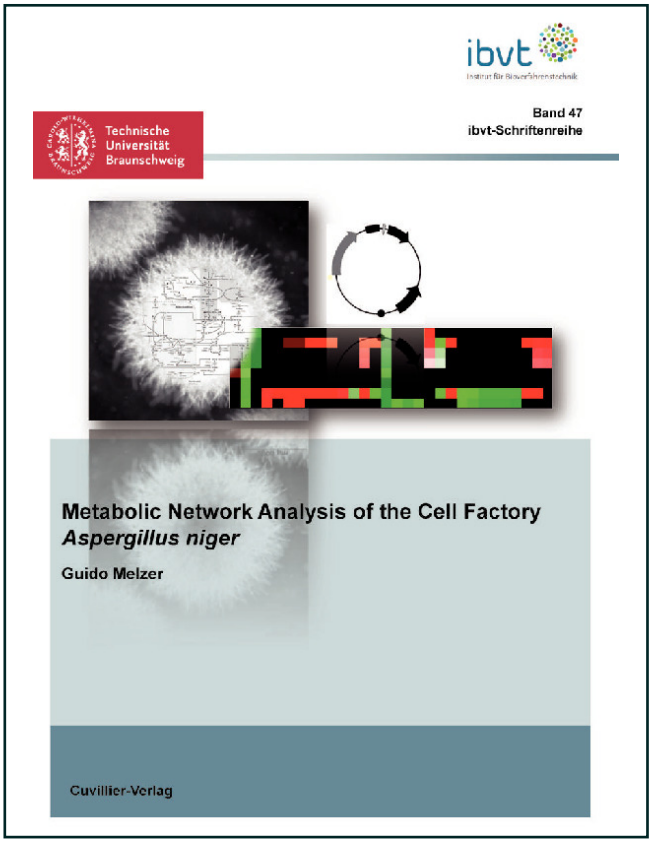
Guido Melzer (Autor)

Metabolic Network Analysis of the Cell Factory Aspergillus niger



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3 Introduction

3.1 General Introduction

Efficient protein production in *Aspergillus niger* requests for superior production strains, whereby metabolic engineering is a powerful strategy for targeted manipulation of the underlying biochemical pathways [1]. In particular, this requires the identification of genetic targets which should be either deleted or amplified to obtain the desired increase of protein production. However, the still limited knowledge on the underlying complex metabolic network and the resulting lack of appropriate strategies to identify genetic targets makes this optimization a difficult task. In this regard, recent studies on *A. niger* including experimental 'omics technologies display important contributions towards systems-oriented understanding of its complex metabolic processes [2-5]. Generally, modelling approaches applying flux balance analysis, metabolic flux analysis, metabolic control analysis or biochemical systems theory became also very popular in the past time and are still of high interest in relevant studies of the metabolic network of *A. niger* [6-10].

Since recent advances in sequencing and bioinformatical technologies [11] the reconstruction of cellular metabolism of many organisms based on information encoded in their genomes have expanded enormously [12-14]. Beyond that, the annotated genome of *A. niger* [15] has enabled the reconstruction of a genome scale metabolic network [2].

This provides the basis for a more systems-oriented investigation of the cellular metabolism of *A. niger* accounting for the high complexity of its metabolic network and selecting promising genes out of many possible candidates. In this regard, the bi-level optimization frameworks OptKnock [16] and OptStrain [17], or the design of deletion strains by prediction of optimum theoretical yield [18] display efficient gene deletion strategies that lead to overproduction of chemicals in microbial systems. They do, however, not provide the prediction of genes to be amplified for superior performance. This rather important information on potential amplification targets can be obtained from ^{13}C metabolic flux analysis [19] as demonstrated successfully for lysine producing *Corynebacterium glutamicum* [20, 21].

However, as in the latter case, such experimental approaches require the availability of appropriate mutants, which can be linked to increased experimental effort, and might not give access to all potentially interesting gene candidates. Additionally, efficient *in silico* methods for simultaneous genome-scale identification of targets to be amplified or deleted are still lacking.

3.2 Objectives of the Thesis

The objectives of the thesis included the development of a modeling framework to exploit the rich information on the genome of *A. niger*, one of the major industrial working horses. For this purpose, a metabolic network should be first constructed from available information extracted from genome scale information, literature data and *in vitro* enzyme studies.
Based on this, *in silico* analysis of the network by the elementary flux mode calculation should be carried out to provide new insights into the metabolic network behaviour. This should consider various nutrient conditions and different relevant products as a basis for new optimization strategies (Figure 1). Based on the simulation results, a new approach could utilize the correlation of pathway fluxes to desired properties of the organism to propose efficient *in silico* design strategies. This novel approach was developed and applied to the industrial cell factories *A. niger* and *C. glutamicum*. Continuous cultivations of *A. niger* were analyzed using stoichiometric flux analysis under varied environmental conditions, to contribute to the validation of the *in silico* approach.

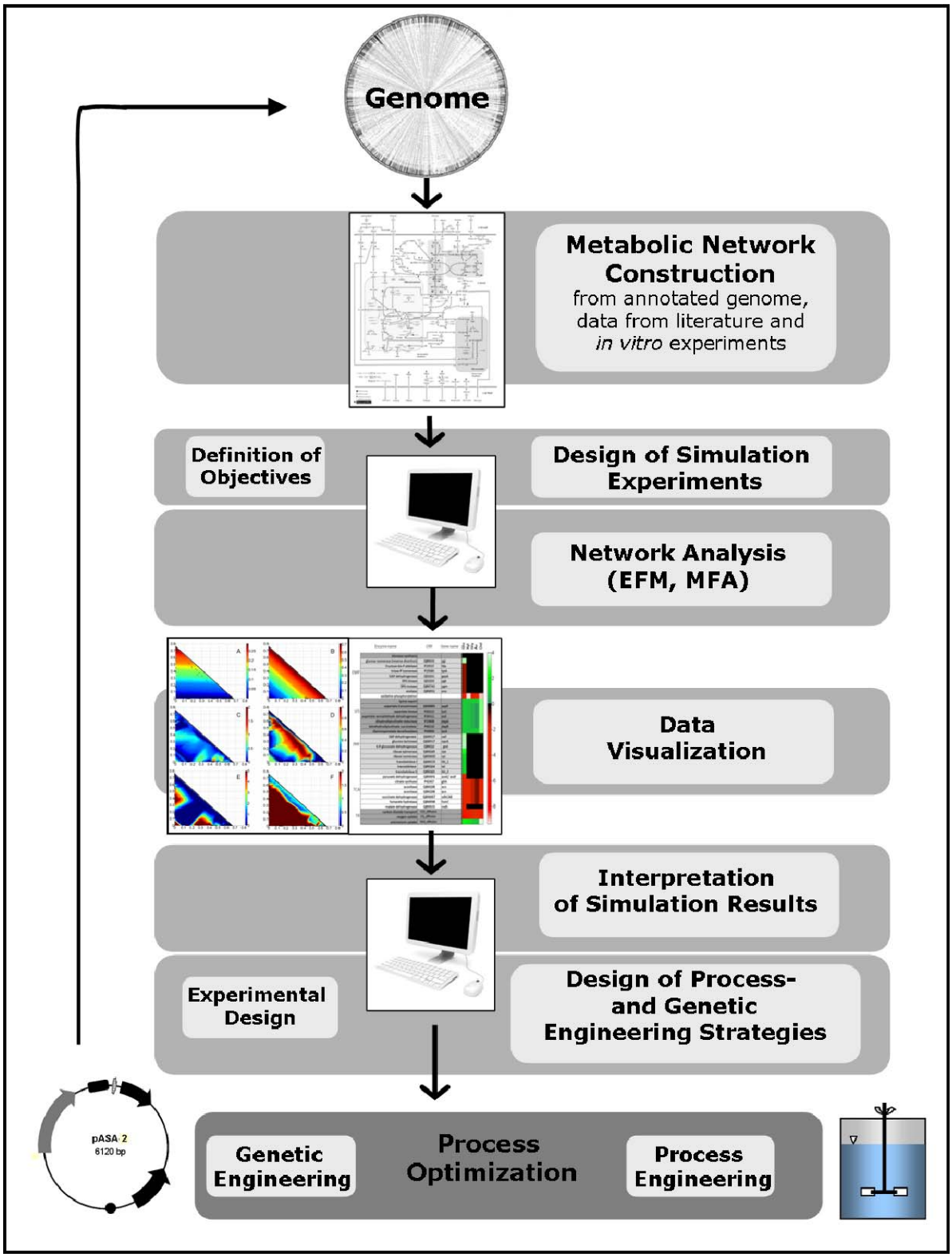

Figure 1: Flow chart visualizing the link between the structure of a metabolic network and the development of process optimization strategies. Abbreviations: MFA: Metabolic Flux Analysis, EFM: Elementary Flux Mode Analysis.

4 Theoretical Background

4.1 Systems Biology

A central goal of systems biology is the understanding of complex biological processes. Beyond exploring the molecular basis of individual reaction pathways, including their mathematical relationships, to comprehensive understanding of functional relationships between parts of the whole cell and more complex systems [22] can be modelled and simulated.

For this, the interactions at a molecular level in the cell, which are causally determined by the environment are investigated by attribution to their underlying processes [23, 24]. Computer simulations, here, provide a tool for understanding of functionality of cellular processes by linking the observed cellular components into functional network operation. This concept was already used in the seventies, where mathematical models were used to describe the complex reaction systems of ethanol production in yeast [25].

A crucial impetus of the enormous progress in recent years was the rapid technical development in modern 'omics technologies such as genomics [26, 27], high throughput metabolomics [28-30], proteomics including RNA-, DNA- and protein microarrays [31-33], high-resolution 2-D gel electrophoresis [34], GC- and LC-MS [35, 36] and fluxomics [19, 37]. Alongside the technical developments, considerable efforts have been made in the mathematical systems biology which includes large collaborative efforts [38].

4.2 *Aspergillus niger* – an Efficient Industrial Host

4.2.1 Biotechnological relevance and application

Aspergillus niger (black mold) is an ubiquitous soil fungus. It has gained high industrial importance for many decades and is one of the most important filamentous fungi used for

biotechnological purposes. *A. niger* has been utilized in industrial fermentations for more than 80 years because of its ability to accumulate and secrete large quantities of metabolites, such as organic acids [39-42], and large amounts of heterologous and homologous proteins [43, 44]. *A. niger* is most suited for producing industrially relevant food-enzymes, such as glucoamylase, chymosine [45-47] or highly glycosylated proteins, such as the β-fructofuranosidase [48]. The production of epoxide hydrolases by *A. niger* provide an important biocatalyst for the synthesis of chiral building blocks for the pharmaceutical, fine and specialty chemical industry [49, 50]. Since the organism is considered to be safe for human health [51], recent efforts to utilize *A. niger* for the production of pharmaceutically relevant heterologous proteins and antibodies have been increased [52, 53].

4.2.2 Genome

Currently, three strains of *Aspergillus niger* have been sequenced. Two of the strains, ATCC 9029 and ATCC 1015 are wild type strains, while *A. niger* ATCC 22343 (CBS 513.88) was isolated after mutagenesis and selection for improved glucoamylase secretion [54]. Most recently, in 2005, the genome of *A. niger* ATCC 1015, a wild type, historic strain was used in research that resulted in the first patented citric acid process that was accepted for sequencing through the US Department of Energy (DOE). Organisms accepted by this program are sequenced by the DOE's Joint Genome Institute (JGI). Another wild type *A. niger* strain, ATCC 9029, was sequenced by Integrated Genomics, an US based company. Finally, ATCC 22343 was sequenced by a Netherlands based company, DSM [15]. Size of the genome and corresponding data between different *A. niger* strains are listed in Table.

Table 1: Genome statistics for *A. niger* ATCC 1015 and ATCC 22343.

	ATCC 1015	ATCC 22343
Chromosomes	8	8
Genes predicted	11,200	14,165
Genome size [MB]	34.85	33.9
Gene length [bp]	1696.1	1572
Transcript length [bp]	1501.3	439.9
Protein length [aa]	484.3	439.9

4.2.3 Carbon core metabolism

A. niger belongs to the filamentous fungi. Its tubular cells, called hyphae, are divided into separate compartments by septa. These contain septal pores providing a connection that allows adjacent cellular compartments to cooperate and coordinate their activities.
Soluble carbohydrates enter the cell by active transport across the fungal membrane. For the saprophytic *A. niger* most carbon in the environment is present as a complex polymer like cellulose, chitin or lignin. Thus it has a wide set of extra-cellular enzymes including cellulases, chitinases, proteases and multi-component lignin degrading enzymes [55-57].
A. niger assimilates nitrogen sources like ammonia and nitrate or amino acids by direct uptake across the hyphal membrane. Moreover, *Aspergillus* is capable to secret many types of secondary metabolites [58] or organic acids [59, 60]. Since the subsequent catabolic pathways are described in detail in relevant literature [61], the chapter highlights the most relevant metabolic pathways implemented in the performed studies.

4.2.3.1 Substrate assimilation and catabolic break down

Mono-saccharides are actively transported into the cell by the MSTA proton symport system, while polyols and fatty acids can be easily transported via the hydrophobic membrane by diffusion [62]. After activation by hexokinases, the phosphorylated sugars are metabolized via the Embden-Meyerhof-Parnas (EMP) pathway, the Pentose-Phosphate pathway (PPP) as well as the 2-keto-3-deoxy-phosphogluconate (KDPG) pathway, whereby the latter pathways play an important role in bacteria, e.g. *Pseudomonas* sp. and *E. coli*, and are absent in *Aspergillus* (Figure 2).