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Filamentous fungi for the production of enzymes, chemicals and materials

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Filamentous fungi have been used for more than a century as versatile and highly productive cell factories. They are used to produce enzymes and small molecule compounds such as antibiotics and organic acids. Filamentous fungi are now also being explored for the production of sustainable materials that can for instance replace plastics. Mutagenesis and genetic modification are used to improve performance of production strains. Single cell technologies and bulk sample analysis are novel strategies to identify genes that can be used for genetic modification of production strains. Such genes may for instance be involved in fungal reproduction and hyphal heterogeneity. These differentiation processes have recently been implicated to affect production of enzymes and small molecule compounds. Finally, use of mixed cultures instead of monocultures can be a strategy to improve production processes.

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Introduction

Filamentous fungi play a crucial role in nature by degrading organic waste. Their filamentous mode of growth enables effective colonization of substrates and provides a large surface to volume ratio facilitating uptake of nutrients. Polymers in organic waste such as (hemi)cellulose and starch first have to be degraded into small molecules before they can be taken up to serve as energy and carbon source. To this end, fungi secrete a wide variety and large amount of enzymes. In addition, fungi secrete primary and secondary metabolites such as organic acids and antimicrobial compounds. Organic acids also function in release of nutrients for fungal growth. For instance, they solubilize soil minerals and can be involved in degradation of cellulose [1]. In addition, organic acids are secreted to lower environmental pH, thereby promoting fungal growth and inhibiting bacterial growth.

Fungal biomass is very abundant in nature illustrated by a single individual of the mushroom forming fungus *Armillaria bulbose* that had colonized about 1000 hectares of forest making it the largest organism on earth [2]. Fungal proliferation in nature is facilitated by the filamentous mode of growth and the secretion capacity of proteins and primary and secondary metabolites. Industry makes use of these properties to produce proteins and small molecule compounds and, recently, mycelium materials as well. Improving fungi as cell factories and their use to produce sustainable materials is the topic of this review. This will include aspects of fungal biology and genetics that have recently been explored to enable/increase production of molecules and materials.

Use of fungi for production of materials

The capacity of fungi to grow on organic material such as plant waste has attracted increasing attention of material sciences. Renewable mycelium-based materials have the potential to replace petroleum-based products such as plastics or could even provide novel materials. These bio-based products could for instance be used as thermal and acoustic insulation and packaging [3,4]. Pure fungal materials are the result of complete degradation of the substrate or are obtained by removing the fungal skin from the surface of a substrate. The properties of the mycelium depend on the substrate, the type of fungus, and growth conditions [5,6,7••]. Even a single genetic modification can affect material properties of the mycelium. For instance, a strain of *Schizophyllum commune* in which the hydrophobin gene *sc3* was inactivated retains more water and produces a more dense mycelium. The latter results in a 3–4 fold increased maximum tensile strength when compared to the wild-type [7••] and shifts the mechanical properties of the mycelium from being similar to those of natural materials to those of thermoplastics.

Composite mycelium materials are obtained by inactivating (e.g. by drying or a heat treatment) the fungus before degradation of the substrate has been completed. Composite mycelium materials have been shown to exhibit properties similar to expanded polystyrene or other foams [3,8,7••,9–11,12••] as well as natural materials such as cork and wood [12••]. Like pure mycelium, mechanical properties of mycelium composites depend on the fungus, substrate, growth conditions, and processing of the

material. In contrast to plastics, mycelium materials can display additional functions such as sorption of metals from liquid waste streams [13] or stimulation of the immune response of animals and human [14].

Use of fungi as cell factories

Mycelium materials are an upcoming field. In contrast, the enormous secretion capacity of fungi is already used by the industry for decades to produce organic acids [15], small molecule drugs [16] and homologous and heterologous proteins [17] (Table 1). Commercial production levels are not available in the public domain implying that reported values are most probably an underestimation. Citric acid has been commercially produced for almost a century with the use of *Aspergillus niger*. Production of this acid by *A. niger* fermentation had already outweighed extraction from citrus fruits close to a hundred years ago [18] and exceeds nowadays 169 g l^{-1} [19]. Other organic acids of industrial interest are for instance gluconic acid, kojic acid, and itaconic acid [15]. The latter acid was listed among the top 12 chemical building blocks and is a sustainable alternative to petroleum-based acrylic acid [20]. Itaconic acid can be produced at a >100 g l^{-1} but the fact that glucose is used as a feedstock makes it still too expensive compared to petrochemical manufactured raw materials. Yet, efforts are being made to reduce its production costs (see e.g. Refs. [20,21]).

Fungal enzymes are used in a wide variety of applications such as in production of food and feed, pulp and paper, textiles, detergents, beverages, and biofuels [22] (Table 1). Production levels of homologous enzymes can exceed 100 g l^{-1} as is the case of cellulases in *Trichoderma* [23]. However, production levels of heterologous proteins are far lower, being limited by protein degradation and inefficient transcription, translation, protein folding, translocation, and/or secretion [24]. Together, production levels of heterologous proteins are still restricted but production of organic acids and homologous enzymes is very efficient. The same holds for small molecule drugs such as antibiotics, statins and steroids. For instance, *Aspergillus terreus* and *Penicillium chrysogenum* produce up to 3 g l^{-1} statins [25] and 60 g l^{-1} penicillin [16], respectively.

Strategies to improve production of molecules and biomass

Industry has used different strategies to improve fungal cell factories. Mutagenesis and screening programs combined with improving fermentation conditions have resulted in strains that produce 100 000 times more penicillin than Fleming's original *Penicillium notatum* strain [26,27]. These strategies also resulted in high organic acid [18] and enzyme [28] production strains. Progress in molecular biology has enabled more directed approaches to solve bottlenecks in product formation that occur at the transcriptional and/or the (post)-translational level. For instance, introduction of a large number of gene copies into the host strain, targeting of constructs to positions in the genome that have a high transcriptional activity, use of strong promoters, and optimization of codon usage have been used to increase protein production. Similarly, use of efficient secretion signals, gene fusion strategies, introduction of *N*-glycosylation sites, increasing expression of the unfolded protein response, and use of protease deficient host strains have been used [28]. The former four strategies can also be used to improve production of small metabolites and biomass. For instance, over expression combined with deletion of genes was used to improve production of itaconic acid in *Ustilago maydis* [29] and *A. niger* [30]. This combined with adjusted culture conditions resulted in 67 g l^{-1} itaconate in the case of *U. maydis* [29]. Although the role of epigenetics in protein secretion and production of primary metabolites is not yet clear, it is now clearly established that epigenetic control has a great impact on expression of secondary metabolite clusters [31]. Clearly, insight in epigenetics can provide leads to improve production of molecules in fungal cell factories. Mixed cultures and

Table 1

Examples of fungi used for the production of enzymes and small molecule compounds

	Applications	Host
Enzymes		
Amylase	Bread making and production of glucose syrup	*Aspergillus niger, Aspergillus oryzae*
Protease	Food, laundry detergent, leather, pharmaceuticals	*Aspergillus niger, Aspergillus clavatus*
Pectinase	Clearing of juices and wine	*Aspergillus niger, Aspergillus oryzae*
Cellulase	Fabric softener	*Trichoderma viride*
Organic acids		
Citric acid	Food, beverages, cosmetics, laundry detergent	*Aspergillus niger*
Gluconic acid	Food, pharmaceuticals and hygienic products	*Aspergillus niger*
Pharmaceuticals		
Penicillin	Antibiotic, mainly Gram-positive bacteria	*Penicillium chrysogenum*
Cephalosporin	Broad spectrum antibiotic	*Acremonium chrysogenum*
Lovastatin	treatment of hypercholesterolemia	*Aspergillus terreus*

the phenomena of sporulation-inhibited secretion and heterogeneity are also novel leads to increase or even enable production of molecules and materials. This will be discussed in the next sections followed by tools that have the potential to contribute to improve production strains.

Mixed cultures to identify novel compounds and to improve production

So far, enzymes and small molecules are routinely produced by fermentation of a single strain. It was recently shown that secretomes and metabolomes can be different when co-cultures instead of mono-cultures are used. Such co-cultures consist of two or more fungal partners [32,33•], or a fungus (fungi) with another microorganism such as a bacterium (bacteria) [34,35,36•]. For instance, co-cultivation of *A. niger* with *Aspergillus oryzae* [33•] or *Streptomyces coelicolor* [35] resulted in a more complex enzyme and secondary metabolite profile, respectively, when compared to the mono-cultures. The *Aspergillus* transcription factor BasR was shown to represent the regulatory node for transduction of the bacterial signal in the fungus. As a result of this transduction, acetylation of chromatin is increased thus activating silent secondary metabolism gene clusters [36•]. Together, these data show that mixed cultures are of interest to identify novel enzymes or compounds and may even be used to optimize production processes such as those of enzyme mixtures that are used for biofuel production.

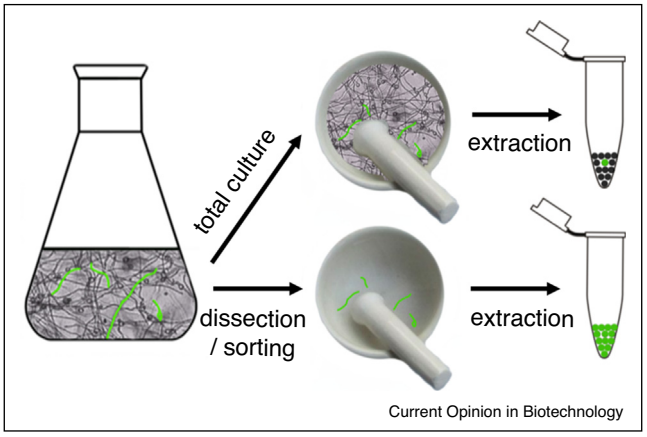
Abolishing (a)sexual reproduction to improve production

It is widely accepted that secondary metabolite production is linked to (a)sexual development [37]. There is now increasing evidence that production of enzymes and biomass are also associated with these developmental processes but in an inverse way [38•,39,40]. Sporulating zones of *A. niger* colonies do not secrete proteins but non-sporulating zones do [41]. Deletion of the sporulation gene *flbA* abolishes sporulation, which is accompanied by protein secretion throughout the colony. FlbA was shown to affect expression of 36 predicted transcription factor genes [42,43]. Inactivation of two of these transcription factor genes, *fum21* and *rpnR*, abolishes production of fumonisin and reduces protein secretion, respectively [44,45]. Notably, the secretion phenotype of *rpnR* was observed in liquid shaken cultures, indicating that sporulation inhibited secretion is an interesting target for improvement of production strains. Future research should reveal which of the 34 other predicted transcription factor genes that act downstream of FlbA are involved in secretion. In this respect, the role of FluG in secretion in *A. niger* is also of interest [46].

Abolishing heterogeneity to improve production

Traditionally, whole cultures are evaluated for production capacity of enzymes or small molecule compounds. However, this ignores the fact that only part of the culture is actually producing the molecule of interest (Figure 1). For instance, expression of genes encoding industrial enzymes is heterogeneous between micro-colonies within the same culture, between zones of an individual micro-colony, and even between hyphae of within a zone of a micro-colony [39]. This implies that expression profiles of the productive part of the mycelium is averaged by the RNA of the non-producing part of the culture (Figure 1). As a result, genes involved in regulatory mechanisms or in the cell biology of a highly producing hypha may easily go undetected. Indeed, differential expression of amylolytic genes in a wild-type *A. niger* strain and a *fluG* deletion strain was observed in particular zones of agar cultures but not within the whole colony [46]. Together, this implies that research should switch from whole culture approaches to single micro-colony, single zone, and single hypha approaches. To this end, one can use particle sorting [47] and laser dissection [48] to capture individual micro-colonies, zones and hyphae. Notably, heterogeneity is even observed within a single hypha [49•]. The most apical compartment is responsible for hyphal extension and secretion and does not dependent on sub apical compartments, at least for growth [49•]. This implies that one should study transcriptomics, proteomics, metabolomics as well as the cell biology of apical compartments to capture in detail which processes make a hypha highly productive. Laser dissection enables isolation of these hyphal tips and allows single compartment expression profiling [48].

Figure 1



Use of whole cultures for extraction of RNA, proteins, or metabolites will result in a mixture of components of producing (indicated in green) and non-producing hyphae. Differential expression within the producing hyphae will be masked by the non-producing cells when the latter form the majority of the population. This can be prevented by sorting or dissecting producing hyphae before extraction.

Genomic approaches

In the previous section particle sorting, laser dissection, and single-cell-omics technology were discussed as tools that

now can be used to further improve fungal production strains. These technologies can be complemented with bulk sample analysis (BSA). BSA tests for an association of variants in the genome, usually single-nucleotide polymorphisms (SNPs), and a phenotype of interest. The technology can be used for collections of natural strains, for segregants of a cross, and for strains resulting from experimental evolution. The decrease in costs of high-throughput sequencing makes that BSA will be more and more used to understand biological mechanisms underlying production of biomass, small molecule compounds and enzymes.

So far, genome-wide association studies of collections of natural strains of fungal cell factories or strains resulting from experimental evolution have not been reported. Yet, it has been used to identify genes involved in organic acid production using segregants of an *A. niger* cross [50]. The parasexual cycle was used to compensate for the absence of a sexual cycle in this industrial cell factory [51•]. Bulk segregant analysis in combination with high-throughput genome sequencing showed that the non-citric acid production phenotype of a UV mutant was caused by a point mutation in *laeA*. This gene encodes a putative methyltransferase-domain protein and is known to be involved in regulation of secondary metabolite production [52]. Recently, *A. niger* genes were identified that were associated with a hyper-production phenotype of a bacterial β-glucosidase whose gene had been placed under control of the glucoamylase *glaA* promoter [53•]. For instance, the mutant screen coupled with whole-genome sequencing revealed the low-affinity glucose transporter MstC. Deletion of its encoding gene significantly improved secretion of the β-glucosidase when it was driven by the *glaA* promoter. Together, these data show that BSA is a powerful tool to identify novel leads that can be used to increase production of homologous and heterologous proteins and probably small molecular weight molecules and biomass as well. Verifying the role of genes in production of molecules or biomass or improving production strains will be more efficient with availability of CRISPR CAS technology. Inactivation of genes by this system has recently been successfully implemented in fungi including cell factories such as *A. oryzae* [54–57].

Conclusions and outlook

Fungi have been used as a cell factory for production of enzymes and small molecule compounds for almost a century. Biomass produced during these production processes has generally been considered a waste stream. This may change in the future since fungal biomass is now being explored as the basis of sustainable biomaterials. The use of mono-cultures to produce enzymes and small molecule compounds may also change in the future. Recently, it has been shown that co-cultivation of different micro-organisms can change the enzyme and small molecule profile of the culture. As such, mixed cultures are not only of interest to identify novel enzymes and small molecule compounds, they can also be used to optimize production processes. For instance, mixed cultures may provide more optimal enzyme mixtures that are for instance used in biofuel production.

Traditionally, production strains have been improved by a black box approach using mutagenesis and screening or by genetic modification focusing on the expression of the gene(s) encoding the product of interest or components of the secretion pathway. It is now clear that differentiation processes such as sporulation and hyphal heterogeneity are also interesting leads to improve production strains. Non-sporulating production strains and strains with reduced heterogeneity are expected to have increased performance. The finding that only part of the hyphae within a culture produces the enzyme or small molecule of interest implies that strategies to identify target genes to improve production processes should be adapted. For instance, single cell approaches have the advantage that differential gene expression is not masked by the contribution of non-producing hyphae within the sample.

Conflict of interest statement

The author declares competing interests. Utrecht University collaborates with the company Mogu.

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