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Metabolic Engineering of Filamentous Fungi

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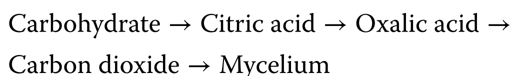
20.1 Introduction

In 1917, the chemist James Currie published a general equation of the metabolism of the filamentous fungus *Aspergillus niger* [1]:



Carbohydrate → Citric acid → Oxalic acid →
Carbon dioxide → Mycelium

He speculated that "This reaction can be controlled to a very considerable extent" by varying the nature and quantity of carbon and nitrogen sources supplied to the medium. Indeed, he could prove his assumption in this groundbreaking publication and concluded that "the conditions most favorable for a high yield of the end-products, carbon dioxide and mycelium, are least favorable for the formation of the intermediate products, citric and oxalic acids" [1]. Currie identified many factors supporting high citric acid production in this study and, thus, laid the foundation for *A. niger* to become the pioneer fungus for industrial organic acid fermentation and the birth of modern biotechnology exploiting filamentous fungi [2]. His publication ends with the following statement:

> The painstaking investigation of all the conditions favoring the production of such substances will lay the only sure foundations for the development of a chemical fermentation industry. It is the hope of the writer that the work here recorded may prove a definite contribution to this much neglected but promising field of scientific endeavor. [1]

A hundred years later, not only *A. niger* but many other filamentous fungi are used as cell factories in very diverse industrial sectors, including chemical, biofuel, textile, pharma, and food industries, to name but a few. The hope of James Currie has, thus, come true. The natural metabolic capacities of filamentous fungi are appreciated as extraordinary diverse, are nowadays much better understood and are purposefully harnessed for the production of primary and secondary metabolites, proteins and enzymes, food and vitamins, and even composite materials and vegan leather (Table 20.1).

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Table 20.1 List of some filamentous fungal cell factories and their products.

Filamentous fungus	Important product(s)
Acremonium chrysogenum	β-Lactam antibiotics (cephalosporins)
Aspergillus niger	Enzymes (glucoamylase, proteases, phytases, glucose oxidase) Organic acids (citric acid, gluconic acid)
Aspergillus oryzae	Enzymes (amylases)
Aspergillus terreus	Enzymes (xylanases) Organic acids (itaconic acid) Secondary metabolites (lovastatin)
Blakeslea trispora	Vitamins (β-carotene)
Fusarium venenatum	Mycoprotein
Ganoderma lucidum	Composite materials (packaging material, construction material) Imitation leather
Penicillium chrysogenum	β-Lactam antibiotics (penicillins) Enzymes (glucose oxidase)
Pleurotus ostreatus	Composite materials (packaging material, construction material)
Thermothelomyces thermophilus	Enzymes (cellulases, phytases, laccases)
Trichoderma reesei	Enzymes (cellulases, hemicellulases)

Note that *T. thermophilus* was formerly named *Myceliophthora thermophila* and *P. chrysogenum* was recently renamed *Penicillium rubens*.
Source: Modified after Cairns et al. [3].

Fungal biotechnology harnessing the metabolic activities of filamentous fungi has, thus, managed to establish itself as an essential platform technology for innumerable branches of industry and shapes our daily life and lifestyle decisively in an invisible way. It offers not only exciting solutions to mankind's efforts to promote the transition from our current petroleum-based economy into a future sustainable bio-based circular economy but also new concepts on how to secure the increasing food demand for a growing human population ([4] and see below).

Many of the naturally existing metabolic activities of filamentous fungi have been elucidated and leveraged in strain optimization programs to obtain more efficient cell factories [5]. If everything seems to be perfect, why, then, consider metabolic engineering of filamentous fungi? The short answers are: (i) Most molecular mechanisms determining the efficiency of substrate utilization and product formation are far from being fully understood; (ii) a confounding factor that limits the productivity of filamentous fungal cell factories is their morphological development under submerged cultivation conditions (Figure 20.1); and (iii) the concept of enlarging the product portfolio within one filamentous fungus to generate a multipurpose cell factory, i.e. a one-size-fits-all solution has not been fully explored yet.

Detailed answers will be given in the following sections accompanying the examples of six established filamentous fungal cell factories: *A. niger, A. oryzae,*

Figure 20.1 Morphologies adopted by filamentous fungi under submerged cultivation conditions, which is the most common fermentation method in industry. Different branching frequencies of the growing mycelium cause the development of different macromorphologies. These are visible to the eye and can range from pellets (lower left), whose diameter can be several hundred micrometer up to several centimeter, over loose clumps (not shown) to dispersed morphologies causing a very viscous culture broth in a bioreactor (lower right). The example of *A. niger* is shown. The diameter of its hyphae is about 3.5–4 μm. Source: Vera Meyer.

A. terreus, P. chrysogenum, T. reesei, and *T. thermophilus*. These species have been selected because their biotechnological products cover most of the product range offered by filamentous fungi. They have also been selected because most metabolic engineering efforts are currently focused on these species.

Despite the progress made, metabolic engineering of filamentous fungi is still in its infancy. Whereas the keyword "Metabolic engineering" coupled with either "*Saccharomyces cerevisiae*" or "*Escherichia coli*" results in about 2000 or 4000 retrieved articles from PubMed in 2019, respectively, the number of articles for filamentous fungi ranges from 5 (*T. thermophilus*), over 40–60 (*A. oryzae, A. terreus, P. chrysogenum, T. reesei*) to about 100 for *A. niger*. One reason for this discrepancy is that the genome sequences of filamentous fungi became available only a decade after the genomes of the model unicellular fungus *S. cerevisiae* (1996, [17]) and the model bacterium *E. coli* (1997, [18]) were released to the public. On top of this, the genomes of filamentous fungi contain far more genes. A filamentous fungal genome usually carries between 9000 and 14 000 genes (Table 20.2), whereas *E. coli* can live with about 4000 [18] and *S. cerevisiae* with about 6000 [17] genes, respectively. Finally, the research communities studying filamentous fungi are considerably small. A recent mapping of research on *A. niger* uncovered a network of about 30 research labs worldwide [2]. By contrast, more than 1800 research labs studying *S. cerevisiae* are registered at the Saccharomyces Genome Database [19].

In the following chapter, the current state of the art of metabolic engineering in filamentous fungi will be discussed. Hereby, the definition of metabolic engineering of the research journal Nature will be used:

> Metabolic engineering is the use of genetic engineering to modify the metabolism of an organism. It can involve the optimization of existing biochemical pathways or the introduction of pathway components, most commonly in bacteria, yeast or plants, with the goal of high-yield production of specific metabolites for medicine or biotechnology. [20]

Table 20.2 Filamentous fungal cell factories with available genome sequence data and CRISPR genome editing tools.

Strain	First genome published	No. of predicted genes	First CRISPR tool published	References
A. niger	2007	~14 000	2015	[6, 7]
A. oryzae	2005	~12 000	2016	[8]
A. terreus	2005	~10 000	—	[9]
P. chrysogenum	2008	~13 000	2016	[10, 11]
T. thermophilus	2011	~9 000	2017	[12, 13]
T. reesei	2008	~9 000	2015	[14, 15]

Note that only the publications reporting a genome sequence or a CRISPR tool for the six fungal cell factories for the first time have been cited here. The reader is directed to [16] for a recent review of the implementation of different CRISPR protocols for filamentous fungi. Note also that *T. thermophilus* was formerly named *M. thermophila* and *P. chrysogenum* was recently renamed *P. rubens*.

This definition already indicates that filamentous fungi play a minor role in the perception of the metabolic engineering community as promising and powerful metabolite producers. However, industrially exploited filamentous fungi are often superior to bacterial and yeast cell factories, regarding robustness to harsh industrial cultivation conditions, metabolic versatility, and secretory capacity [4].

The focus of the chapter will, thus, be on the development and implementation of genetic tools for filamentous fungi (Section 20.2), the establishment of metabolic and regulatory models (Section 20.3), engineering strategies for improved substrate utilization (Section 20.4), enhanced product formation (Section 20.5), and new product developments (Section 20.6). The chapter concludes with a discussion of current strategies on how to engineer and control the development of macromorphologies in filamentous fungi (Section 20.7) and will finally discuss new developments in the metabolic engineering of filamentous fungi in the near future (Section 20.8).

20.2 Development and Implementation of Genetic and Genome Tools

Molecular studies with filamentous fungi have long been considered as difficult and painstaking because of their low growth rate compared to bacteria and yeast, a lack of efficient genetic transformation systems, the nonexistence of versatile dominant or auxotrophic selection markers, and poor transformation rates. Diametrically opposed to this is the wish of bioengineers who consider

fast and efficient genetic manipulation tools as a fundamental prerequisite for metabolic engineering. Consequently, unicellular and easier to handle bacterial and yeast systems tend to be the default, although filamentous fungal systems may be the rational choice for many applications [4].

Fortunately, the last 10–15 years have witnessed a revolution in molecular tools and technologies for filamentous fungi. Several efficient transformation techniques, a broad range of selection markers, and a variety of constitutive or inducible expression systems are available nowadays for filamentous fungal cell factories [21, 22], making them easy to handle for trained fungal staff. In addition, the lengthy procedure of screening for homologous transformants within the set of transformants gained has been streamlined by the implementation of recipient strains which are defective in nonhomologous recombination [23]. Hence, knock-out, knock-in, gene replacement, and conditional gene expression of any gene of interest has become routine and a genetically modified filamentous fungus carrying the intended genetic modification can be obtained within approximately a week. Simultaneous expression of all genes belonging to a complete biosynthetic pathway via a polycistronic expression cassette has also been shown to be feasible in filamentous fungi and used to produce bioactive secondary metabolites, such as the antibiotics penicillin and enniatin, as well as the insecticides austinoids in different *Aspergilli* [24–27].

In addition, genome sequences have been published for hundreds of filamentous fungi, including the most relevant industrial cell factories (Table 20.2) and the genomic datasets for filamentous fungi are continuously expanding and being made accessible for the research community by databases such as FungiDB [28, 29], MycoCosm [30], and Ensembl [31]. The genomes of 17 different *A. niger* strains, for example, have been sequenced since the first *A. niger* genome became available in 2007 [6, 32–39]. The availability of this wealth of genome sequence data combined with the most recent implementation of a rich and diverse set of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genome editing protocols for filamentous fungi (Table 20.2), which are also compatible with microtiter plate methods [40], will probably elicit a new era in genetic and metabolic engineering for filamentous fungi. Fast and efficient introduction of targeted single-point or large chromosomal mutations or rewiring of a biosynthetic pathway in filamentous fungi is no longer fantasy, but reality.

However, the challenge of today is the quality of genome data for filamentous fungi, which can vary greatly. If of poor quality, the outcome of comparative genomics studies is impaired as is the efficiency of genetic and metabolic engineering efforts. Therefore, the research community advocates several recommendations and protocols to the individual researchers in order to enable sustainable use and reuse of published genomics data and, more broadly speaking, of transcriptomics, proteomics, and metabolomics data [4, 41, 42]. Unfortunately, a gold standard genome (i.e. a reference genome that is near error-free and near gapless and can be used to map the genomes of closely related organisms) has not been published yet for filamentous fungi. However, a gold standard genome for a lab strain of *A. niger* is likely to become released soon [43].

20.3 Metabolic and Regulatory Models

Next to gene annotation and genetic engineering, metabolic and regulatory models are of crucial importance for metabolic engineering approaches. They predict how genetic and ambient medium perturbations impact growth and metabolite production and can, thus, guide metabolic engineering efforts.

Genome sequences generally form the basis for draft genome-scale metabolic models (GEMs), where genes become assigned to metabolic pathways and experimental data integrated into a structured framework. The probably best and, in a community-driven effort, continuously curated fungal GEM is available for the yeast model *S. cerevisiae*, which can even be used to predict phenotypic traits of single point mutations [48]. The best and validated GEM for a filamentous fungus available so far is for *A. niger* (Table 20.3). The power of this community-driven GEM is that it integrates the experimental knowledge from nearly 900 publications, thus, representing the best experimentally supported model currently available for a filamentous fungus. It is a true consensus model as it integrates information from former models (e.g. [49]). Furthermore, it provides individual strain-specific models for the most commonly used *A. niger* strains (CBS 513.88 and ATCC 1015) and can be used easily for gene-protein association studies based on genomic and transcriptomic data [44]. It is notable that existing GEMs can be used as a template to reconstruct a GEM for related species [50]. Older GEMs for *A. niger* have, thus, been used to develop GEMs for *P. chrysogenum* [46] and *T. reesei* [51] (Table 20.3). A convenient software suite for the semiautomated reconstruction, simulation and curation of GEMs is the RAVEN toolbox, which is freely available at GitHub [52]. An alternative is the metabolic model reconstruction algorithm CoReCo, which has been recently improved and used for *T. reesei* and 55 other fungi [53]. The code for this pipeline can be downloaded from GitHub [54] or the BioModels database [55].

A confounding factor when harnessing genome data is the fact that a considerably high number of genes is "hypothetical" and lack functional predictions.

Table 20.3 Filamentous fungal cell factories with curated genome-scale metabolic models (GEMs).

Strain	Year	GEM	References
A. niger	2018	Covers 2320 reactions and 1325 genes	[44]
A. oryzae	2014	Covers 2453 reactions and 820 genes	[45]
A. terreus	2014	Covers 2401 reactions and 794 genes	[45]
P. chrysogenum	2013	Covers 1471 reactions and 1006 genes	[46]
T. thermophilus	—	—	—
T. reesei	2016	Covers 3926 reactions and 697 genes	[45]

Note that only the most recent models are referred to. The reader is directed to [47] for a recent review of the current state of genome scale reconstruction in filamentous fungi.

Recent estimates suggest that between 40 and 50% of the genes in a filamentous fungal genome are hypothetical [4]. Furthermore, only 2–10% of the genes with predicted functions have been experimentally studied in filamentous fungi and, thus, have a verified function [29, 56]. This renders thousands of genes within a single filamentous fungal genome uncharacterized, a reconstructed GEM considerably incomplete, i.e. with gaps, dead-end reactions and dead-end metabolites, and our general understanding of filamentous fungal biology, therefore, far from comprehensive. Even for the model *S. cerevisiae*, 21% of its predicted genes (i.e. about 1400 genes) still have dubious functional predictions in 2019, which is 22 years after the release of its genome sequence and despite a research community with more 1800 labs worldwide [57].

One powerful solution for this challenge is the interrogation of gene expression networks based on hundreds of transcriptomic datasets available for filamentous fungi. This wealth of data can be harnessed to improve gene annotations and to assign gene function predictions. The underlying hypothesis is the so-called "guilt-by-association" approach, which is rooted in the assumption that genes which are frequently coexpressed during growth, development, metabolite production, and under diverse environmental conditions or during genetic perturbations are likely to function in the same or closely related biological processes or pathways [59]. A meta-analysis of 283 transcriptomics experiments publicly available for *A. niger*, for example, generated coexpression networks for 9579 genes, which are about 65% of all genes present in the *A. niger* CBS 513.88 genome [58]. This dataset was coupled with gene ontology enrichment analyses [60] and allowed to predict biological processes including metabolic and regulatory functions for 9263 of *A. niger* genes (Figure 20.2a). Remarkably, this approach assigned processes to 2970 hypothetical genes, which is about 50% of all hypothetical genes predicted in the genome of *A. niger*. These predictions, therefore, give, for the first time, the opportunity to link hypothetical genes with known metabolic and cellular processes (Figure 20.2b). This compendium can, thus, be used for hypothesis generation on a variety of conceptual levels and can generate shortlists of yet unstudied genes that can be investigated further under certain conditions to decipher their function.

Taken together, many GEMs are currently available for filamentous cell factories and can be used to simulate biomass accumulation and metabolite production. Nevertheless, GEMs for filamentous fungi need continuous quality improvement. They are considerably limited as they rely on current insights into genome data and gene function predictions. Older models, for example, for *T. reesei*, exhibited stoichiometrically unrealistic yields (more carbon was available in the biomass than was available from the carbon source) but newer models contain more relevant biochemical pathways and stoichiometrically unrealistic yields are no longer observed [53]. However, in the most recent model for *T. reesei*, the condition where the highest protein production and secretion is experimentally observed, the GEM predictions showed the highest variation and predicted higher growth rates than were experimentally measured. Hence, there is still a long way to go to until *in silico* GEMs can fully predict the growth and product formation of filamentous fungi.

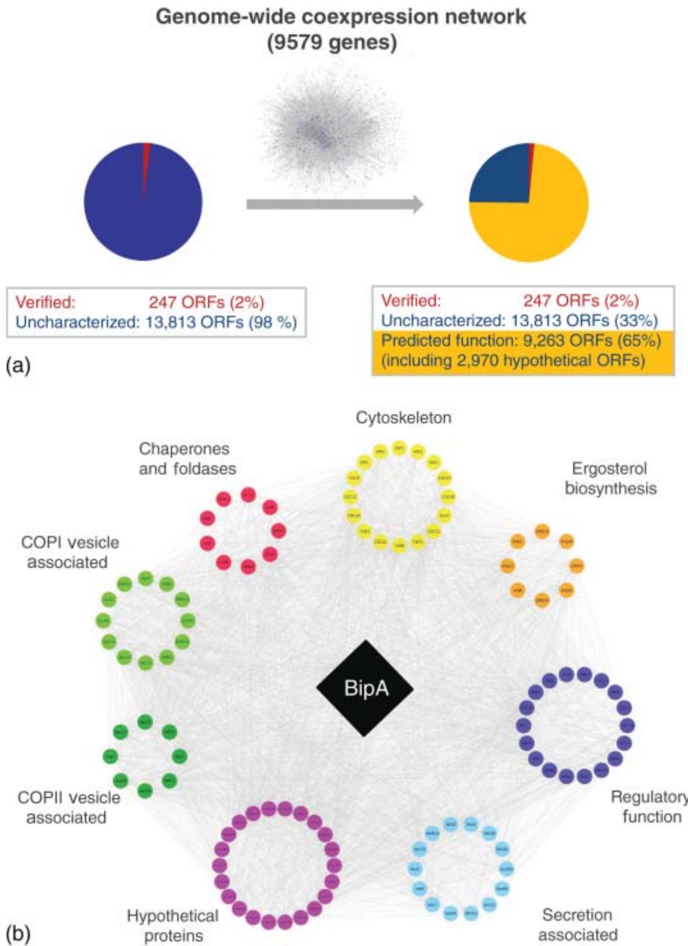

Figure 20.2 Gene coexpression networks uncover highly connected genes, which are functionally related, members of the same metabolic or regulatory pathway and controlled by the same transcriptional regulatory program. (a) The global gene coexpression network obtained for *A. niger* covers 9579 genes and allows functional predictions for thousands of genes including hypothetical ones. (b) Coexpression sub-networks can be interrogated for any gene of interest. The example of the chaperon BipA is shown, which is of central importance for high protein secretion in *A. niger*. Genes are represented by circles, with positive and negative correlations depicted by grey and red lines, respectively. BipA is given in a black diamond box. The BipA encoding gene is highly connected with genes with functions in protein folding (chaperones and foldases), protein transport (COPI- and COPII-vesicle transport, cytoskeleton), plasma membrane biosynthesis (ergosterol biosynthesis), and other secretion and regulatory-associated proteins. The strength of this approach is that several hypothetical proteins have been identified to probably play a role in protein secretion, which is not possible based only on gene content information. Source: Note that parts of this figure have been reproduced and modified from Schäpe et al. [59], CC BY 4.0.

20.4 Engineering Strategies for Improved Substrate Utilization

During evolution, filamentous fungi have learned to live and feed on various polymeric substances and efficiently decompose organic matter [61]. Polysaccharides from plant biomass are among the preferred carbon sources, perhaps because the kingdom of plants concentrates most of the biomass on Earth (450 gigatons of carbon out of a total of 550 gigatons of carbon) [62]. Whereas humans first ingest and then digest, filamentous fungi have learned to first digest through extracellular hydrolysis of the polymers and then ingest the resulting low molecular weight degradation products. For this purpose, enzymes such as cellulases, amylases, pectinases, and inulases, to name but a few, become secreted into the surrounding medium and hydrolyze plant polysaccharides, such as cellulose, starch, pectin, and inulin. The degradation products are usually mono- and oligosaccharides and are taken up into cells with the help of specific sugar transporters present in their plasma membranes. The high and very efficient degradation potential of plant biomass makes filamentous fungi very interesting as a source of enzymes for several industries, including food and feed, pulp and paper, pharmaceutical and chemical industries, and places filamentous fungal cell factories in a central position for the sustainable production of biofuels and chemicals [4].

The enzymes involved in carbohydrate degradation have been classified into families in the Carbohydrate-Active enzyme database (CAZy, [63]). A recent comparative genomics analysis uncovered that the predicted enzyme set for plant polysaccharide degradation in *A. niger*, *A. oryzae*, *P. chrysogenum*, and *T. reesei* harbors 200, 242, 174, and 119 enzymes, respectively, whereas only 30 can be found in *S. cerevisiae* [64]. It is generally thought that the presence of a gene predicted to encode carbohydrate-degrading enzyme correlates with the ability of fungi to grow on a specific carbon source [65]. However, it seems that extra copies of an enzyme do not necessarily result in faster growth or degradation but rather correlate with the phylogenetic relationship between different species [65].

CAZyme families are controlled by orthologous transcription factors, some of which are present in almost all filamentous fungi (e.g. Ace1, CreA, ClrA, ClrB, GaaR, XlnR), whereas others are only present in few filamentous fungi (e.g. AmyR, InuR, Ace2 [66]). An important underlying molecular mechanism of CAZyme gene expression is the inducer-dependent activation of their corresponding transcription factors. The inducing compound is a mono- or disaccharide or a derivative thereof which becomes liberated from the specific plant polysaccharide by the respective fungal enzyme. Upon uptake, the inducer activates the substrate-specific transcription factor (e.g. cellobiose, the degradation product of cellulose, activates the cellulase regulator ClrA which, in turn, activates ClrB). A comprehensive overview of fungal CAZymes and the complex regulatory machinery behind ensuring their expression can be found in two recent reviews [66, 67].

Several approaches have been followed to increase substrate utilization, i.e. to increase the secretion of the enzyme set to fully degrade the plant biomass. This is a major challenge, as the composition of plant biomass usually varies due to the use of different agricultural waste streams, pretreatments or impurities. The enzyme cocktails secreted by filamentous fungi, therefore, vary considerably. Although this can be appreciated as a high natural metabolic flexibility, a lot of effort is invested by academic and industrial groups to genetically engineer filamentous fungi to produce specific CAZymes independently of these variations, i.e. to obtain a defined set of enzymes at high yields. These genetic engineering approaches involve, for example, targeted deletions of transcriptional repressors (e.g. Ace1 and Rce1 in *T. reesei*, [68, 69]) or overexpression of transcriptional activators (e.g. AmyR in *A. niger* [70], ManR in *A. oryzae* [71], and Xyr1 in *T. reesei* and *T. thermophilus*, respectively [72, 73]). However, as overexpression of AmyR or Xyr1 does not cause inducer-independent CAZyme gene expression, several approaches have been followed to generate synthetic transcription factors, which constitutively bind to their target gene promoters. One successful example is the constitutive overexpression of a hybrid transcription factor in *T. reesei*, which contained DNA-binding domains from both Cre1 and Xyr1 [74]. Another very interesting approach follows the rational overexpression of epigenetic regulators, which are thought to result in more loosely packed chromatin and, thus, the easier access of transcription factors to their target genes. The overexpression of predicted chromatin remodelers (e.g. *N*-acetyltransferase or methyltransferase) indeed improved cellulase gene expression in *T. reesei* [75, 76]. The interested reader is referred to a most recent review which covers the research literature up to 2018 [67] for more genetic engineering examples regarding the optimization of fungal plant biomass degradation. Thereafter, *A. oryzae* has been reprogrammed to produce a cellulolytic enzyme cocktail at high yield. This metabolically engineered strain produces all three enzyme activities, a cellobiohydrolase, an endoglucanase, and β-glucosidase activity, respectively, to fully degrade cellulose to glucose, whereby each cellulase gene was constitutively expressed from multiple gene copies. The resulting strain displayed a 40-fold higher cellulase activity than the progenitor strain containing single copies of the respective genes [77].

Interestingly, the growth of *T. reesei* on cellulose is not only inducer-dependent but also light-dependent. *T. reesei* has photoreceptors which sense blue light and, in turn, alter cellulase gene expression tenfold and more. A remarkably high 75% of glycoside hydrolases seem to display blue light-dependent gene regulation and a lot is already known regarding how this is achieved on the molecular level. A recent comprehensive review is recommended for further reading [78].

Although this phenomenon has not yet been studied at all in *A. niger, A. oryzae, A. terreus,* or *T. thermophilus*, an impact of light on secondary metabolite production in *P. chrysogenum* has been reported (see Section 20.6). It might come as no surprise that the metabolism of fungi – like in humans – has adapted to light and darkness during evolution. Still, this phenomenon is nearly unexplored in filamentous fungal cell factories, except for *T. reesei*. A phylogenetic analysis with a particular focus on representative genomes of major filamentous fungi has recently identified opsin-encoding genes in *A. niger, A. terreus, and P. chrysogenum*. These fungal opsins are probably functional homologs of

bacterial green-light sensory rhodopsins, suggesting that light-sensing systems also exist in these cell factories [79]. In any case, the current insights, although very minor, have already provided important implications for research and strain improvement programs [78]. On the one hand, good laboratory praxis should ensure controlled light conditions and avoid light pulses during fungal cultivations. This can be achieved by constant light in shakers or glass vessel bioreactors which are run in labs where the light is always on. This will ensure consistent data from gene regulation and multiomics studies. On the other hand, large-scale industrial production with filamentous fungi occurs in darkness due to cultivation in stainless steel fermenters. Therefore, one might consider blinding filamentous fungal cell factories by deleting genes encoding light-sensing proteins. Genetic engineering approaches of blinded strains at lab-scale level would, therefore, more likely result in production strains which – with respect to light and darkness – would behave more predictively at a large-scale level.

20.5 Engineering Strategies for Enhanced Product Formation

The product portfolio of filamentous fungi is as diverse as their abilities to grow on different organic carbon sources. Figure 20.3 highlights some central catabolic routes and main products derived thereof which are harnessed in fungal biotechnology. These products involve primary metabolites (organic acids), macromolecules (proteins), or secondary metabolites (polyketides, nonribosomal peptides, terpenes, alkaloids). Some examples selected and current metabolic engineering strategies to increase their titer or redirect metabolic fluxes into other pathways will be discussed in the following section.

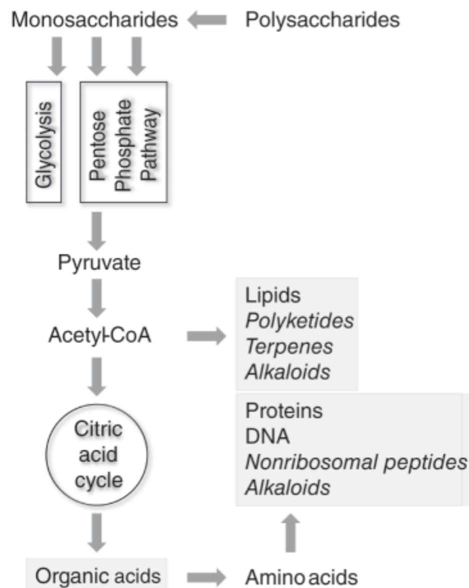

Figure 20.3 A simplified model of carbon catabolism in filamentous fungi when cultivated on polysaccharides. The main product classes are summarized in grey boxes; products of the secondary metabolism are indicated in italics. Acetyl-CoA provides the link between primary and secondary metabolism. For simplicity, the currency metabolites ATP and NAD(P)H are not indicated in the figure. Note that most filamentous fungi also secrete proteases and lipases, enabling them to also grow on other polymeric carbon sources, such as proteins and lipids.

20.5.1 *Aspergillus niger*

Citric acid, an intermediate of the citric acid cycle, is the most important bulk product in the organic acid industry worldwide. It has been produced mainly with *A. niger* and used as a flavoring agent, acidifier, and chelating agent in food, pharma, and chemical industries for the last 100 years [2]. Approximately 80% of the worldwide production of citric acid is realized by submerged fermentation of *A. niger*, whereby yields of $0.95\,g\,g^{-1}$ glucose are achieved [80]. Although this is already close to the theoretical yield ($1.067\,g\,g^{-1}$ glucose), several strain optimization programs are ongoing to achieve or even exceed the physiological limit, for example by uncoupling fermentation from biomass formation, which is principally feasible as shown for the bacterium *Thermotoga maritima* producing hydrogen-biofuel [81]. Systems metabolic engineering to rationally redesign *A. niger*'s citric acid production capacities is generally possible due to the wealth of *A. niger* multiomics data and a well-curated GEM available. Comparative genomic studies with the 17 *A. niger* genomes available, some of which are citric acid producers, some of which are enzyme producers, have already unveiled unique genes or single nucleotide polymorphisms [32], which relate to the phenotypic traits of the production strains.

A recently published review lists 18 metabolic engineering approaches dedicated to improving citric acid production in *A. niger* [82], a few of which will be highlighted here. Dynamic flux balance analysis on time-course data during batch fermentation of the citric acid-producing strain *A. niger* ATCC 1015, for example, has recently uncovered that phosphate-limitation is a key factor that induces citric acid production [83]. A proteomics approach with a focus on membrane-associated proteins has identified two new high-affinity glucose transporters (MstG, MstH) and one rhamnose transporter (RhtA) which can be used to optimize substrate uptake in *A. niger* [84, 85]. Deletion of an α-glucosidase (*agdA*) coupled with overexpression of the glucoamylase-encoding gene *glaA* improved substrate utilization when cultivated on liquefied corn starch and, thus, productivity [86]. Finally, ATP-mediated feedback inhibition of the glycolytic enzyme phosphofructokinase became attenuated by replacing the ATP-producing cytochrome-dependent respiration with an alternative oxidase (Aox1), which still enables reoxidation of NADH but does not produce ATP [87]. These and further studies highlight why *A. niger* is such an excellent citric acid producer: (i) Efficient carbon source utilization, (ii) high glycolytic flux due to relief of ATP and citrate feedback inhibition, (iii) high anaplerotic activities which refill the citric acid cycle upon depletion, (iv) low citrate degrading enzyme activities, and (v) alternative respiratory pathways. Even more improvements of citric acid production became feasible with the recent identification of the citrate exporter CexA [88] and its targeted overexpression via the inducible synthetic Tet-on gene switch [89]. The future challenge will be to integrate all these individual gene modifications into one chassis strain that carries all relevant modifications in a synergistic manner, which, when referring to James Currie, results in minimum biomass but maximum citric acid production. The importance of cell compartmentalization for citric acid production has also not been fully explored yet. The canonical view, so far, is that the citric acid cycle

runs mainly in mitochondria, as the citrate synthase CitA is localized there. However, it has recently been proposed that part of the citrate could also be synthesized in the cytosol by a cytosolic localized CitB enzyme, which would be accompanied by less ATP production and, hence, reduced feedback inhibition [90]; a hypothesis worth studying further.

A. niger has recently gained lots of interest as a potential new producer of other organic acids, such as itaconic and galactaric acid. Itaconate could replace petroleum-based polyacrylic acid, which is a precursor for the polymer industry (absorbent polymers, polyester resins, synthetic latex) and galactarate could replace the current petroleum-based polyethylene terephthalate (PET) used for plastic production [90, 91].

Itaconate stems from the citric acid cycle, where citric acid becomes metabolized to *cis*-aconitate, which is then converted to itaconate by the enzyme cis-aconitate decarboxylase CadA [92]. This pathway is common for *A. terreus* but not naturally present in *A. niger* because it lacks CadA and a *cis*-aconitate transporter MttA, which transports *cis*-aconitate out of the mitochondrium into the cytoplasm where *cis*-aconitate becomes eventually decarboxylated to itaconate by CadA [93]. Surprisingly, rewiring of the metabolism of *A. niger* toward itaconate does not turn it into a superior itaconate producer. This is currently assumed because of unwanted itaconate conversion into either itaconate methyl-ester or full oxidation into pyruvate and acetyl-CoA [94]. Hence, more research is necessary to establish *A. niger* as an itaconate producer in which product degradation has been successfully prevented.

The rewiring of *A. niger* to overproduce galactarate, which was achieved by several single- and multiplexed CRISPR approaches, seems very promising. In brief, an engineered *A. niger* strain was established that was capable of hydrolyzing pectin (a component of the plant primary cell wall and most abundant in sugar beet pulp and citrus processing waste streams) to D-galacturonate, which was further oxidized to galactarate with titers of $12\,g\,l^{-1}$ [40, 91]. Interestingly, D-galacturonate has also been shown to be convertible by a genetically engineered *A. niger* strain to L-ascorbate, i.e. vitamin C [95]. Although only $170\,mg\,l^{-1}$ vitamin C was obtained, which is far below of what can be achieved with bacterial cell factories, this can be considered as an important breakthrough study. This is the first report of a metabolically engineered filamentous fungus for vitamin C production in a one-step fermentation process on citrus peel waste.

Last but not the least, *A. niger* is one of the most commonly exploited cell factories for protein and enzyme production because of its extraordinarily high secretion capacities. The most abundant enzyme secreted by *A. niger* is glucoamylase, which has applications in industries based on starch, for example, the food, feed, biofuel and chemical industries. Up to $30\,g\,l^{-1}$ can be achieved during industrial production [96]. *A. niger* is also used for the production of other enzymes, including cellulases, pectinases, proteases, catalases, and phytases [2, 5, 97]. A common theme between citric acid and protein production is surprisingly oxygen limitation. It was shown for both processes that limited oxygen supply favors high production in the strict aerobic fungus *A. niger* [98, 99], i.e. an inverse correlation between citric acid/protein production and cell growth can be found. Notably, a high specific protein production rate is also

achieved at relatively low growth rates in *T. reesei* [53]. A recent multiomics analysis with a focus on glucoamylase production integrated transcriptomics, metabolomics and GEM simulations reported that this is probably achieved through several metabolic mechanisms: (i) An increased flux through glycolysis, which probably generates more amino acid precursors for protein production; (ii) reduced fatty acid and ribosome biogenesis and, thus, reduced growth; and (iii) increased flux through the glyoxylate bypass to reduce NADH formation from the citric acid cycle and to maintain the cellular redox balance [99]. The general view is that once growth is limited, more reducing equivalents NADH and NADPH, and precursors can be channeled into glucoamylase production. A comparative transcriptomics analysis of *A. niger* strains forced to overexpress and secrete glucoamylase uncovered that *A. niger* benefits from a very flexible transcriptional machinery that ensures *A. niger* adapts to the burden of high protein loads. Under this circumstance, *A. niger* increases the transcription of secretory pathway genes involved in protein folding in the endoplasmic reticulum (ER) and protein trafficking from the ER over the Golgi to the plasma membrane (see Figure 20.2b). In addition, expression of genes less required for growth and survival under this condition become decreased [100]. This phenomenon is called "Repression under secretion stress" (RESS) and was first discovered in *T. reesei* [101]. *A. niger* can, thus, fall back on a very efficient regulatory and metabolic machinery that balances cellular capacities with the necessary. The canonical view is that the extraordinary capacity of *A. niger* and other filamentous fungi for protein secretion is linked to their hyphal tip growth mode, a relationship that will be discussed in more detail in Section 20.7.

20.5.2 *Aspergillus oryzae*

A. oryzae has traditionally been used for the production of Asian food and beverages for over a thousand years and was awarded the title as the national microorganism of Japan (Koku-kin) by the Brewing Society of Japan in 2006 [102]. The efficacy of *A. oryzae* as a protein producer and secretor is also rooted in a sophisticated transcriptional control machinery that enables high-level production and secretion of amylolytic enzymes when cultivated on starch-rich sources, such as rice and soybeans [103]. *A. oryzae* also gained lots of interest recently as a cell factory for biofuel production, as its genome contains many cellulolytic and xylanolytic enzymes [64]. First transcriptomics insights have already uncovered that the conserved transcriptional regulator XlnR seems to play a central role in the regulation of cellulolytic gene expression than in other filamentous fungi [104], thus, being an excellent target for future metabolic engineering approaches. A successful recent metabolic engineering approach focusing specifically on multiple cellulolytic genes which increased their secretion 40-fold [77] was described in Section 20.4.

A. oryzae is, furthermore, of biotechnological interest because it is the exclusive producer of the secondary metabolite kojic acid, which is basically a by-product during rice and soybean fermentation. However, because of its high biocompatibility and antioxidant activity, it is applied in cosmetics as a skin-lightener. This is due to its potent tyrosinase inhibition activity in the

synthesis of melanin [105]. It is, moreover, of interest as a building block for biodegradable plastics [106, 107]. It is assumed that all enzymes necessary for synthesizing kojic acid are encoded in a gene cluster which comprises 14 genes, one of which encodes the pathway-specific transcription factor KojR [108]. Targeted overexpression of KojR combined with overexpression of three cellulolytic genes enabled *A. oryzae* to produce kojic acid directly from cellulose instead of from starch [106]. However, the kojic acid titer achieved on cellulose was about 100-fold lower when compared to glucose as a carbon source ($26\,g\,l^{-1}$). Hence, further optimization efforts are necessary. Nevertheless, the use of cheap and renewable carbon sources derived from waste streams containing cellulose might establish *A. oryzae* as an attractive cell factory for kojic acid-based biodegradable plastics in the near future.

Finally, another promising platform chemical which can be produced with *A. oryzae* and that could serve as a driver to consolidate bioeconomy is malic acid. This organic acid stems from the citric acid cycle and has manifold applications in the food (acidulant, flavor enhancer), chemical (polyester resins), and pharmaceutical (acidulant) industries [109]. Several microbial cell factories from bacterial, yeast, or filamentous fungal origin have been genetically engineered during the last few years to produce this platform chemical. The approaches followed the biochemical routes which have been genetically engineered, and the yields which have been achieved in the different organisms have recently been summarized in [110]. *A. oryzae* is among the strains with the greatest potential, which fostered several metabolic engineering efforts. Lately, a producing strain was reported that displayed high malate titer ($127\,g\,l^{-1}$) and malate yield ($0.9\,g\,g^{-1}$ corn starch) and produced much less succinate, which is the unwanted byproduct in this process [111]. This was achieved by synergistically targeting carbon and redox metabolism and included 12 genetic modifications in total which were introduced into *A. oryzae*. These involved overexpression of amylolytic genes, overexpression of the malate-producing enzyme fumarase, increased expression of the glyoxylate shunt to bypass succinate, downregulation of the malate-degrading enzyme citrate synthase, and introduction of a NADH oxidase of bacterial origin to improve the redox balance by decreasing the $NADH/NAD^+$ ratio, to name but a few [111]. The significant increase in malate production with a simultaneous decrease of the by-product succinate improved the productivity of this process considerably and is, thus, an important step forward to a sustainable production of this platform chemical in *A. oryzae*.

20.5.3 *Aspergillus terreus*

A. terreus is a well-established cell factory for the production of the organic acid itaconate and the polyketide lovastatin. The former is of interest for the polymer industry [112], as described in Section 20.5.1, while the latter is applied in medicine as a cholesterol-lowering drug for the treatment of cardiovascular diseases. It has been marketed under the trade name Mevacor since the late 1980s [113]. Lovastatin also serves as a starter molecule for manufacturing semisynthetic statins. One example is simvastatin, the second leading statin in the market and traded under the name Zocor [114].

Interestingly, the biosynthetic routes for both itaconate and lovastatin are encoded in adjacent biosynthetic gene clusters in the genome of *A. terreus*. However, a biosynthetic co-occurrence of both has never been reported, probably because they are not under the control of a common regulatory mechanism [115]. This is thought to be because itaconate stems from the primary metabolism, whereas lovastatin is from the secondary metabolism. It has, therefore, been proposed that strains which have been selected during strain development programs for itaconate manufacturing are poor producers of lovastatin and *vice versa*. The maximal titers which can be reached for itaconate in *A. terreus* are $140\,g\,l^{-1}$ but only about $1\,g\,l^{-1}$ for lovastatin [115].

Reports of genetic and metabolic engineering of both metabolites in *A. terreus* are sparse. Most efforts are devoted to process optimization, in order to identify optimum carbon and nitrogen sources, trace elements, pH conditions, oxygen supply, and macromorphologies. The interested reader may consult [112, 115] for a detailed overview of bioprocess-related studies. Another review which can be recommended for further reading is a comparative analysis of citric acid production in *A. niger* and itaconate production in *A. terreus* regarding common or dissimilar metabolic and regulatory mechanisms important to ensure their high level production in both cell factories [90].

Current efforts in metabolic engineering concerning *A. terreus* aim to reroute lovastatin biosynthesis in order to obtain high amounts of one of its biosynthetic intermediates, monacolin J. This is basically the preferred precursor for semisynthetic simvastatin production and not lovastatin [116]. The classic production process of simvastatin has, so far, integrated several steps: (i) Lovastatin production through *A. terreus* fermentation; (ii) lovastatin extraction and purification from the biomass of *A. terreus*; (iii) enzymatic hydrolysis of lovastatin to monacolin J; and (iv) chemical transformation of monacolin J to simvastatin [117]. An improved *A. terreus* strain was engineered recently that allowed a single-step bioproduction of monacolin J through deleting the gene encoding the last enzymatic step of lovastatin biosynthesis (*lovD*) and constitutive overexpressing of the gene encoding the pathway-specific transcription factor LovE. This enabled high-level production of monacolin J ($5.5\,g\,l^{-1}$), which is far above all published efforts to establish monacolin J production in heterologous hosts, such as *S. cerevisiae* ($75\,mg\,l^{-1}$) or *Pichia pastoris* ($600\,mg\,l^{-1}$) [117].

20.5.4 *Penicillium chrysogenum*

P. chrysogenum (renamed *P. rubens*) is important for antibiotics production and is the main cell factory producing β-lactams, such as penicillin and semisynthetic derivatives thereof. Penicillin is a secondary metabolite which is naturally produced during late stages of growth of *P. chrysogenum*. Its biosynthetic pathway is encoded in a biosynthetic gene cluster which comprise three genes. The core gene is a nonribosomal peptide synthase. Current production strains have undergone multiple classic strain improvement programs over the last 70 years, since *P. chrysogenum* was established as a β-lactam production strain in 1943. These strains can contain up to 50 copies of the penicillin gene cluster and are able to produce up to $55\,g\,l^{-1}$ penicillin [118].

Strain improvement programs were based on random mutagenesis using UV or mustard gas and considerably altered the metabolic fluxes in *P. chrysogenum* [118, 119]. Most recent attempts aim to comprehensively understand the genetic, regulatory, and metabolic mechanisms that were rewired in order to reconstruct high-production strains by targeted genome breeding in the near future. Genome breeding is a well-established approach which has been successfully applied for the first time for the amino acid-producing bacterial cell factory *Corynebacterium glutamicum* [120]. Basically, mutations useful for production become identified in a set of different low- and high-producing classical mutant strains through genome sequencing and integrated multiomics analyses. The relevant mutations become, thereafter, systemically introduced into a wild-type genome to obtain a genetically streamlined strain that carries only useful mutations.

A comparative genomic and metabolomic analysis of three *P. chrysogenum* strains with low or high penicillin titers led to the following conclusions [119]: Firstly, 2500 mutations have been introduced into high-level *P. chrysogenum* penicillin production strains over the last 70 years when compared to the progenitor wild-type strain. Secondly, the epigenetic and light-sensing regulator complex Velvet with its central methyltransferase LaeA and the scaffold protein VelA have been repeatedly targeted. And thirdly, *P. chrysogenum* seems to be very flexible in redirecting nitrogen, i.e. amino acids, from one nonribosomal peptide biosynthetic route to another. If the penicillin route is blocked, for example, other nonribosomal peptides are produced by *P. chrysogenum*, such as roquefortines, meleagrin, or chrysogenins [119]. A similar redirection phenomenon is also observed in the bacterial antibiotics producer *Streptomyces coelicolor* [121]. This might suggest that nonribosomal peptides could be viewed as a flexible set of nitrogen storage molecules under a high flux of carbon and sufficient intracellular amino acid availability. A hypothesis worth studying further.

The penicillin biosynthetic route in *P. chrysogenum* has lately been reprogrammed toward an industrial pravastatin production process [122]. Pravastatin (trade name Pravachol) is an interesting alternative to simvastatin (Zocor) because of different structural, bioavailability and pharmacokinetic properties [123]. A one-step fermentative production of pravastatin in *P. chrysogenum* was achieved by (i) deleting the penicillin gene cluster, i.e. generation of a β-lactam-free platform strain, (ii) random introduction of the compactin gene cluster from *Penicillium citrinum* in the genome, and (iii) expression of a fusion protein containing an stereoselectively evolved compactin hydroxylase from *Amycolatopsis* and a reductase as a redox partner from *Rhodococcus*. This eventually resulted in a production strain that achieved titers of $6\,g\,l^{-1}$ pravastatin [122]. The readers may consult a recent review for more engineering examples focusing on *P. chrysogenum* and strategies which are followed for this cell factory to activate or silence biosynthetic gene clusters [124].

20.5.5 *Trichoderma reesei*

The main biotechnological importance of *T. reesei* is attributed to its cellulase and hemicellulase enzymes, which are key to converting lignocellulosic biomass

into biofuel. Lignocellulose is composed of cellulose, hemicellulose and lignin and is a waste product of agriculture (e.g. straw, bagasse, corn stover) and forestry (sawdust). Whereas the originally isolated wild-type strain (QM6a) is a poor (hemi)cellulase producer and secretor, classic strain development yielded the Rutgers strain Rut-C30 with $30\,g\,l^{-1}$ in the 1980s. Hypersecreting strains were obtained by directed evolution at the dawn of the twenty-first century, achieving cellulase titers of $100\,g\,l^{-1}$ [125]. These are the highest titers ever reported for protein secretion and exceed by 10–10 000-fold what can be achieved nowadays with bacterial, yeast or mammalian cell factories. Protein secretion titers in these cell factories are usually in the order of $mg\,l^{-1}$ to only a few $g\,l^{-1}$ [126–128]. Such an extraordinary high capacity of filamentous fungi, such as *T. reesei, A. niger,* and *A. oryzae*, exploited in biotechnology for protein secretion can be attributed to several factors: (i) An effective protein secretion machinery is a prerequisite for fast hyphal growth (see Section 20.7); (ii) the saprophobic lifestyle of filamentous fungi on plant biomass is only possible due to efficient secretion of high amounts of extracellularly active enzymes; and (iii) the RESS phenomenon, which was first described in *T. reesei* but has also been documented in *A. niger* ([101], see Section 20.5.1), ensures efficient down-regulation of genes less important for survival during fast colonization of dead plant material. The underlying metabolic and regulatory machineries enabling *T. reesei* to transcribe and express (hemi)cellulose encoding genes efficiently and genetic engineering strategies to make (hemi)cellulase secretion inducer-independent have been discussed in Section 20.4.

Thermostable cellulases are one major goal in current lignocellulose degradation processes [129] and have inspired protein engineering efforts in *T. reesei* to obtain cellulases which are functional at higher temperatures. In general, high process temperatures are preferred in biorefineries as they reduce the viscosity and solubility of lignocellulosic biomass and thus increase reaction rates [129]. Using protein structure and stability predictions, chimeric enzymes were designed based on a cellulase from *T. reesei* and its thermostable homologs from two other filamentous fungi (*Talaromyces emersonii* and *Chaetomium thermophilum*). This approach eventually enabled the improvement of the thermostability of the *T. reesei* enzyme by up to 3 °C [130]. Lytic polysaccharide monooxygenases are auxiliary enzymes that accelerate the breakdown of cellulose, chitin and starch by oxidative cleavage of glycosidic bonds [131]. It has been shown that, when added to purified *T. reesei* cellulolytic enzyme cocktails, they improve the hydrolysis of lignocellulose considerably [132]. This observation has led to the development of the new commercial cellulase cocktails Cellic CTec2 and Cellic Ctec3 [133]. A thermostable lytic polysaccharide monooxygenase from the filamentous fungus *Talaromyces cellulolyticus* has recently been heterologously expressed in *T. reesei* and, indeed, improved *T. reesei*'s degradation efficiency of cellulose and delignified corncob residues [134].

T. reesei is also of interest for the production of the organic acid galactarate. A QMA6a-derived strain, in which its intrinsically encoded degradation pathway for galactarate was eliminated and a bacterial galacturonate dehydrogenase gene was introduced, resulted in a strain which produced $20\,g\,l^{-1}$ galactarate directly

from pectin [135]. A recently metabolically engineered *S. cerevisiae* platform strain obtained 8 g l^{-1} from citrus peel waste [136].

20.5.6 *Thermothelomyces thermophilus*

The biotechnological application of the thermophilic *T. thermophilus* is primarily associated with its ability to produce and secrete thermostable cellulolytic enzymes. It is an emerging filamentous fungal cell factory and was previously known as *M. thermophila*. The *T. thermophilus* strain ATCC 42464 is accepted as the general wild-type strain in academia, whereas the proprietary mature enzyme production strain C1 is used in industry [137]. Production levels up to 100 g l^{-1} cellulases are possible using the C1 strain, with the additional advantage of low viscosity levels during fermentation. The first commercial product was CeluStar CL, which was granted the GRAS status by the FDA in 2009 [138]. It is currently being improved by industry to become a producer for biologics, such as vaccines, therapeutic enzymes, proteins and biosimilars [139]. In addition, first very promising metabolic engineering studies show impressively how *T. thermophilus* can be reprogrammed to generate platform chemicals, such as fumarate and malate, directly from renewable feedstocks.

So far, fumaric acid has been produced with filamentous fungi from the genus *Rhizopus*, which naturally employ the cytosolic reductive citric acid cycle under nitrogen-limiting conditions. Fumarate is of interest as an acidulant and antioxidant in the food and beverage industries and for the manufacturing of synthetic resins and biodegradable polymers to replace petroleum-based processes [140]. Optimization of medium composition and process-relevant parameters increased the fumarate titers in natural *Rhizopus* strains up to 40 g l^{-1} [141, 142]. Metabolically engineered *E. coli* strains reached 28 g l^{-1} on glucose and 42 g l^{-1} on glycerol as a carbon source [143, 144], an engineered *S. cerevisiae* strain 6 g l^{-1} [145] and an engineered strain of the yeast *Torulopsis glabrata* 33 g l^{-1} [146]. A recent CRISPR-based metabolic engineering effort in *T. thermophilus* achieved 17 g l^{-1} fumarate and involved simultaneous optimization of organic acid transport in and out of the mitochondria, overexpression of fumarase to increase the flux from malate to fumarate and deletion of the fumarate-degrading enzyme fumarate reductase [147]. Conceptually in a similar approach but tackling other phenomena such as CO_2-fixation through pyruvate carboxylase and mitochondrial transport systems, a malate-overproducing strain was generated that produced 200 g l^{-1} malate from crystalline cellulose and 110 g l^{-1} from corncob [148]. These data are promising for the future establishment of *T. thermophilus* as an efficient cell factory for platform chemical production directly from plant waste streams.

20.6 Engineering Strategies for the Production of New-to-Nature Compounds

Filamentous fungi produce a wide range of secondary metabolites, which are all derived from acetyl-CoA as the critical initial building block (Figure 20.3).

Table 20.4 Selected secondary metabolites from filamentous fungi and their applications.

Compound	Application	References
β-Lactams	Penicillins and cephalosporins account for more than 30% of the global antibiotics market	[149]
Cyclosporin	Immunosuppressant that avoids organ rejection in transplant surgery	[150]
Echinocandins	Caspofungin, micafungin, and anidulafungin used for the treatment of *Candida* infections	[151]
Griseofulvin	Antifungal used for the treatment of skin infections	[152]
Mycophenolic acid	Immunosuppressant that avoids organ rejection in transplant surgery and is traded as CellCept	[153]
Myriocin	Chemical analog thereof is used to treat multiple sclerosis; approved in 2018 as Gylenia	[154]
Statins	Lovastatin, simvastatin and pravastatin are used to treat cardiovascular diseases by lowering cholesterol levels	[113]

These compounds not only differ in structure but also in their bioactivities, which can be antibacterial, antifungal, insecticidal, antiparasitic, or cytotoxic, to name but a few. Some currently traded important pharmaceuticals from filamentous fungi and their medicinal applications are summarized in Table 20.4. Metabolic engineering strategies to improve the production of some of these have been discussed in Section 20.5.

Genome mining of hundreds of filamentous fungal genomes disclosed that the number of predicted biosynthetic gene clusters by far exceeds the number of known fungal secondary metabolites and suggests that millions of fungal metabolites await their discovery, many of which will have potential pharmaceutical applications [4, 58, 155]. The reader is referred to [156, 157] for more information on how to identify and harness this untapped resource by integrating multiomics studies and implementing technological advances, such as microfluidics, next-generation 3D-bioprinting and controlled cocultivation.

Notably, the impressively high structural diversity of the secondary metabolites discovered so far suggest that they can already serve as very interesting lead structures for the development of a broad repertoire of new-to-nature compounds. These novel compounds might have new bioactivities or improved bioavailability, higher stabilities and better pharmacokinetics compared to related drugs used currently or could even serve as better precursors for semisynthetic routes for new drugs [158]. Combined with the power of filamentous fungal cell factories established already in which pathway engineering ensures their efficient diversification, new production processes might become feasible. In the following, the conceptual strategy for the diversification of fungal secondary metabolites will, thus, be illustrated along with the example of fungal cyclodepsipeptides (CDPs), which are cyclic nonribosomal peptides composed of alternating units of amino acids and α-hydroxy acids.

Enniatin, beauvericin, bassianolide, and PF1022 belong to the class of CDPs and they exhibit antibacterial, antifungal, insecticidal, anthelmintic, or even anticancer activities and are, thus, of great interest to the pharmaceutical industry [158]. Two CDPs have already been commercialized: fusafungine (a mixture of enniatins) for the treatment of bacterial throat infections and emodepside (a semisynthetic derivative of PF1022A), which is used as anthelmintic compound in veterinary medicine [159]. Expression of the nonribosomal peptide synthetase encoding gene *esyn1* from *Fusarium oxysporum* under the control of the synthetic Tet-on gene switch in *A. niger* and optimization of the medium composition resulted in enniatin B production up to 4.5 g l^{-1} during fed-batch bioreactor cultivations [160, 161]. Tet-on-driven polycistronic expression of the *esyn1* and the *kivR* gene encoding an enzyme generating the α-hydroxy acid precursor molecule resulted in 40% of the product titer [25]. This proved for the first time that polycistronic secondary metabolite biosynthesis is possible in *A. niger* and, furthermore, suggested that the KivR enzyme catalyzes the rate-limiting step in enniatin B biosynthesis.

Remarkably, *A. niger* was shown to be not only a superior expression host for enniatin B but also for beauvericin and bassianolide, by producing the highest titers ever reported for bacterial, yeast, or fungal hosts [162]. It was, furthermore, demonstrated that *A. niger* is an ideal platform strain for the production of new-to-nature CDPs, which were obtained by designing chimeric CDP synthetases by either the swapping of enzyme modules, domains, or subunits thereof [163, 164]. Feeding alternative α-hydroxy acid precursors also allowed the synthesis of novel CDP derivatives up to 1 g l^{-1} [162]. Most importantly, some of the new-to-nature CDPs displayed considerably higher bioactivities compared to their parental CDPs and reference drugs [164]. Hence, the currently available engineering toolbox for *A. niger* and its high metabolic flux toward amino acids and α-hydroxy acids can be harnessed to produce nonribosomal peptides with natural or novel structures at industrial relevant titers.

20.7 Engineering Strategies for Controlled Macromorphologies

The macromorphology of filamentous fungi adopted during submerged cultivation in bioreactors is very critical for product titers. The formation of pellets, loose clumps, or dispersed macromorphologies (Figure 20.1) result from various interacting phenomena [3].

In brief, spores are used as inoculum. After an initial period of spore swelling in which spores break metabolic dormancy, a complex developmental program is initiated that ensures that germ tubes are formed. An intricate intracellular interplay and coordinated regulation of polarity proteins (which target growth exclusively to the newly formed tip, e.g. cell end markers, formins, polarisome), cytoskeletal elements (tubulin, actin), and vesicle transport proteins (which ensure trafficking of proteins within vesicles from the ER over the Golgi to the plasma membrane along the cytoskeletal tracks, e.g. SNARE proteins,

GTPases, myosin) ensure that vesicles accumulate at the tip of the germ tubes, where they eventually fuse with the plasma membrane. This results in plasma membrane extension, which causes the germ tube to elongate and form long, thread-like cells termed hyphae. Part of the vesicular cargo is devoted to cell wall synthesizing proteins, which remain embedded in the plasma membrane and secure the biosynthesis of chitin and glucans. Another part is released into the external environment and is mainly composed of hydrolytic enzymes, whose function is to degrade organic matter, i.e. amylases, cellulases, proteases, and lipases, to name but a few. As growth continues, hyphae start to form cross-walls (called septa) and branches and, eventually, a mycelium is formed. Hence, it is thought that strains with more hyphal tips might have the theoretical potential to secrete more proteins as more exit routes are available.

From a cell biological perspective, this is an oversimplified view of what is known so far (an estimated number of 2000 proteins are thought to participate in hyphal growth and development in *Aspergillus* [165]) and the reader may consult the following reviews for further reading [166–168]. The cell biological knowledge generated so far inspired several targeted strain optimization efforts. It was shown, for example, that the downregulation of chitin synthase genes in *A. niger* and *P. chrysogenum* elevated citric acid (40%) or penicillin titers (40%), respectively [169, 170]. In another study, cell cycle genes were upregulated in *A. oryzae* and increased malate titers by about 50%. Furthermore, a hyperbranching *A. niger* strain was generated that accumulated the same biomass during cultivation while it was hyperbranching compared to the wild-type [171]. This hyperbranching strain was then further genetically engineered to overexpress glucoamylase. By putting transcriptional control of the *glaA* gene under the metabolism-independent Tet-on gene switch, 400% higher secretion of the glucoamylase was achieved [172]. Smaller pellets and smaller dispersed mycelia were observed in all four cases when compared to the respective progenitor strains.

From a process engineering perspective, it is known that the first hours after spore inoculation will already be influential for the development of the final macromorphologies. This depends on the spore titer used, their ability to coagulate or not, the medium composition, the pH of the medium, and the agitation speed of the bioreactor. The frequency of hyphal branching is also decisive – the higher the branching frequency, the higher the tendency to form pellets. Notably, dispersed mycelia or loose clumps can also agglomerate during later stages of fermentation and form pellets, whereas pellets can fragment into smaller entities due to high shear forces. The mechanistic basis for the formation of pellets or dispersed mycelia is, thus, attributed to hyphal extension and branching rates, pellet fragmentation rates, and bioreactor parameters, which are now increasingly modeled [173–176].

Pelleted or dispersed macromorphologies have both advantages and disadvantages. Pellets are more resistant to shear stress and cause low viscosity of the liquid phase. However, transport of oxygen and substrate to the core area of larger pellets is limited by the hyphal network, which, in turn, may limit growth, viability, and, finally, product formation. By contrast, dispersed mycelia grow rapidly and are less limited regarding nutrient transport but cause a higher medium viscosity, thus, a lower volumetric gas-liquid mass transfer. Dispersed mycelia are

also more susceptible to shear stress [3]. Importantly, a canonical view on how fungal macromorphologies are coupled with product formation is missing. On the one hand, this is due to the fact that many reports in the literature contradict each other [3, 115]. On the other hand, only a limited number of systematic attempts have been undertaken so far to (i) understand the genetic and metabolic network driving hyphal growth and the evolution of macromorphologies, (ii) to analyze at which levels the macromorphological structures interact with the bioreactor environment, and (iii) to measure to what extent these interactions feedback to the cells and activate or repress certain metabolic activities.

However, several tools have recently been implemented to measure and quantify macromorphological parameters under submerged cultivation conditions which are important prerequisites for future attempts to model and engineer fungal macromorphologies in an integrated manner. The MPD pipeline [177], for example, measures hundreds of macromorphological structures from microscopic images by quantitatively assessing filamentous fungal cultures, which usually consist of both dispersed and pelleted forms (Figure 20.4a). It, thus, gives a quantitative measurement of culture heterogeneity. Furthermore, it automatically generates key Euclidian parameters for individual fungal structures, such as projected area, circularity, aspect ratio, and surface roughness, and calculates the dimensionless morphology number MN, which varies between 0 (a one-dimensional line) and 1 (a perfect circle) [178].

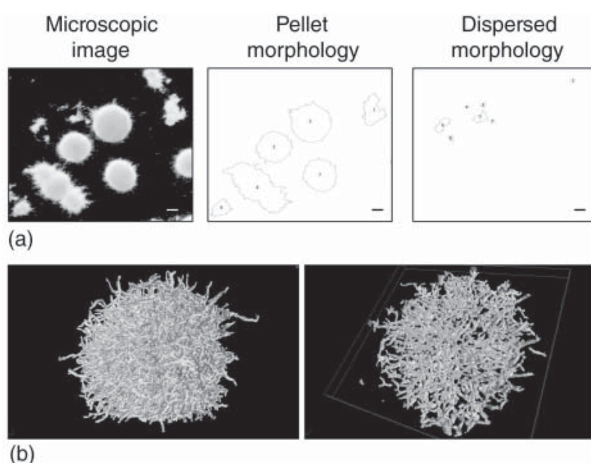

Figure 20.4 Image analysis tools to quantify fungal macromorphologies. (a) The MPD pipeline [177] uses microscopic raw images (left panel, the scale bar = 500 μm) to automatically assess pellets and disperse structures. Structures are depicted as outlines indexed with a unique number (red), enabling simple assessment of automated calls by the end user. Processed outlines of fungal structures passing default definitions of pelleted (≥500 μm^2) and dispersed (<500 μm^2 and ≥9 μm^2) structures are shown. (b) A three-dimensional X-ray microcomputed tomography image of an *A. niger* pellet. Left panel: projection of a whole pellet, right panel: projection of a central slice with a depth of 25 μm. Source: Note that (a) has been reproduced from Cairns, et al. [177], CC BY 4.0. Images presented in (b) are courtesy of Heiko Briesen and Stefan Schmideder.

An integrated approach implementing the gene coexpression network available for *A. niger* [58] (see Figure 20.1), CRISPR technology and the MPD pipeline has already identified a gene (*alpD*) whose tunable Tet-on-based gene expression caused a protein hypersecretion phenotype, which was correlated with increased dispersed mycelia, and both decreased pellet diameter and *MN* [177]. This is in good agreement with the available literature data for *A. niger*, which implies that a low *MN* value (i.e. a dispersed morphology) seems to correlate positively with protein production, whereas a high *MN* value (i.e. a pellet morphology) seems to correlate with citric acid production [3].

Most importantly, neither bright-field nor confocal laser-scanning or electron microscopy are able to analyze the 3D structure of pellets in a nondestructive way, making it difficult to analyze the growth in inner pellet regions and potential autolysis due to nutrient and oxygen limitation. This limitation has recently been overcome by successful application of X-ray microcomputed tomography (μCT) on freeze-dried pellets from *A. niger* and *P. chrysogenum* [179, 180]. This technique allows one to investigate the spatial distribution of hyphae within pellets (Figure 20.4b) and to measure effective diffusivity of oxygen or nutrients into, within and out of pellets [180]. It is now possible to calculate morphological properties, including hyphal length, number of tips, number of branches, hyphal growth unit, porosity, and hyphal average diameter directly from μCT data. Calculations are possible, for the first time, which quantify, for example, that a pellet of *A. niger* (*P. chrysogenum*) with a diameter of about 600 (1000) μm consists of a total hyphal length of about 1.4 m (2.9 m) and provides about 15 000 (20 000) tips [179]. The latter information is of great importance for protein production in filamentous fungi, because it is generally assumed that protein secretion occurs predominantly at the tip. However, recent reports also demonstrated that amylases colocalize at septal junctions in *A. niger* and *A. oryzae*, indicating that secretion may additionally occur at septa [181, 182]. In any case, it now becomes feasible to adjust the number of tips by genetic means and relate the macromorphological structures gained to the productivities measured. Both the MPD and the μCT pipeline, therefore, provide important tools toward future multiscale modeling which couples the evolution of macromorphological heterogeneities of filamentous microorganisms with oxygen supply and product formation.

20.8 Future of the Field

A pioneering and pivotal role of filamentous fungi for biotechnology is undisputed. As cell factories, they dominate the organic acid and enzyme market, occupy a key position in the production of antibiotics and drugs, and adopt important niches as cell factories for food and beverages. Importantly, they emerge as cell factories for the production of composite materials and animal-free leather, the latter of which will probably become a disruptive technology. A chemist, James Currie, laid the foundation for fungal biotechnology. Now, a century later, biologists, chemists, bioengineers, process engineers, and material scientists collaborate to harness and merge cell biological data, multiomics data, synthetic biology tools, and bioprocess data and integrate these

synergistically to metabolically engineer filamentous fungi for new and better products produced in a sustainable way. Investigations are no longer painstaking, as was the practice at Currie's time, but benefit from the different perspectives, know-how, tools, and technologies of these disciplines and enable important breakthroughs. Current research trends are devoted to understanding and engineering fungal heterogeneities during submerged cultivation for improved product formation by minimizing nonproducing cells, the development of filamentous fungal genomes devoid of unwanted genes and gene clusters with the long-term perspectives to generate minimal genomes, the identification of new secondary metabolites by integrated bioinformatics, multiomics and high throughput screening pipelines and the development of controlled cocultivation devices from microfluidic to larger scale to identify and produce secondary metabolites hidden in the genomes of filamentous fungi [2, 3, 157, 183].

The physicist Niels Bohr (1885–1962) once said: "Prediction is very difficult, especially about the future." A more forward-looking and pragmatic attitude is credited to the economist Peter Drucker (1909–2005), who said: "The best way to predict your future is to create it." How can filamentous fungal cell factories and bioengineers working on their metabolic improvements shape our future in the next decades? Here are some potential scenarios.

The concept of a "one-size-fits-all" solution, i.e. the establishment of multipurpose cell factories, is within reach. *A. niger* has been shown to be an efficient producer of citric acid, gluconic acid, galactaric acid, vitamin C, nonribosomal peptide-based drugs, antibodies, and enzymes, some of which have been discussed in this chapter. As it grows very fast on waste biomass based on starch, cellulose, or proteins, it could provide a platform strain for diverting carbon from many wastes toward diverse chemical and consumer products. As it tolerates extreme cultivation conditions intolerable to most bacterial or yeast cell factories, for example, a broad spectrum of pH (2–10), temperature (10–50 °C), salinity (0–34%), and water activity (0.6–1) [97], it has the advantage of being a robust cell factory perfectly adapted to harsh industrial cultivation conditions. Remarkably, it can also withstand hostile conditions in outer space and is the most common fungal isolate of the International Space Station. When mankind is about to travel to new planets, *A. niger* could become an essential companion for the autonomous production of food, vitamins, renewable chemicals, enzymes, antibiotics, drugs and proper waste management during space travel. A very recent review has discussed for the first time the opportunities for fungal biotechnology in outer space [184].

A further scenario for new, better and smarter consumer products is based on a strong collaboration of biotechnologists with artists and designers. Fungi are champi(gn)ons; this has not only been recognized by scientists working on the application of filamentous fungi for 100 years but also by artists and designers who started to exploit filamentous fungi for the production of sustainable textiles, biomaterials, and leather for the last 10 years [185, 186]. Stronger mutual collaborations in the future will ensure that the scientific, art, and design communities will feed and sustain each other and enable "outside the box" thinking for new innovative breakthroughs. In the sense of Peter Drucker, this will create novel and unprecedented avenues for filamentous fungi as cell factories in years to come.

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