enzymes.

3.2. More efficient COPII machinery

Secretory proteins and enzymes destined for secretion follow a conventional endoplasmic reticulum (ER)-Golgi secretory route. Once properly folded and glycosylated in the ER, secretory proteins are packed into coat protein complex II (COPII)-coated vesicles and transported to the Golgi complex. The COPII core machinery comprises five proteins; Sar1, a secretion-related RAS1 GTPase and two subcomplexes, Sec23/24 and Sec13/31 (Brandizzi and Barlowe 2013). The assembly of COPII coat is initiated by Sar1 activation in which inactive and soluble Sar1-GDP is converted into the active and membrane bound form of Sar1-GTP by a guanine nucleotide exchange factor (GEF), Sec12, which is an ER resident membrane protein (Barlowe and Schekman 1993). Activated Sar1 then recruits the 'inner layer', Sec23/24, through binding to Sec23 (Bi et al. 2002). Sec24 serves as a main COPII adaptor that specifically recognizes ER export signals in cargo (Brandizzi and Barlowe 2013). Finally the 'outer layer', Sec13/31, is recruited and formed into a cage structure and COPII-coated vesicle budding is completed by membrane curvature and fission (Brandizzi and Barlowe 2013). Although the five proteins (Sar1, Sec23/24 and

Sec13/31) are the minimal core that can form COPII vesicles from membranes *in vitro* (Barlowe et al. 1994; Matsuoka et al. 1998), other elements are also required such as Sec16 that facilitates COPII vesicle formation at the ER (D'Arcangelo et al. 2013).

One of the reasons why *A. niger* possesses such an outstanding secretion capacity could be an efficient packaging of cargo via the COPII machinery. Although the information about COPII-coated vesicle transport available for *A. niger* is sparse, our transcriptomic study using the glucoamylase (GlaA) overproducing strains hints to a role of COPII machinery under high secretion conditions. By comparing the transcriptomes of a GlaA over-producing strain to a wild-type strain (Chapter 4), it was found that the expression levels of several COPII-related genes were induced. These induced genes include the five COPII core genes (Sar1, Sec23/24 and Sec13/31), Sec16 as well as four ER vesicle proteins (Ervs, -29, -14, -41, -46). These four Ervs are ER resident proteins that are packed into COPII vesicles; Erv29 is involved in trafficking of multiple soluble cargo proteins, Erv14 is required for the ER exit of many integral membrane proteins, Erv41 and Erv46 forms a complex to be involved in the membrane fusion stage of the ER to the Golgi transport (D'Arcangelo et al. 2013; Otte et al. 2001). In total 16 of the 26 genes related to the COPII vesicle transport were differentially expressed in the GlaA overexpression strain, making it one of the most significantly enriched Gene Ontology (GO) (Chapter 4, Table A4). Although this data does not directly indicate that the COPII machinery is more efficient compared to others, it can show at least that *A. niger* can modulate its secretory machinery (or at least COPII machinery) depending on the loads of secretion which may enable this fungus to secrete high. Further studies such as the investigation of the role of cargo recruiting proteins (Sec24 and Erv proteins) or COPII-coated vesicle formation in *A. niger* could give more insights into the role of cargo recruitment in relation to efficient protein secretion.

3.3. Heterogeneity and super-secreting hyphae in *A. niger*

The two methods most often used to grow filamentous fungi are on agar plates or in liquid cultures (shake flasks or bioreactors). In *A. niger* liquid cultures, inoculation with fungal spores results in the formation of micro-pellets with a diameter size up to a few millimeter. On plates, the fungal colony can be divided into several zones covering the centre of the colony towards the edge of the colony (Levin et al. 2007). Heterogeneity in *A. niger* is observed at the colony level (zonal difference), pellet level as well as at the hyphal level with respect to growth, gene expression and protein secretion (de Bekker et al. 2011a; de Bekker et al. 2011b; Krijgsheld et al. 2012; Levin et al. 2007; Vinck et al. 2011; Vinck et al. 2005; Wösten et al. 1991). Only a limited region of a plate colony can grow and secrete

proteins namely the central and periphery zone of the colony, however, glucoamylase is secreted only at the periphery zone (Wösten et al. 1991). Secretome analysis revealed that the secreted proteins are different between zones of macro-colonies (Krijgsheld et al. 2012). Even within one zone, the peripheral zone, the secretion and expression of genes are different (Vinck et al. 2011; Vinck et al. 2005). By using a reporter strain that expresses GFP under the control of the glucoamylase promoter (P*glaA*) it was demonstrated that there are two different populations of hyphae present at the periphery of the colony: one population that has high and another one with low GFP fluorescence (Vinck et al. 2005). Similar results were obtained using different promoters, *aguA*, *faeA* or *aamA* (Vinck et al. 2011). Surprisingly, even among neighboring hyphae, gene expression profiles are different (de Bekker et al. 2011a).

Heterogeneity has also been observed at the level of micro-pellets. Statistical analysis of the population of cell pellets indicated two different populations with respect to size and gene expression using GFP reporter strains under control of P*glaA*. Larger pellets with an average diameter of 595 µm represented 61% of the cultures, while smaller pellets with an average diameter of 505 µm accounted for 39% (de Bekker et al. 2011b). A high fluorescent population was observed in 32% whereas 68% showed a low fluorescence (de Bekker et al. 2011b). The ratio of highly or lowly expressed populations was not the same but different among different strains e.g. P*faeA*, P*aamA* (de Bekker et al. 2011b). In addition, heterogeneous mRNA accumulation was shown within the pellets showing zonal differences (de Bekker et al. 2011b). Heterogeneity of macro-colonies can also be found in other filamentous fungi such as *Neurospora crassa* and *A. oryzae* (Kasuga and Glass 2008; Masai et al. 2006; Moukha et al. 1993), however, studies are not as detailed as in *A. niger* (see above); only the difference in zonal gene expression or secretion was studied.

So what can possibly make *A. niger* a better secretor than the others? In *A. niger*, different populations of hyphae seem to be present that may act as high or low producers. It will be of interest to examine whether the heterogeneity on the hyphal level is also found in other fungi. If a higher percentage of so called ‘super secreting hyphae’ is only found in *A. niger* this might explain the difference. Yet another aspect to study the process in more detail is to try to convert all hyphae in a culture into super secreting hyphae and thereby further improve protein production. To address this, it is important to establish the molecular mechanism by which a normal hypha is converted into a ‘super secreting hypha’.

4. The future is on the dynamics

Once a secretory protein is translated, it has to travel through the secretory pathway (see above). It may be possible that *A. niger* has a higher rate of secretion compared to other

fungi. To address that, a kinetic study of protein synthesis and secretion needs to be followed in the future as has been performed in *T. reesei*. Using *in vivo* labeling experiments, the average synthesis time and secretion time of CBHI cellobiohydrolase were measured as 4 min and 11 min respectively in *T. reesei* (Pakula et al. 2000). Secretion time of *T. reesei* was somewhat slower compared to the yeast, *S. cerevisiae* in which invertase transport time was 5 min (Novick et al. 1981; Pakula et al. 2000). Since the kinetic data for the protein synthesis and secretion in filamentous fungi are scarce, it will be of interest to determine the kinetics and compare the results among fungi in the future.

Several recent studies have indicated the important interplay between endocytosis and exocytosis in relation to protein secretion. It is proposed that exocytosis occurs at the hyphal tips in filamentous fungi, whereas endocytosis occurs behind the tip (Taheri-Talesh et al. 2008). Exocytosis is visualized using GFP-tagged reporters (GFP-SynA in *A. nidulans* (Taheri-Talesh et al. 2008), AmyB-GFP in *A. oryzae* (Hayakawa et al. 2011) or GFP-SncA in *A. niger (Kwon et al. 2013b)*) showing the signals along the hyphae but more pronounced at the hyphal tips (Chapter 3 & 5). The endocytic machinery was shown to be a ring-like structure that is excluded from the hyphal apex and localized a few μm behind the growing apex (Araujo-Bazan et al. 2008; Kwon et al. 2013b). Polarized secretion and growth require the delivery of cell wall biosynthetic enzymes as well as the delivery of extracellular enzymes via secretory vesicles. The retrieval and recycling of the excess membrane used for the delivery via secretory vesicles is necessary and is taken care of via endocytic processes. Further analysis of a turn-over rate for endo/exocytosis will provide important information with respect to the secretion capacity.

Secretory related organelles e.g. ER, Golgi and secretory vesicles are most important serving as the folding, modification and delivering station, respectively. The localization of these organelles is abundantly present through the hyphae especially in the apical area (Carvalho et al. 2011b; Markina-Inarrairaegui et al. 2013; Pantazopoulou and Penalva 2009; Pinar et al. 2013). In the past few years, these organelles have been successfully labeled with fluorescent markers and following their localization and dynamics under conditions of high protein production could give further insights into the mechanism by which filamentous fungi including *A. niger* secrete high amounts of proteins.

5. Aim and Thesis Outline

The research described in this thesis aims to get more fundamental insights in the molecular mechanisms used by *Aspergillus niger* in relation to control morphology and protein secretion. Knowledge on these two aspects is highly relevant to further optimization of *A. niger* as a cell factory.

Chapter 1 introduces what is important in relation to product formation in fungal biotechnology and mechanisms are discussed to explain the high secretion capacity of *A. niger*. In **Chapter 2**, the functional analysis of all six Rho GTPases encoded in *A. niger* (RacA, CftA, RhoA, RhoB, RhoC, RhoD) is described and revealed that they exert distinct and overlapping functions during the life cycle. Interestingly, a comparison of the function of Rho-GTPases among Aspergilli (in particular the comparison between *A. niger* and *A. nidulans*) revealed interesting differences (Harris 2011). **Chapter 3** describes a follow-up study of *racA* (Chapter 2) to elucidate the impact of morphology on protein production. This study clearly showed that simply making more hyphal tips did not result in an increase of protein production levels. In **Chapter 4,** the transcriptomic effect of overexpression of a secreted enzyme (glucoamaylase) is described. It is shown that overexpression of glucoamylase induced many genes that are part of the unfolded protein response. A comparison of this dataset with other datasets in which *A. niger* was triggered to induce an unfolded protein response allowed to define a core set of genes that appear to be involved in dealing with misfolded proteins or high secretion loads. In **Chapter 5,** seven genes encoding putative *A. niger* orthologs that are known to function in key aspects of the protein secretion machinery in *S. cerevisiae* were analyzed. A reporter strain was constructed in which secretory vesicles are visualized by labeling a specific vesicle-SNARE (v-SNARE) with GFP giving GFP-v-SNARE. Using that strain, the protein secretion process in wild-type and mutants was visualized and analyzed. This study revealed that the exocyst-mediated vesicle transport is only partially conserved between *S. cerevisiae* and *A. niger*.