Carbon starvation in the filamentous fungus Aspergillus niger

Nitsche, B.M.

Summary and discussion

In industrial biotechnology, *Aspergillus niger* is well known as a versatile cell factory with a naturally high secretion capacity (Pel *et al.*, 2007) which is commonly exploited for large scale production of a wide range of organic acids, enzymes and proteins. To optimize production processes, several cellular activities that determine product yields have been intensively studied during the last decades, including, for example, the unfolded protein response and protease secretion (Peberdy, 1994; MacKenzie *et al.*, 2005; Jørgensen *et al.*, 2009; Mattern *et al.*, 1992; Braaksma *et al.*, 2009). Many fungal bioprocesses are operated as fed-batch cultures (Zustiak *et al.*, 2008) preventing catabolite repression by ensuring a nutrient-limited growth regime. Nutrient limitation, however, induces diverse catabolic processes that can negatively affect product yields for example by product hydrolysis or disintegration of hyphal integrity resulting in decreasing active biomass fractions.

As nutrient limitation can be life-threatening, complex responses have evolved that ultimately lead to fungal self-propagation. Endogenous recycling and extracellular hydrolysis are thought to liberate building blocks and energy to fuel asexual development during carbon starvation. Despite the negative effect on product yields, there has been no previous comprehensive investigation of carbon starvation in the industrially important filamentous fungus *A. niger*. We therefore followed an approach to comprehensively study carbon starvation in *A. niger* during submerged cultivation, which is described in this thesis and is discussed as follows: (I) Establishment of computational resources for omics data analysis and interpretation in chapters 2 and 3; (II) Cultivation of *A. niger*, data generation, analysis and interpretation in chapter 4; (III) Investigation of a candidate pathway with strong transcriptional induction during carbon starvation by molecular genetic approaches in chapter 5.

Genome-wide transcriptional analysis has great potential to provide system-level insights into cellular responses, but requires an unbiased, high-throughput statistical approach for data analysis, interpretation and eventually generation of hypothesis. The application of open source and open access computational tools is desirable at universities in particular, and can be considered beneficial as they are freely distributed and actively developed by leading experts from within the scientific community. However, they can be less intuitive and depend on a command-line-interface, as it is the case for R/Bioconductor (R-Team, 2008; Gentleman *et al.*, 2004) used in this thesis for transcriptome data analysis. Therefore, chapter 2 was intended as an introduction to transcriptome data analysis using R/Bioconductor, by providing a step-by-step tutorial analyzing two public transcriptome datasets of *A. niger* (Nitsche *et al.*, 2012b). Experience has shown that this approach is suited to bring biologists into contact with R/Bioconductor, even allowing them to analyze their own datasets.

Following the initial phase of data analysis, including the identification of differentially or co-expressed gene sets, omics data should be biologically interpreted to e.g. drive the generation of new hypotheses. A powerful, high-throughput and unbiased approach is enrichment analysis of functional annotations (B. H. J. v. d. Berg *et al.*, 2009). Amongst the most commonly used functional annotations for enrichment analysis is Gene Ontology (GO) annotation (Ashburner *et al.*, 2000). In chapter 3, this valuable resource for functional analysis of omics data was extended to the genus *Aspergillus* by mapping the *A. nidulans* GO annotation to all Aspergilli with published genome sequences and implementing the web application FetGOat (Fisher's exact test Gene Ontology annotation tool, http://www.broadinstitute.org/fetgoat/index.html) for GO enrichment analysis. Both the annotation and FetGOat were used in two case studies to re-analyze two public datasets, demonstrating their applicability, ease of use and potential to generate new hypotheses (Nitsche *et al.*, 2011).

Having set up the computational tools for omics data analysis, genome-wide transcriptional profiles of carbon starving submerged cultures of *A. niger* were established, analyzed and interpreted in chapter 4. The experimental setup involved submerged batch cultivation in carbon-limited minimal medium applying bioreactor technology which ensured high reproducibility, monitoring and control of physiology and macromorphology. The major morphological responses to carbon starvation were the emergence of empty hyphal compartments (hyphal ghosts), secondary (cryptic) growth of thin non-branching hyphae and subsequently formation of asexual developmental structures. These morphological hallmarks suggest that secondary growth and conidiation are fuelled by recycling of resources derived from emptying hyphal compartments. Importantly, microscopic analysis has shown no indication of hydrolytic weakening and fragmentation of fungal cell walls. Principally, resources from empty compartments could be thought to be directly recycled by neighboring compartments via an endogenous route or first leak into the culture broth where they are subsequently taken up by growing hyphae.

Transcriptomic data analysis showed that throughout the course of carbon starvation, approximately 50% of the transcripts were differentially expressed. The numbers of up- and downregulated genes were easy to compare. In order to identify major transcriptionally induced and repressed cellular processes, GO annotation enrichment analysis was applied for sets of up- and downregulated genes. The repressed processes were primarily related to transcription, translation and respiration which reflect the physiological adaptation to carbon starvation and energy deprivation. On the contrary, the major transcriptionally induced processes included catabolic and reproductive pathways. As such, autophagy and conidiation were most dominantly induced. Furthermore, analysis focusing on carbohydrases and proteases revealed strong transcriptional induction of several secreted and non-secreted hydrolases.

More than 30 autophagy-related genes have been identified to date in yeast and filamentous fungi (Kanki *et al.*, 2011; Xie *et al.*, 2007). For 23 of these genes, ortholog candidates were identified in *A. niger* and all except one were transcriptionally induced during carbon starvation. This concerted induction of the autophagy pathway gave rise to questions about the role of autophagy during carbon starvation. Does autophagy protect against the formation of empty hyphal compartments or does it promote these processes? Is autophagy required for effective elongation of thin hyphae during the secondary (cryptic) growth phase under carbon starvation or not? In looking for these answers, three genes (*atg*1, *atg*8 and *atg*17), that have been predicted to be essential for efficient autophagy in other orgnamisms (Tsukada *et al.*, 1993; Matsuura *et al.*, 1997; Cheong *et al.*, 2005; Kabeya *et al.*, 2005), were deleted in *A. niger* wild-type and fluorescent reporter strains. Phenotypic characterization of the mutant strains indicated that *atg*1 and *atg*8 are essential for efficient autophagy in *A. niger*, whereas deletion of *atg*17 had little to no effect. Analysis of the Δ*atg*1 and Δ*atg*8 mutant strains during submerged growth in carbon-limited batch cultures showed that the morphogenetic adaptation to carbon starvation, including the emergence of empty hyphal compartments and secondary growth of thinner non-branching hyphae, was accelerated when autophagy was impaired. Furthermore, we demonstrated that mitochondrial turnover in response to carbon depletion was severely impaired in both Δ*atg*1 and Δ*atg*8 mutants. This suggests that autophagy thus delays cell death during carbon starvation by organelle turnover which is important for physiological adaptation during carbon starvation. Further investigation will be required to elucidate the effect of impaired autophagy in *A. niger* on product yields. For example, it has been observed in *Penicillium chrysogenum* that the impairment of autophagy delays cell degeneration and results in enhanced production levels (Bartoszewska *et al.*, 2011a).

It has proved difficult to answer how hyphal ghosts are formed. Either hyphal compartments lyse meaning that their content leaks into the culture broth or they are actively evacuated by neighboring compartments which thus resembles endogenous recycling. Although secretome data indicated that there was no considerable accumulation of intracellular proteins in the culture broth during carbon starvation, immediate hydrolysis of leaked cytosolic proteins cannot be excluded. However, an accelerated emergence of empty compartments in the Δ*atg*1 and Δ*atg*8 mutants suggests that endogenous recycling by neighboring compartments does not occur via autophagic processes.

This study is the first systems level investigation of carbon starvation in the filamentous fungus *A. niger*. While we followed a single lead from the transcriptomic data analysis, there are many more processes remaining to be investigated and the dataset obtained forms a comprehensive framework for future investigations of the complex cellular responses.