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*Review*

# Protein expression and secretion by filamentous fungi

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In the search for optimal platforms for protein expression and secretion, filamentous fungi in principle provide some of the best microbial cell factories. They are inherently endowed with the ability to secrete proteins. Fungi belonging to *Aspergillus* and *Trichoderma* species are well-studied for industrial production of proteins and enzymes. Our understanding of these organisms at the level of transcription, translation, post-translational processing and the secretory pathways has improved significantly in recent years. Despite this, the ability of these fungal secretion platforms has not yet been able to match their intrinsic secretion capacity to produce foreign proteins. Details of the molecular mechanisms of the secretory pathways in filamentous fungi are emerging. This knowledge can be gainfully employed to enhance protein production in filamentous fungi, particularly in the secretion of heterologous proteins of value.

**Keywords.** Aspergilli; fusion proteins; heterologous expression; secretion pathways; unfolded protein response

## 1. Introduction

Filamentous fungi in nature express and secrete large amounts of enzymes to scavenge nutrients from their surroundings. The saprophytic phenotype and biosynthetic potential of these organisms are widely exploited in white biotechnology (Meyer 2008; Meyer *et al.* 2016; Cairns *et al.* 2019a). Around fifty percent of all the industrial enzymes are produced by filamentous fungi – and with production levels easily reaching tens of grams per liter. Over the years, it has become apparent that filamentous fungi offer certain clear advantages compared to other protein expression systems (Nevalainen *et al.* 2005; Nevalainen and Peterson 2014). However, unlike the bacterial (such as *E. coli*) and yeast (such as *Pichia*; Juturu and Wu 2018) systems, filamentous fungal expression platforms are 'work in progress' because of their complexity. The experience gained thus far includes – relatively inexpensive media and fermentation scale-up expertise, the ability of post-transcriptional and post-translational modifications (particularly useful in expressing eukaryotic gene products), their GRAS (generally regarded as safe) status and the quickly expanding molecular/ genetic tools to engineer relevant strains.

There is vigorous on-going research in the area of fungal protein secretion involving both homologous and heterologous polypeptide products. A comprehensive list of proteins (both homologous and heterologous) expressed or expressed and secreted by key filamentous fungal hosts is listed in table 1. Many excellent reviews have also appeared addressing different aspects of the recombinant protein production by filamentous fungi (Lubertozzi and Keasling 2009; Su *et al.* 2012; Ward 2012; Lambertz *et al.* 2014; Reilly *et al.* 2016; Baker 2018; Damasio *et al.* 2019). To date, homologous proteins are expressed and secreted at least 10–1000 times more efficiently than the heterologous proteins by these fungi. This limitation has led researchers to explore every single aspect of mycelial expression and secretion apparatus (Sun and Su 2019). The complex and elegantly regulated process of protein expression and secretion can be divided into three broad stages shown below (figure 1). Most components


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**Table 1.** Recombinant proteins expressed and produced in filamentous fungi

| Host | Protein expressed (originally from)* |
|---|---|
| *A. niger* (including var. *awamori*) | Mammals: Chymosin (Bo), GM-CSF (Hu), Immunoglobulin G1 (Hu), Interferon-α-2 (Hu), Interleukin-6 (Hu), Lactoferrin (Hu), Pancreatic phospholipase A2 (Po), Prochymosin (Bo), α-1-Proteinase inhibitor (Hu), Tissue plasminogen activator (Hu)<br>Fungi: Arabinofuranosidase (As), Aspartyl protease (Mm), Catalase (As), Glucoamylase (An), Glucoamylase (As), Glucose oxidase (As), Laccase (Tv), Lipase (Tl), Manganese peroxidase (Pc), Pectin lyase (An), Peroxidase (Pe), Triglyceride lipase (Mm), Xylanase (As)<br>Bacteria: Dockerin (Ct), Enterotoxin subunit B (Ec), β-Galactosidase (Ec), β-Glucuronidase (Ec), Hydrolase (Tf)<br>Others: Cell surface glycoprotein (Bm), α-Galactosidase (Ct), Green fluorescence protein (Av), Lysozyme (Ov), Pyranose dehydrogenase (Am), Thaumatin (Td), Thaumatin II (Td) |
| *A. nidulans* | Mammals: Chymosin (Bo), Interferon-α-2 (Hu), Interleukin-6 (Hu), Lactoferrin (Hu), Prochymosin B (Bo), Tissue plasminogen activator (Hu)<br>Fungi: Carboxypeptidase (Ao), Glucoamylase (An), Peroxidase (Pe), Taka-amylase A (Ao), Tryprostatin B (Af)<br>Bacteria: Endoglucanase (Cf), Enterotoxin subunit B (Ec), β-Galactosidase (Ec)<br>Others: VP6 protein (Ro) |
| *A. oryzae* | Mammals: Chymosin (Bo), Lactoferrin (Hu), Lysozyme (Hu)<br>Fungi: Aspartyl protease (Mm), Cellulase (Hs), β-Galactosidase (As), Laccase (Ms), Lipase (Ca), Lipase (Tl), Phytase (Ps), Protease (Rh), Triglyceride lipase (Mm)<br>Others: Endoglucanase (Rs), Neoculin (Cl) |
| *T. reesie* | Mammals: CbhI-Fab fusion antibody (Hu), Chymosin (Bo), Erythropoietin (Hu), α-Galactosidase (Hu), Obestatin (Hu)<br>Fungi: Acid phosphatase (An), Aminopeptidase (As), Cinnamoyl esterase (Pi), Endochitinase (Th), Endoglucanase V (Hs), β-Glucanase (Ts), Glucoamylase P (Ho), Glucoamylase (Hr), β-Glucosidase (Te, Pd), Hydrophobin (Ai), Laccase (Ma, Tr), Lipase (An, Tl, Ta), (endo-1, 4) Mannanase (Ts), Xylanase (Hs, Ac, Px)<br>Bacteria: Xylanase (Nf)<br>Others: Endopeptidase B (Ba), Laccase (Pr) |

Ac, *A. nainiana*; Af, *A. fumigatus*; Ai, *A. nidulans* Am, *A. meleagris*; An, *A. niger*; Ao, *A. oryzae*; As, *Aspergillus* sp.; Av, *A. victoria*; Ba, Barley; Bm *B. microplus*; Bo, Bovine; Ca, *Candida* sp.; Cf *C. fimi*; Cl *C. latifolia*; Ct, *C. thermocellum*; Ec, *E. coli*; Hr, *H. resinae*; Hs, *Humicola* sp.; Hu, Human; Ma M *albomyces*; Mm M *miehei*; Ms, *Myceliopthora* sp.; Nf, *N. flexuosa*; Ov, Ovine; Pc, *P. chrysosporium*; Pd, *P.decumbens*; Pe *P. eryngii*; Pi *P. equis*; Po, Porcine; Pr *P. radiata*; Ps, *Peniophora* sp.; Px, *P. oxalicum*; Rh, *Rhizomucor* sp.; Ro, Rotavirus; Rs, *R. speratus* (termite); Ta *T. thermophilus*; Td *T. daniellii*; Te, *T. emersonii*; Tf, *T. fusca*; Th, *T. harzianum*; Tl, *T. lanuginosus*; Tr, *Trametes sp.*; Ts, *Trichoderma* sp.; Tv, *T. versicolor*.

Data collated from Nevalainen *et al.* 2005; Sharma *et al.* 2009; Fleissner and Dersch 2010; Su *et al.* 2012; Druzhinina and Kubicek 2016 and back references.

of the first stage (i.e. expression) are well-documented but the complexities of the latter two stages are being teased out. While the tools already in place are briskly summarized here, this review emphasizes the recent research related to protein modification, maturation and secretion events (figure 1) in filamentous fungi.

In this review, we examine different aspects of fungal platforms for protein production including – the strain selection and development, morphology and culture conditions, designing and altering promoters, secretory signals and pathways, maturation of the nascent polypeptide in the endoplasmic reticulum (ER), hypersecretion stress and unfolded protein response (UPR), protein processing and modification in the Golgi and the subsequent secretion apparatus. The contribution of omics approaches in providing new insights and novel targets are also covered. Among filamentous fungal protein production factories, major focus has been on strains belonging to *Aspergillus* (Fleissner and Dersch 2010) and to a lesser extent on *Trichoderma* (a prolific cellulase producer; Rantasalo *et al.* 2019) species (table 1). Within the genus *Aspergillus,* however, *Aspergillus niger* has occupied center stage both in terms of basic and applied research (Cairns *et al.* 2018). In fact *A niger* and *Aspergillus oryzae* are the two industrially important enzyme/protein producers; these two alone account for more than 50% of the total industrial enzymes produced from filamentous fungi (AMFEP 2015). Much research on protein expression/secretion involves these two Aspergilli with *Aspergillus nidulans* (has a sexual cycle and is amenable to classical genetics) serving as the laboratory model. Accordingly, more recent research (after 2015) on Aspergilli as hosts for protein production and secretion is highlighted.

## 2. Strategies to improve protein production/secretion:

### 2.1 *Strain selection and development*

*Classic tools:* Most of the early work on developing amylase producing strains of *Aspergillus* species involved random mutagenesis and screening (Weenink *et al.* 2006). While this approach has been reasonably successful, the absence of traditional genetic crossing in *A. niger* has not been possible due to the lack of sexual cycle. It was therefore difficult to map and recombine mutants and eliminate deleterious background mutations. Parasexual recombination is an alternative but is not widely exploited for strain improvement because of the tedium. With high throughput DNA sequencing and decreasing cost of sequencing, it is now possible to simply sequence the genome of the high secreting strain and compare it with the wild type reference genome sequence, to identify all of the useful mutations. Judicious combination of mutagenesis, comparative genomics, and functional genomics should allow a better understanding of the desired phenotype. The genome sequence of three strains of the same species, the wild type citric acid producer *A. niger* ATCC 1015, the industrial enzyme producer *A. niger* CBS 513.88 and the laboratory strain *A. niger* NRRL3, are now available for such analyses (Brandl and Andersen 2017). A comparative metabolomics analysis identified enhanced flux through the pentose phosphate pathway as a general feature of Aspergilli expressing recombinant proteins. A comparative genomics study along with transcriptome analysis of *A. niger* towards metabolic engineering for citrate production was recently reported (Yin *et al.* 2017a). Identifying the critical gene sets for optimal productivity and morphology is feasible from omics data (Cairns *et al.* 2019a). Rational strain engineering could encompass such a global view (see 'Insights through omics analysis' section below) as well as a reductionist approach by knocking out single relevant genes. Protease deficient strains (to prevent protease degradation of heterologous proteins) have been obtained both by mutagenesis and screening and by direct gene knockout strategies (Reilly *et al.* 2016).

Generating recombinant fungal strains is often vital to achieving industrial-level production. Transformation of filamentous fungi was successfully achieved in the 1980s. Autonomously replicating plasmids, that provide a successful alternative to integrative transformation in yeast, is not common and very rarely used in filamentous fungal genetic recombination work. The incoming DNA is integrated into the host genome via random integration. Ectopic or heterologous recombination is the predominant mode of integration in filamentous fungi. Homologous recombination is better accomplished by employing strains deficient in the non-homologous-end-joining (NHEJ) pathway (Ninomiya *et al.* 2004) and/or employing extended flanking sequences. Significant progress has been made in the transformation of Aspergilli in terms of protocols for DNA entry and markers for selection (Lakshmi Prabha and Punekar 2004). The tools of this trade include markers for selection and integration and expression vectors with suitable promoters. A range of both nutritional (including bidirectional ones) and dominant (antibiotic or antimetabolite resistance)

markers are available for selection (Dave *et al.* 2015). Targeted genetic changes such as gene disruption, gene replacement, locus-specific integration and precise genome editing in filamentous fungi are currently possible. For instance, the *pyrG* locus was targeted to improve the integration of the gene of interest in *A. niger* (Arentshorst *et al.* 2015).

A precise gene editing has become feasible through the CRISPR-Cas9 technology. To date, this genome editing system has been established for a variety of filamentous fungi (Schuster and Kahmann 2019). Recently, a gene-targeting method that provides selectable, iterative, and ultimately the marker-free generation of genomic deletions and insertions was reported. This method was validated by targeting the phenotypic marker *albA* and subsequently the *glaA* and *mstC* loci of *A. niger* (Leynaud-Kieffer *et al.* 2019). While excellent (near 100 percent) gene-editing efficiency was observed across these three loci, this may not be the case at all loci as the essentiality and accessibility do influence the genomic modification.

*Media engineering and fermentation:* It is well known that the fungal secretome profile is unique on different growth media (Benoit *et al.* 2015). Presence of complex nitrogen source(s) induces the proteolytic enzymes (Farnell *et al.* 2012) and this could influence the stability of heterologously expressed proteins. Strategies to address protease degradation may include – a) the manipulation of bioprocessing parameters, b) use/ addition of protease inhibitors, or c) the construction of protease-deficient strains (Zoglowek *et al.* 2015). The *A. niger* acidic protease activities are significantly lower in a growth medium set above pH 5.0. The conventional medium and growth optimization approach for protein production is a good first step. Shake flask conditions are less favorable for protein production (when compared to controlled bioreactor cultures) as such growth is associated with lower-level expression of ER-resident chaperones and foldases and higher levels of vacuolar proteases (Lu *et al.* 2010). Submerged cultures in batch (or fed-batch) mode are usually employed for enzyme secretion/fermentation. An alternative and potentially useful strategy is continuous processing such as through chemostats. A further modification of this chemostat cultivation is perfusion or retentostat cultivation. Two promoters that are suitable for *A. niger* continuous cultivation of industrially relevant products were recently described (Wanka *et al.* 2016). The fungal morphology may be affected by the nature of the growth medium and the genetic network. Growth and protein secretion are coupled at the hyphal tip and any changes in the morphology like hyper-branching phenotype could impact secretion in submerged cultures (te Biesebeke *et al.* 2005a; Cairns *et al.* 2019a). Hence, modelling fungal morphologies and growth kinetics have assumed importance in improving protein expression and secretion. Recent developments in the omics studies are rapidly uncovering relevant genes for optimized morphology, secretion and productivity (see below).

It is an empirical fact that the amounts of enzymes secreted by filamentous fungi in solid-state culture are large and frequently exceed the quantities secreted in submerged culture (Iwashita 2002). But molecular biology of solid-state culture mycelia has attracted less attention. Different control mechanisms regulate glucoamylase and protease gene transcription in *A. oryzae* in solid-state and submerged fermentation (te Biesebeke *et al.* 2005b). Solid-state specific gene expression was observed for *glaB* and *pepA* genes of *A. oryzae* and the relevant promoter elements were identified. A solid-state culture-specific release of enzymes from the cell wall was also noted during the production of β-glucosidases in *Aspergillus kawachii*. An extracellular soluble polysaccharide was concerned with the location and stability of this *bglA* gene product. Clearly, the secretion of this enzyme through the cell wall layer was efficient in solid-state culture but was greatly reduced in submerged culture. A number of small secreted proteins (SSPs) are identified and their expression is species-specific within *Aspergillus* species (Valette *et al.* 2017; Vivek-Ananth *et al.* 2018). Hydrophobic surface binding SSPs like hydrophobins and HsbA may be involved in recruiting degradative enzymes to the straw surface; in fact, these SSPs are strongly induced by the switch from glucose to wheat straw. HsbA is a small protein that is able to recruit lytic enzymes to the surface of hydrophobic surfaces and promote their degradation (Ohtaki *et al.* 2006). Solid-state fermentation simulates the natural habitat of filamentous fungi while understanding of genome-wide gene expression and the regulation of gene expression of lignocellulose-degrading enzymes in ascomycete fungi during solid-state culture are limited. More recently, a transcription factor (Atf1) that regulates the expression of cellulase and xylanase genes during solid-state fermentation, was reported (Zhao *et al.* 2019).

### 2.2 *Promoters and protein expression systems*

Typically an expression cassette for the expression and secretion of a protein on demand contains – a strong promoter, a secretion signal peptide followed by the

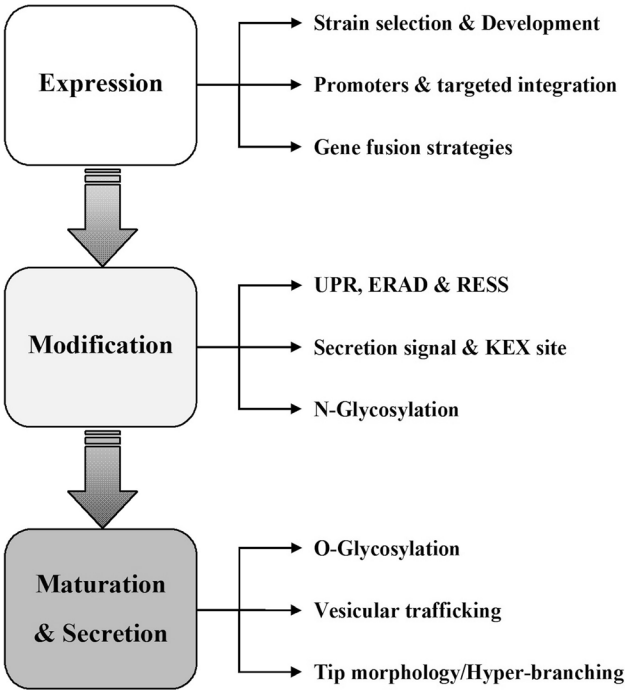


**Figure 1.** Overall flow diagram for fungal protein expression and secretion. The metabolic pathway for protein secretion in filamentous fungi consists of three broad stages – beginning with the gene of interest and culminating in the secretion of the mature protein. Various events and strategies to optimize them are also shown.

open reading frame (or cDNA) coding for the polypeptide of interest. Such a cassette is constructed with a suitable marker for selection and relevant flanking sequences to assist locus-specific homologous recombination. Long flanking sequences are necessary to achieve gene targeting in filamentous fungi; even then a large number of transformants need to be screened to pick the right recombinant. Mutants with a defective NHEJ pathway are very efficient recipient strains for gene-targeting approaches, where homologous recombination frequencies reach up to 100 percent. In order to overcome the disadvantages of permanently NHEJ-defective strains, transiently *kusA* disrupted strain of *A. niger* was developed (Carvalho *et al.* 2010). Integration of the expression construct at a transcriptionally active region is important as this facilitates a higher level of transcription and improves mRNA stability. Protein production also increases (a) by optimizing the codon usage and (b) with gene copy number but displays a leveling effect. The transcription factor availability often limits the extent of gene expression. It is well recognized that a robust promoter along with its cognate transcription factor(s) are minimally required for successful and efficient expression of any protein.

*Fungal promoters:* Fungal promoters are a prerequisite to the expression of both homologous and heterologous proteins. A variety of constitutive and inducible promoters are available for use (Fleissner and Dersch 2010). As a general rule, homologous promoters offer better expression yields than the heterologous ones. The prevalent gene promoters available for protein production in filamentous fungi include those of *adhA*, *alcA*, *alcC*, *aldA*, *amdS*, *amyA*, *amyB*, *aphA*, *exlA*, *gdhA*, *glaA*, *glaA1*, *gpdA*, *oliC*, *pkiA*, *sodM*, *sucA*, *tef1* and *tpiA* (Ward 2012; Fitz *et al.* 2018); couple of useful promoters developed in our group are from *A. niger agaA* and *citA* genes (Dave and Punekar 2011). Among these, *gpdA*, *adhA*, *gdhA* promoters are well-studied and highly used constitutive fungal promoters. Inducible promoters such as *glaA, cbhI, alcA* have also found application in driving the heterologous protein expression in fungi (Kluge *et al.* 2018). While such native inducible promoters are under the control of carbon and nitrogen regulation, promoters that are inducible by specific inducer molecules are also reported (Fitz *et al.* 2018). For instance, pantothenate inducible promoter fused to *Trichoderma reesei* β-glucosidase showed a clear induction of expression at low amounts (0.1 and 1.0 mM) of pantothenic acid. Also, the promoter of *bphA* (benzoate para-hydroxylase) from *A. niger* can respond to the presence of benzoic acid (Antunes *et al.* 2016). In order to further fine-tune protein expression, metabolism-independent promoters were designed and developed. Examples of such tunable promoters include a thiamine promoter system in *A. oryzae* (Shoji *et al.* 2005), the human estrogen receptor system in *A. nidulans* and *A. niger*, and a system based on *E. coli* tetracycline resistance operon gene expression in different *Aspergillus* species (Kluge *et al.* 2018). The Tet-On/Tet-Off system is best suited for the expression of heterologous proteins and to study conditional expression and titration of protein products in filamentous fungi (Meyer *et al.* 2011). Novel promoters uniquely adapted to specific growth and/or medium conditions have also been established for *A. niger*. These include the *PcitA* (Dave and Punekar 2011) the promoter of a Krebs cycle gene and *Pgas* (Yin *et al.* 2017b) an inducible promoter that functions efficiently during growth in acidic pH. Two highly active promoters and native secretion signals were derived from *anafp* (antifungal protein) and *hfbD* (putative hydrophobin) genes, for protein production during extremely low growth rates of *A. niger* (Wanka *et al.* 2016).

*Cis-regulatory elements:* While they have high utility value, filamentous fungal promoters are less

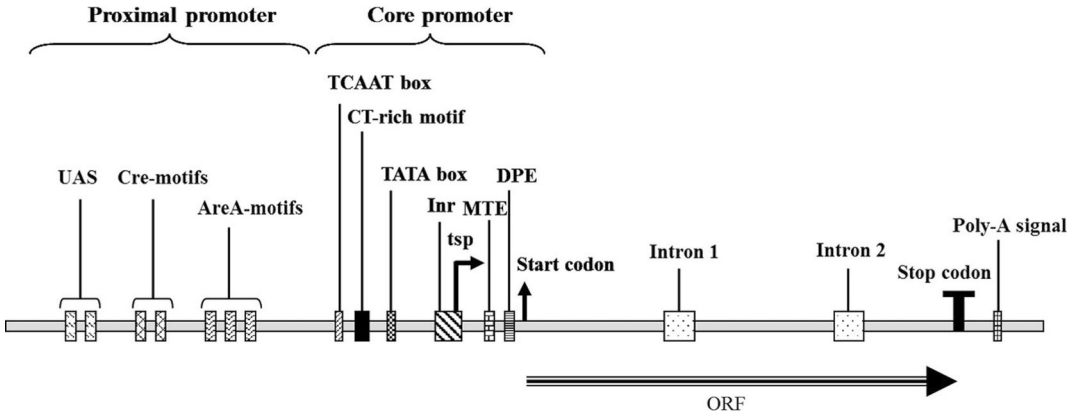


**Figure 2.** Structural features of a representative fungal gene. The typical filamentous fungal gene with two introns and various potential *cis* regulatory elements is depicted. Of the various core promoter elements only TATA box, CCAAT motifs, CT rich regions are functionally well characterized in filamentous fungi. The proximal promoter elements for AreA and CreA binding are shown. Other pathway specific elements like upstream activator sequence (UAS) may be present in some genes.

characterized and poorly defined. In comparison, yeast and mammalian promoters are better defined in terms of their composition of *cis*-regulatory elements (Gasch *et al.* 2004). Similar promoter elements from filamentous fungi are postulated but their functional characterization is often lacking. A promoter typically consists of 'core promoter' and 'proximal promoter'. Core promoter comprises sequences (70–80 bp) immediately surrounding the transcription start point (tsp). It is recognized by the basal transcriptional machinery to initiate transcription. Upstream of the core promoter (~ 250 bp or more) represents the proximal promoter region and contains sequences important for transcriptional regulation (binding sites for transcriptional factors and enhancers or suppressors). These sequences are collectively called promoter elements (figure 2). The role of downstream regulatory elements, if any, is very little understood.

Among core promoter elements only TATA box, CCAAT motifs, CT rich regions have been functionally characterized in filamentous fungi. Other elements (like Inr, DPE, MTE, BRE and CpG islands) found mainly in higher eukaryotes are less known. Contrary to the common misconception, the majority of core fungal promoters do not contain a TATA box. While CCAAT motifs are not always important for function, the introduction of multiple copies of this motif in the *glaA* promoter improved gene expression in *A. niger* (Liu *et al.* 2003). The promoter function in *Aspergillus* was improved by incorporating multiple copies of a *cis*-acting element (Minetoki *et al.* 2003). However, potential titration of a common *trans*-acting element was also noted in this high expression. Upstream of the tsp, most fungal gene (for example, *gpdA*, *oliC*, *trpC*, *citA* and *agaA*) promoters contain a pyrimidine (CT) rich stretch. These CT-rich sequences were particularly noticeable in genes lacking the TATAAA and CCAAT motifs and in highly expressed (particularly housekeeping) genes, thus suggesting their role as possible promoter elements.

Proximal promoter elements, found proximal to core promoter region, enhance, suppress or modulate the adjacent gene expression according to the environmental status of an organism. These can be global or pathway-specific; and in response to stimulus transcriptional factors bind to these elements and bring about the required changes in gene expression. The carbon and nitrogen regulation/repression in filamentous fungi provide excellent examples of well-studied proximal promoter elements. Carbon catabolite repression, well known in yeast, is also extensively studied in Aspergilli. A wide domain carbon repression gene *creA* was identified and it encodes a DNA binding protein having two zinc finger domains. Putative CreA binding sites are reported but the signal transduction mechanism (including *creA*) is expected to be different and complex in filamentous fungi. In Aspergilli, *areA* encodes a positively acting global regulatory protein (AreA) that turns on numerous nitrogen metabolic genes. AreA is a GATA-like transcriptional factor that binds to consensus sequence HGATAR in the proximal promoter region of nitrogen-regulated genes. Apart from elements that respond to carbon catabolite repression and nitrogen metabolite repression, the key pH-responsive transcriptional factor PacC (encoded by *pacC* gene) binds to conserved sequence 5'-GCCARG-3' in the target gene.

The literature on the characterization of filamentous fungal promoter elements is non-exhaustive. Nevertheless, the promoter toolbox for recombinant gene expression is continuously being upgraded (Fitz *et al.* 2018). A transcription activator element of *glaA* promoter was identified and a multiple copy insertion of this element led to improved heterologous gene expression (Liu *et al.* 2003; Minetoki *et al.* 2003). Similarly, a 'gpd box' in the *A. niger gpdA* promoter was characterized. Multiple 'gpd box' insertions (up to 3 copies) within the *gpdA* promoter also resulted in improved protein expression. A decrease in the expression was observed when further copies were incorporated (Zhang *et al.* 2018). A decrease in gene transcription/expression is known to occur beyond certain copies of regulatory elements due to the titration effect of transcription factors (see below; Brewster *et al.* 2014). In another approach, the repressor sites in the promoter were replaced by transcription activator elements to achieve increased gene expression (Zou *et al.* 2012). Synthetic expression systems by rational design are also being developed. Here the synthetic trans-activators comprise a DNA-binding domain, a heterologous regulatory domain and an activation domain. We have already noted the example of Tet-On/Tet-Off system above. Recently, a synthetic expression system that is functional in a broad spectrum of fungal species without the need for host-dependent optimization was described. It consists of two expression cassettes, the first providing a weak, but the constitutive level of a synthetic transcription factor (sTF), and the second enabling strong, at will tunable expression of the target gene via the sTF-dependent promoter (Rantasalo *et al.* 2018). In their design, the TATA box containing core promoters of genes that were highly transcribed under various growth conditions were chosen.

*Trans-regulatory elements:* The regulation of gene expression may be achieved by appropriate transcription factors. The Cys2His2 type transcription factor CreA from Aspergilli (or its homolog in other fungi) is the main transcriptional repressor contributing to carbon catabolite repression. Elimination of carbon repression due to defective CreA and/or CreB (a ubiquitin-specific protease involved in this repression), or their orthologs are known to result in an increased expression of carbohydrate active enzymes in filamentous fungi. In some cases, however, the expression might require the presence of active transcriptional activators along with their cognate inducing metabolites. Deletion of *creA* alone may not suffice to increase the secreted enzyme production. In Aspergilli, *areA* encodes a positively acting global regulatory protein AreA, which unlike CreA, turns on numerous nitrogen metabolic genes in the absence of preferred nitrogen sources (ammonium and glutamine). NmrA, a negative acting transcriptional factor, presents an additional mechanism to regulate the expression of AreA (Andrianopoulos *et al.* 1998).

The diversity in the control of transcription factor activities could be exploited to modulate transcriptional regulation in industrial fungal cultures (Alazi and Ram 2018). The titration effect of transcription factors due to multiple copies of promoters or *cis*-regulatory elements could be overcome by overexpression of respective transcription factors. However, the regulatory network details decide the final outcome. Second, one could select/generate a constitutively active transcription factor. Such transcription factor variants may be identified via classical mutagenesis and screening approaches or through rational recombinant DNA tools. For instance, XlnRV756F (a variant of XlnR) was identified in *A. niger* by employing a forward genetic screen. Deletion and/or down-regulation of select repressors may similarly be designed. Lastly, elevating the intracellular inducer levels is another effective approach; cellobiose can be made to accumulate (and induce fungal cellulases!) by deleting/ eliminating the β-glucosidase activity.

### 2.3 *Gene fusion strategies*

*Use of pre- and pro-peptides:* Heterologous and homologous protein expression is best achieved in filamentous fungi by employing a strong promoter before the codon-optimized open reading frame. Secretion of the expressed protein is a different matter, however. The classical secretion pathway recognizes a secretion signal ahead of the mature polypeptide (see below). In principle, a host can be made to secrete any protein (homologous or heterologous) as long as the protein has a secretion signal at its N-terminus that is recognized by the host secretion pathway. Homologous signals are best recognized by the host while heterologous signal peptides may be poorly processed; this can result in poor expression and yield. Heterologous fungal secretory sequences are generally functional across other filamentous fungi; secretory signals from nonfungal sources do not function in a fungal expression platform. Accordingly, there have been serious attempts to create in-frame fusion constructs with sequences of (a) the secretion signal, (b) a pro-peptide or (c) highly expressed host protein as a carrier. The

classic proof of concept study of this kind was the controlled expression and secretion of bovine chymosin in *A. nidulans* (Cullen *et al.* 1987). Wherein, the transcriptional, translational, and secretory control regions of the *A. niger* glucoamylase gene were functionally coupled to either prochymosin or preprochymosin cDNA. Subsequently, many attempts are made to exploit such a strategy. For example, a construct was designed to secrete human α-galactosidase A under the *cbhI* promoter plus CbhI secretion signal and CbhI carrier fusion (Smith *et al.* 2014); the fusion protein was proteolytically cleaved at a Kex2 site during secretion by *T. reesei*. Whereas the intracellular expression was effective, secretion stress (including UPR and ER degradation; see below) remained a bottleneck. Yin *et al.*, (2015) heterologously expressed an α-amylase gene from *Penicillium* sp. in *A. oryzae* with the strong host *amyB* promoter as an *amyB* fusion separated by a Kex2 site. The hIFNb was expressed in *Aspergillus unguis* with the help of *A. nidulans Pgpd* and the glucoamylase signal peptide from *Aspergillus awamori* (Madhavan *et al.* 2017).

*Linkers and Kex sites:* Fusing heterologous protein to host carrier protein enhances its expression, secretion and stability, and prevents its degradation. Glucoamylase in *A. niger* and cellobiohydrolase in *T. reesei* is highly expressed proteins and constitute around 60% of the total secreted proteins (Lu *et al.* 2010; Rantasalo *et al.* 2019). They are best exploited as carrier proteins for heterologous protein expression in the two respective fungal hosts. In general, expression of heterologous proteins is optimally achieved by fusing the sequence with the strong promoter and native secretion signal of the host. *Malbranchea cinnamomea* xylanase, *Penicillium citrinum* and *A. oryzae* β-glucosidase sequences when fused to *A. niger citA* promoter could be readily expressed and secreted by *A. niger* (unpublished data). All three cases are examples of heterologous secretory sequences that functioned well in *A. niger*. However, the same *M. cinnamomea* xylanase secretion signal when fused to EGFP sequence, either directly or via a linker, did not function. While EGFP folding in the ER lumen may be an issue, it appears that even subtle differences between secretory peptides can affect protein secretion. A direct fusion of functional domains without a linker may be undesirable because of the misfolding of the fusion proteins and/or low yield in protein production. The choice and use of a linker for fusion is an important but underexplored area of research (Chen *et al.* 2013). Linkers usually contain small and hydrophilic amino acids like alanine, glycine and serine (Elleuche 2015). One can also add a cleavable site so that after fusion protein is targeted to the secretory pathway, homologous protein-linker can be cleaved leaving heterologous protein for secretion. The Kex2/KexB serine protease can be used to cleave fusion constructs by deliberately introducing Kex2/KexB sites. This Golgi resident serine protease cleaves at Lys/Arg-Arg site, so introducing such a cleavage site or modifying cleavage site is one of the strategies researchers have employed to enhance secretion. There is scope to improve the efficiency of such cutting. For example, modification of the sequence before the Kex2 site increases the cutting efficiency in *A. niger* (Wang and Ward 2015) and *A. oryzae* (Koseki *et al.* 2017) leading to higher secretion rates.

### 2.4 *Secretory signals and pathways*

Limitation in heterologous protein secretion is largely due to the complexity of the filamentous fungal system. Filamentous fungi have a well-developed pathway for the secretion of proteins. It starts with the transport of mRNA to the cytoplasm and endoplasmic reticulum (ER) where ribosomes translate the message into a polypeptide. The polypeptides so formed will undergo some posttranslational modifications like glycosylation. This is followed by transport of these polypeptides to Golgi bodies, where further modification and folding will take place. These proteins are transported to the membrane and then secreted out. What is known so far about the protein secretory pathways in filamentous fungi is summarized in figure 3.

*The classical pathway:* The mRNA is translated into polypeptide and this polypeptide is targeted to ER through either the SRP (signal recognition particle – a protein-RNA complex)-dependent or the SRP-independent mechanisms. In the SRP-dependent mechanism signal sequence in the polypeptide is detected by SRPs, targeted to the cytosolic face of ER and then attached to the peptide translocation complex in ER. Then the SRP dissociates and further elongation of the peptide is carried out by the ribosome along with the translocation of growing peptide inside the ER lumen till full protein is synthesized. The signal sequence is removed in the ER lumen by a signal peptidase. In the SRP-independent mechanism, the growing polypeptide chain interacts with cytosolic HSP70 chaperone and co-chaperones and is transported to the ER membrane where it binds with SEC complex which acts as membrane receptor. This will translocate the growing polypeptide into the ER lumen for further processing.

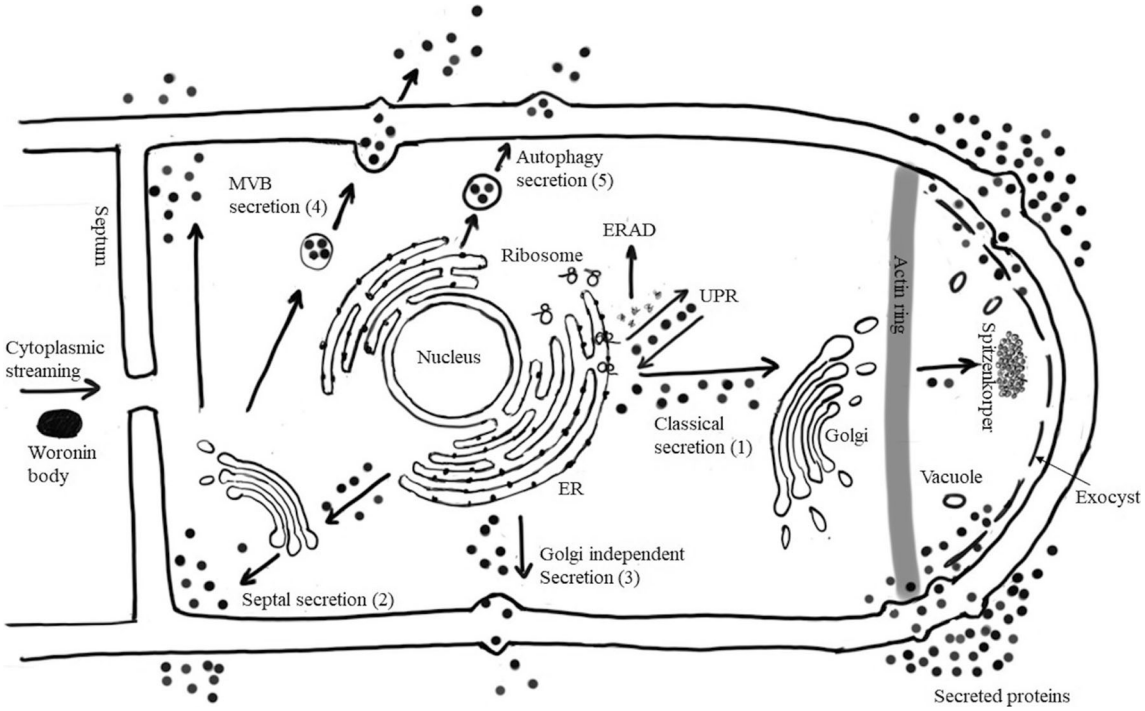


**Figure 3.** Secretory pathways in filamentous fungi. A representation of the protein secretion pathway, as well as its components in the mycelial tip region of a filamentous fungus is shown. While the picture is not exhaustive, the following modes of secretion are exemplified: (1) Conventional classical secretion through the ER (Conesa *et al.* 2001), (2) septal secretion (Hayakawa *et al.* 2011; Fiedler *et al.* 2018a), (3) Golgi-independent secretion (Shoji *et al.* 2014), (4) multi-vesicular bodies (MVB) secretion (Shoji *et al.* 2014) and (5) autophagy secretion (Miura and Ueda 2018).

The well-studied SRP-dependent mechanism exploits the presence of a secretory signal in the polypeptide to be secreted. The secretory signal is an N-terminal amino acid sequence in a polypeptide; it is recognized by SRPs and targets the polypeptide to the secretory pathway. Experimentally validated signal peptides on average are of 15–36 amino acids long sequences at the beginning of the protein destined for secretion. Signal peptides comprise the N-terminal region with positively charged residues, a central hydrophobic core region and the C-terminal region with polar residues. Highly secreted proteins like *T. reesei* cellobiohydrolase and *A. niger* glucoamylase are endowed with efficient signal peptides. Such secretion signals can be fused to heterologous protein for better secretion. The signal peptide is cleaved by a resident ER signal peptidase to release the mature protein. Even subtle differences between secretory signals can affect protein secretion. The first successful use of a synthetic signal was demonstrated in *A. nidulans* to secrete human interferon α2 and bacterial endoglucanase (Gwynne *et al.* 1987). The *A. niger* GlaA signal peptide is much more effective in directing α-galactosidase production than its own signal sequence (Xu *et al.* 2018). Non-native secretory signal peptides may cause changes to the secondary structure of the protein and lead to secretion impairment. In sum, the efficiency of secretion likely depends on the accurate match between the secretory signal, recombinant protein and the SRP system of the heterologous host (Zoglowek *et al.* 2015). Using two different experimental approaches, around 200 proteins with a predicted signal peptide were identified to be secreted proteins in *A. niger* (Tsang *et al.* 2009; Braaksma *et al.* 2010). It was noted that the prediction of a signal peptide does not guarantee that the protein is actually secreted and two proteins were found to be secreted via a non-classical route (see below).

*Unconventional protein secretion:* Growth and conventional protein secretion are coupled at the hyphal tip. The journey of the protein begins at the ER and further gets processed in the Golgi. The secretory proteins packaged into vesicles bud from Golgi travel along microtubules and actin filaments to the extending hyphal apex and aggregate in the Spitzenkörper. There they become tethered to the plasma membrane by the exocyst and release the vesicle cargo (figure 3). However, many examples of extracellular proteins are being reported that do not have secretory signals nor follow the classical pathway (Shoji *et al.* 2014; Rabouille

2017; Miura and Ueda 2018). Unconventional protein secretion (UPS) comprises cargos that reach the plasma membrane by bypassing the Golgi despite entering the ER. The UPS is mostly triggered by ER stress and may involve GRASP (Golgi reassembly stacking protein). In some cases, they are transported to the cell membrane via alternative routes bypassing both ER and the Golgi. The examples of fungal proteins that go through UPS are the chitin synthases of *Neurospora crassa*, a cellobiase from *Termitomyces clypeatus* and PepN from *A. niger*. But the secretion of PepN is independent of the autophagy machinery in *A. niger* (Burggraaf *et al.* 2016). Recent research work suggests that, when normal secretion and tip growth is impacted, fungal protein secretion may also occur at/ through the septal junctions (Hayakawa *et al.* 2011; Fiedler *et al.* 2018a) and this could be of industrial relevance. Both UPS and septal secretion pathways represent future avenues for strain engineering efforts (Cairns *et al.* 2019a). Unconventional secretion of chitinase Cts1 from *Ustilago maydis* occurs via the fragmentation zone and circumvents the endomembrane system for its secretion. Clearly, such a system could provide the unique possibility of producing heterologous proteins secreted without N-glycosylation (Reindl *et al.* 2019).

Intracellular proteins can also get secreted via vesicle and organelle engineering approaches. In 'peroxicretion' proteins can be forcibly targeted to peroxisomes; in turn, peroxisomes themselves are tailored (through v-SNARE) to be transported to the plasma membrane for fusion (Sagt *et al.* 2009; Fleissner and Dersch 2010). GFP was released into the extracellular space through this artificial secretion pathway.

### 2.5 *Role of ER in secretion and ER stress*

Most of the secretory protein production in eukaryotic cells gets processed through the ER. This is the folding factory where the quality control of proteins entering the conventional secretory pathway is coordinated (Almanza *et al.* 2019; Benham 2019). The folding of the nascent polypeptide is assisted by a number of specific ER-resident proteins like foldases, chaperones and protein disulfide isomerases assisted by the thioredoxin system. Correctly folded proteins destined for secretion pass through a specific subdomain of the ER known as transitional ER sites (or ER exit sites). The aberrant proteins in *A. oryzae* accumulate toward basal hyphae while the secretion of properly folded proteins continues through ER exit sites towards the hyphal tip (Kimura *et al.* 2010). As far as defining all the ER components of the secretion pathway we are still in the 'mapping phase' and the omics approaches with different fungi are providing some insights (see below).

*Over-expression of ER components:* Efforts to increase the production of heterologous proteins by individual overexpression of resident ER proteins (like chaperones, foldases, lectins, lectin-chaperones, *nucleotide* exchange factors, co-chaperones, and the ER-specific protein degrading machinery) have met with mixed results (Geysens *et al.* 2009; Ward 2012; Heimel 2015; Sun and Su 2019). Three secretion pathway-related genes were overexpressed to enhance the secretion of *A. niger* glucose oxidase in *T. reesei*. Overexpression of *snc1* (SNC1 is a v-SNARE protein acting in the last step of protein secretion – vesicle fusion from Spitzenkörper to the plasma membrane), *hec1* and *bip1* genes was found beneficial (Wu *et al.* 2017). Also, overexpression of one component gene affected the expression of the other two, suggesting a complex regulation of the secretion pathway – co-overexpression of multiple components may not give an additive effect! Clearly, specific features such as glycosylation sites, hydrophobic patches, or disulfide bonds in the protein to be secreted require different ER-resident proteins to be upregulated. The sophisticated protein quality check within ER ensures that only correctly folded proteins are ultimately escorted to the cell exterior. When this system is overwhelmed by cellular demand and/or stressed due to unfolded/misfolded proteins, specific ER-stress signaling pathways, collectively known as the unfolded protein response (UPR), are upregulated.

*Secretion stress and UPR:* Swamping of the cellular protein secretory pathway leads to ER stress. This is countered in two ways namely a) by inducing UPR and b) by ER-associated protein degradation (ERAD). Genomic analysis of the secretion stress response in *A. niger* identified genes mainly belonging to UPR such as *pdiA*, *prpA*, *bipA*, *clxA*, and *lhsA* (Guillemette *et al.* 2007). Some of these gene products adopt a thioredoxin fold which each contains one active CGHC catalytic motif – the thioredoxin reductase pathway is required to ensure correct disulfide formation and for efficient trafficking of secreted proteins (Poet *et al.* 2017). In a genomic study, these and additional genes involved in UPR were found to be upregulated (Geysens *et al.* 2009). The UPR induction is under the control of *hacA* gene product – a transcription factor. This *trans* regulator recognizes and binds to UPR elements in the promoters of target genes. The *A. niger* UPR pathway was constitutively induced by

expressing the activated form of this transcription factor. While the native protein secretion was unaffected by *hacA* expression, heterologous protein production (of *Trametes versicolor* laccase and bovine preprochymosin) was significantly improved in this fungus (Valkonen *et al.* 2003). In another study with *A. oryzae*, the secreted protein MsdS (1,2-α-D-mannosidase) and its mutant (lower thermostability) form were expressed at different levels (Yokota *et al.* 2017). At moderate expression levels the wild-type MsdS was secreted but the mutant was not. Also, the mutant MsdS accumulated in the mycelia when the *hrdA* gene (involved in the ERAD) was deleted. The ER quality check is such that misfolded (MsdS) and highly expressed endogenous secretory proteins (like α-amylase) act synergistically in UPR induction.

UPR is closely linked to the ERAD pathway. In ER-associated protein degradation, the misfolded proteins in the ER lumen are returned through a translocon back to the cytoplasm, ubiquitinated and degraded by the proteasome (figure 3). Increased intracellular levels of GlaGus (glucoamylase-glucuronidase fusion protein) were observed in *A. niger* when ERAD pathway genes (*hrdC* and *derA*) were deleted (Carvalho *et al.* 2011). These findings suggest that a delay in protein degradation may have occurred. Through double deletion mutations in ERAD (*derA*) and autophagy (*atg1* or *atg8*), it was shown that autophagy is not an alternative to ERAD in eliminating misfolded proteins from the ER in *A. niger* (Burggraaf and Ram 2016). There is also evidence for a unique feedback regulation called repression under secretion stress (RESS) (Guillemette *et al.* 2007; Sun and Su 2019). Excess secretory pressure on the ER leads to inhibition of transcription of secreted proteins thereby reducing stress on the secretory pathway.

### 2.6 *The Golgi and glycosylation pathways*

For most secreted proteins, post-translational modification by glycosylation is an important part of protein maturation. Glycosylation is important in protein folding, localization and stability; it also serves as information for ER quality check and protein degradation. Protein glycosylation comes in two varieties – the N- and O-glycosylation. The first glycosylation of fungal proteins occurs in the lumen of the ER (N-glycosylation on β-amide nitrogen of Asn) and this is followed up by further processing of N-glycans in the Golgi (O-glycosylation on hydroxyl groups of Ser or Thr) (Geysens *et al.* 2009). Intensive O- and N-glycosylation and different glycoforms are shown for CbhI and other *Trichoderma* secretory proteins. Under-glycosylated proteins undergo the ER quality check; the non-native proteins are directed to the retrograde transport and then degraded by ERAD pathway. Incorrect glycosylation of fusion proteins interferes with proper protein folding. Defects in protein O-glycosylation may result in the up-regulation of the stress response and RESS along with interactions between O- and N-glycosylation (Kruszewska *et al.* 2008). The structure of the glycans decorated on the antibodies heterologously expressed in *A. niger* and *Pichia pastoris* is of the high-mannose type. In some aspects, filamentous fungi are superior to yeast in protein glycosylation (Nagaraj *et al.* 2009). Glycoengineering and the expression of therapeutic glycoproteins in humanized fungi have shown some promise (Gerngross 2004). At least with Cbh expression, the engineering (addition or deletion) of glycosylation sites on the protein appears to be a better strategy than optimizing the glycosylation system of the heterologous host (Zoglowek *et al.* 2015).

Elegant genetic dissection of the secretory route for an inducible, highly glycosylated fungal inulinase (InuA) in *A. nidulans* was recently reported (Hernández-González *et al.* 2018). Various mutations affecting different intracellular membranous compartments were used to investigate the route by which this protein reaches the extracellular medium. The core-N-glycosylation of InuA occurs in the ER and it is hyperglycosylated in the Golgi. Also, it was observed that early endosomes are specifically required for InuA exocytosis. Another study indicates that *A. niger* responds to forced transcription of secretory enzymes by increasing post-Golgi carriers to accommodate the cargo overload (Fiedler *et al.* 2018a).

## 3. Insights through Omics analysis

We are in the process of describing the plethora of players involved in the filamentous fungal secretion pathway. The global omics approaches with different fungi are uncovering these details both at the transcriptome and proteome level (Damasio *et al.* 2019). The *Aspergillus* secretome (Tsang *et al.* 2009; Braaksma *et al.* 2010; Lu *et al.* 2010), secretory pathway (Punt *et al.* 2001; Geysens *et al.* 2009; Schalén *et al.* 2016) and ER stress response (Guillemette *et al.* 2007) genes were targeted for study. FlbA (sporulation protein) was recently predicted to affect the expression of 36 putative transcription factor genes. Of these, *rpnR*

is involved in proteotoxic stress resistance and impacts protein secretion when *A. niger* is grown on xylose (Aerts *et al.* 2019). A quantitative proteome profiling of *N. crassa* revealed components of the protein processing and export pathways (Liu *et al.* 2020). As an example, CWH43, a putative ceramide conjugation protein that localizes in the ER, modulates the production of cellulases.

*Discovering new targets involved in protein production:* A coordinated regulation of the *A. niger* secretory pathway was deciphered when the fungus was grown in carbon-limited chemostat cultures. Increased protein secretion by *A. niger* on maltose was associated with transcriptional upregulation of more than 90 genes encoding elements of the secretory pathway; these gene products are either ER-resident proteins or are involved in vesicle trafficking between ER and Golgi (Jørgensen *et al.* 2009). The transcriptional response resembles that of UPR due to ER stress and involves elements of protein translocation to ER, folding, N-glycosylation as well as protein quality control. Clearly, the secretory pathway adjusts to the secretion demand of the endogenous glycoproteins (like extracellular enzymes) in response to carbon source availability. One such upregulated gene is *arfA* – encoding a small GTPase (Fiedler *et al.* 2018b). A loss of function/ gain of function analysis (using Tet-On system) showed that of *arfA* is an essential gene and regulates GlaA secretion, tip morphology and actin ring position in *A. niger*. A genome-wide co-expression network of *A. niger* genes uncovered connections between extracellular protein and citric acid titers. Of the three Golgi associated ArfA regulatory proteins studied, expression of AgeB and GeaB is linked to growth and protein production (Cairns *et al.* 2019b). A family of secretion-related small GTPase-encoding genes from *A. niger* was identified (Punt *et al.* 2001). While *srgA* (gene coding a small GTPase) is not essential for growth, SrgA mutants displayed increased hyphal diameter, unusual apical branching and decreased protein secretion when grown on glucose. The protein secretory pathway may have both glucose regulation dependent and independent components. Deletion of *racA* (gene coding for the Rho-GTPase) results in *A. niger* biomass with about 20 percent more hyphal tips (hyper-branching phenotype) (Fiedler *et al.* 2018a). Concomitantly, a 4-fold increase in GlaA secretion was noted. Of the 14 upregulated genes, known to play a role in protein secretion in *A. nidulans*, *rabD* (encoding a Rab GTPase) over-expression resulted in a 40 percent increase in secretion (Schalén *et al.* 2016). While some manipulations of the ER-resident proteins improved the secretion potential of fungal cell factories our understanding of the secretory pathway is far from complete. However, modifying fungal micromorphology and hyper-branching is a promising avenue to improve protein secretion in many filamentous fungi.

The genus *Aspergillus*, because of its enormous relevance to Biotechnology, is of critical importance. It has received much attention as many genomes have been sequenced for comparison of genotypes and phenotypes (de Vries *et al.* 2017; Vesth *et al.* 2018; He *et al.* 2019). For instance, many ORFs with predicted functions in redox pathways, protein transport, protein modification and protein secretion were uniquely present in an *A. niger* strain with high protein secretion capacity. One such gene was *tupA* – with a role in higher protein (GlaA) secretion and poor conidiation (Sui *et al.* 2020).

*Genetic and genomic screens for protein production:* Constitutively active transcription factor variants may be generated and selected/ identified via classical mutagenesis and screening approaches or through rational recombinant DNA tools. Improved protein expression may be sought through mutations in *creA* locus (or in the corresponding *cis*-regulatory element(s)) by generating strains with the relief of carbon catabolite repression. Similarly, *areA* mutants with favorable features of nitrogen metabolite repression could be screened/selected for. More recently, with the advent of affordable and fast sequencing of fungal genomes, robust forward genetic screens are feasible (Baker 2018). At present only a few examples of this approach are known. For instance, $XlnR^{V756F}$ (a variant of XlnR) was identified in *A. niger* through such a screen. Deletion and/or down-regulation of select repressors may similarly be designed. Several point mutations in AraR (transcriptional regulator of arabinan degradation), obtained with forward genetic screen, resulted in the constitutive expression of its target genes (*abfA*, *abfB*, and *abfC*) (Alazi and Ram 2018). GaaX as a repressor specific to the regulation of pectin catabolism was also picked up through forward genetic screening (Niu *et al.* 2017). Lastly, a forward genetics screen coupled with whole-genome resequencing led to the identification of MstC (a low-affinity glucose transporter) as a novel target. Deletion of *mstC* significantly improved the secretion of recombinant proteins driven by a glucoamylase promoter in *A. niger* (Reilly *et al.* 2018). So far, MstC is the only example identified by a forward genetic screen that is not a *trans*-acting regulatory element. However, the role of *mstC* in the secretion pathway remains to be

**Table 2.** Patents related to improving protein production in filamentous fungi

| Strategy exploited for improvement | Patent no. |
|---|---|
| **Strain selection and development** | |
| Recombinant dimorphic fungal cell | US20030134353 |
| Methods for producing polypeptides in Aspergillus mutant cells | US20050153397 |
| Recombinant vector capable of increasing secretion of koji mold protease | US20090130711 |
| Evolution of whole cells and organisms by recursive sequence recombination | US20120252681 |
| Method for improved protein production in filamentous fungi | US2015307862 |
| Host cell capable of producing enzymes useful for degradation of lignocellulosic material | US20150329843 |
| A kind of *Aspergillus niger* strain and its application available for protein production | CN107384813A |
| Filamentous fungi with improved protein production | WO2018093752A1 |
| **Fungal promoters and trans elements** | |
| Method for producing a recombinant protein | US20030040047 |
| Method for production of secreted proteins in fungi | US20040115790 |
| Transcription factors for cellulosic enzyme production | US20140220641 |
| Mutant cells for protein secretion and lignocellulose degradation | US20150247152 |
| **Fusion proteins** | |
| Production and secretion of proteins of bacterial origin in filamentous fungi | WO/1997/027306 |
| DNA sequences, vectors, and fusion polypeptides to increase secretion of desired polypeptides from filamentous fungi | US20030224489 |
| Expression of glycosyltransferase in Aspergillus | US20050164351 |
| KEX2 Cleavage regions of recombinant fusion proteins | US20140024067 |
| Methods of increasing secretion of polypeptides having biological activity | US20150044756 |
| **Secretory signals and pathways** | |
| Signal sequence trapping | US20080058214 |
| Use of an aspartic protease (NS24) signal sequence for heterologous protein expression | US20110027830 |
| Unconventional protein secretion | US20140227727 |
| Filamentous fungi mutants and use thereof | JP 2018019622 |
| Fungal chaperone proteins | WO/2019/185535 |
| **Role of ER in secretion** | |
| Production of secreted proteins by filamentous fungi | US20130189733 |
| Over-expression of foldases and chaperones improves protein production | US20140087443 |

established. Recently, Jps1 was identified as a novel component of unconventional secretion to enable the export of chitinase in *Ustilago maydis* (Reindl *et al.* 2020). Forward genetic screens and whole-genome sequencing are expected to become regular tools to discover novel targets in protein expression/ secretion by filamentous fungi.

## 4. Bottlenecks in protein secretion by fungal cell factories

Fungal protein secretion is a complex and highly regulated process. Of the different stages in the secretion of a protein (figure 1), gene expression is the best understood. Attempts have been made to express a range of proteins (both homologous and heterologous; table 1) with some success. In general, homologous proteins are better expressed/secreted by a filamentous fungus (Nevalainen and Peterson 2014). Aspergilli (and *A. niger* in particular) hold much promise to be the protein expression workhorses of the future. Their ability for intracellular expression is well demonstrated but the consistent secretion of the expressed protein remains a challenge. Many factors contribute to efficient protein secretion while individual aspects of some of them have received patent coverage (table 2). Despite all that accumulated knowledge, it is not yet possible to achieve comparable levels of expression and secretion of two different proteins (more so with the heterologous ones!) in the same host. For instance, when AbfA and Cbhl enzymes from *A. fumigatus* were heterologously expressed in *A. nidulans*, the extracellular proteome profiles were different (Zubieta *et al.* 2018). Apparently, the context of the protein sequence (such as the number of cysteines and the number of N-glycosylation sites) impacts the secretion level of that protein.

It is important to achieve integration of heterologous gene(s) at a transcriptionally active region of the host genome; this facilitates higher transcription and improves mRNA stability. While the conventional

secretory pathway through ER and Golgi is reasonably defined, the other channels of protein secretion (UPS such as septal secretion) are just beginning to be explored. They may be valuable in the secretion of proteins that have to bypass the classical pathway and glycosylation events. A better understanding of the ER lumen environment is the key to the proper folding of proteins, especially the heterologous ones. As of now, the fusion protein route seems to be the best option for heterologous protein production in filamentous fungi. Significantly secreted proteins like GlaA of *Aspergillus* and CbhI of *Trichoderma* serve as the best templates to fuse with heterologous target proteins. Proper processing of foreign proteins through ER lumen, Golgi, endosomes through to the plasma membrane of the host remains a challenge. Many players of the pathway leading to UPR, ERAD, RESS and vesicular trafficking are being uncovered. Our understanding of how these components are integrated into the sophisticated quality control system of protein secretion continues to evolve. A key challenge in producing heterologous proteins in filamentous fungi is their proper glycosylation. Both ER and Golgi are involved in this complex processing. It appears that manipulating the glycosylation sites on the protein to be expressed is an easier option than engineering the host glycosylation machinery. Fungal protein secretion is also closely linked to mycelial morphology, hyper-branching and tip density. A detailed study of hyphal compartments may be useful in designing better protein secretion strategies (Tegelaar and Wösten 2017).

## 5. Envoi

Protein synthesis and secretion extract major costs from the filamentous fungal host. An upper bound on secretion capacity means that secretion of major host proteins may have to be attenuated/compromised. Expression of most homologous and many heterologous proteins in fungi is now possible and this chassis works well (tables 1 and 2). However, bespoken protein secretion continues to be a challenge and fungal expression platforms for secretion are still 'work in progress'. Considering the complexities, it may be best to treat the entire fungal protein secretion process as a metabolic pathway for optimization. Pending a full definition of the secretory pathway itself, tinkering with individual genes and discrete changes in the genome may serve. This will be somewhat of a trial and error (albeit with many interesting examples cataloged in this review) than the desirable rational approach. Metabolic engineering of fungal primary metabolism to produce industrial value metabolites has already arrived (Yin *et al.* 2017a; Chroumpi *et al.* 2020). But engineering protein secretion yet awaits a detailed understanding of the players and pathways. Rapid progress is being made in this direction and taming the filamentous fungal protein secretion apparatus is on the horizon.

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