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MINI-REVIEW

Heterologous protein production in filamentous fungi

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Abstract

Filamentous fungi are able to produce a wide range of valuable proteins and enzymes for many industrial applications. Recent advances in fungal genomics and experimental technologies are rapidly changing the approaches for the development and use of filamentous fungi as hosts for the production of both homologous and heterologous proteins. In this review, we highlight the benefits and challenges of using filamentous fungi for the production of heterologous proteins. We review various techniques commonly employed to improve the heterologous protein production in filamentous fungi, such as strong and inducible promoters, codon optimization, more efficient signal peptides for secretion, carrier proteins, engineering of glycosylation sites, regulation of the unfolded protein response and endoplasmic reticulum associated protein degradation, optimization of the intracellular transport process, regulation of unconventional protein secretion, and construction of protease-deficient strains.

Key points

- *This review updates the knowledge on heterologous protein production in filamentous fungi.*
- *Several fungal cell factories and potential candidates are discussed.*
- *Insights into improving heterologous gene expression are given.*

Keywords Filamentous fungi · Heterologous protein production · Fungal cell factories · Gene regulation

Introduction

Filamentous fungi have been used to produce a wide range of valuable compounds for centuries. With the advent of biotechnology and molecular (synthetic) biology, they have been exploited as hosts for the production of primary and secondary metabolites, and homologous and heterologous proteins which are worth several billion dollars per year (Cairns et al. 2018). Filamentous fungi have several advantages over other microorganisms in terms of homologous protein secretion. For instance, they have a natural ability to secrete a variety of proteins in large quantities, efficient folding, post-translational modifications, inexpensive cultivation, and easy induction (Madhavan et al. 2022). Furthermore, industrial fermentation systems are well established for several species. However, heterologous protein production in filamentous fungi is far from optimal and there is still scope for further improvement (Meyer et al. 2016). At present, compared to homologous proteins, the level of heterologous protein production is significantly lower (Li et al. 2022). In case of the industrial workhorse *Aspergillus niger*, for example, the production level of a homologous protein has been reported to be up to 400 times higher than a heterologous protein (Li et al. 2022).

Mainly species of the genera *Aspergillus*, *Trichoderma*, *Penicillium*, and *Myceliophthora*, and to a lesser extent *Fusarium*, *Rhizopus*, or *Mucor*, are being used for large-scale protein production (Meyer et al. 2020). Furthermore, species such as *A. niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*, or *Trichoderma reesei* have a Generally Recognized As Safe (GRAS) status, facilitating their industrial applications. In fact, according to the list of enzymes compiled by the Association of Manufactures and Formulators of Enzymes Products updated in May 2015, the main fungal hosts used

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for the production of industrial enzymes are *Aspergillus* and *Trichoderma* species, followed by *Penicillium* (AMFEP, https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf). Many enzymes derived from these species have been applied in several industrial areas such as food and feed, biofuels and biochemicals, pharmaceutics, pulp and paper, detergents, textile, waste management, and/or agricultural industries (Kalra et al. 2020). In this review, we aim to highlight recent advances in fungal cell factory research and development, with a particular focus on the approaches used for improving the production of heterologous proteins in filamentous fungi, which are summarized in Fig. 1.

Main fungi used as protein cell factories

Aspergillus

Species in the genus *Aspergillus* are relevant to diverse fields such as biomedicine, bioenergy, health, and biotechnology. This is a large and diverse genus comprising ~ 400 recognized species including the industrially important *A. niger*, *A. oryzae*, *Aspergillus sojae*, *Aspergillus tubingensis*, *Aspergillus terreus*, and *Aspergillus unguis*, and some pathogenic species such as *Aspergillus fumigatus* and *Aspergillus flavus*, which are harmful to animals and/or plants (Romero et al. 2021). *Aspergillus* species are effective decomposers of organic substrates, as they are generally very efficient producers of extracellular enzymes. Moreover, *Aspergillus* species have become very suitable fungal cell factories at the industrial level due to their good ability to produce and secrete native and heterologous proteins, organic acids, and secondary metabolites (Ntana et al. 2020). In fact, among the commercial enzymes used in the food industry that are produced in filamentous fungi, more than 50% are produced in aspergilli (https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf).

A. niger is widely used as an industrial workhorse for the production of organic acids (particularly citric acid), proteins, and for basic genetic research (Yang et al. 2017; Lübeck and Lübeck 2022). In the last decades, much progress has been made for protein production using *A. niger*, with increasing titers of secreted proteins. Additionally, *A. niger* and other aspergilli have been harnessed for biosynthesis of diverse enzyme cocktails (Cairns et al. 2018). As examples, phytases, which are used to improve the nutritional content of animal feed, were first marketed in

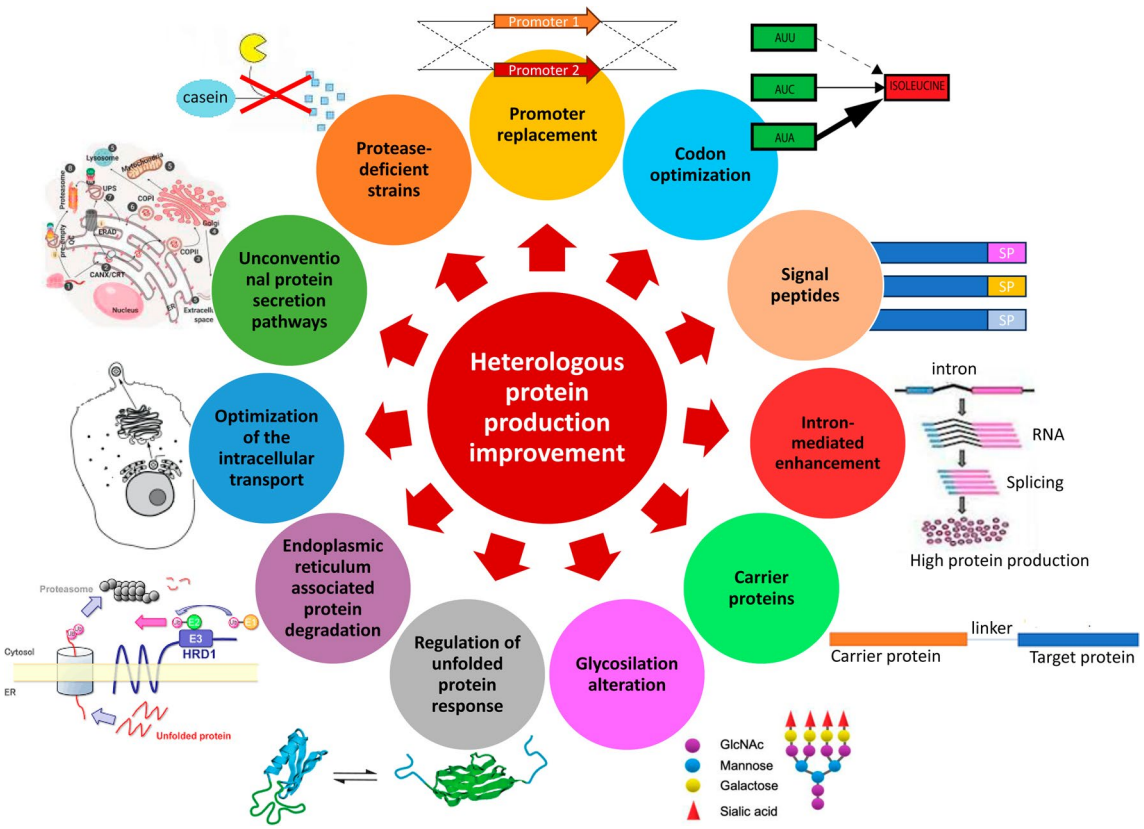

Fig. 1 Approaches used for improving the production of heterologous proteins in filamentous fungi

1991 (Jatuwong et al. 2020). The biotechnological market of phytases is estimated to be around €150 million per year, with *A. niger* being one of the most commonly used microorganisms for their production (Haefner et al. 2005). Moreover, the production level of recombinant *Myceliophthora thermophila* trehalase and *Penicillium citrinum* nuclease P1 achieved 1698.83 U/ml (Dong et al. 2020) and 77.6 U/ml (Chen et al. 2019), respectively, in *A. niger*, and the yield of human erythropoietin was 73.9 mg/l, which is a 41-fold improvement over the original yield (Rojas-Sánchez et al. 2020).

A. oryzae is together with *A. niger* one of the preferred hosts for the production of heterologous proteins due to its excellent protein secretion capabilities. Its use is also considered safe as it has been used in fermented cuisine (miso, soy sauce, or douchi) for more than 10 centuries (Jin et al. 2021). One of the advantages of *A. oryzae* is that it secretes large amounts of enzymes but produces very few secondary metabolites under typical cultural conditions (Meng et al. 2022). The β-glucosidase (BGL) from the lower termite *Neotermes koshunensis*, a well-known cellulose decomposer, was successfully produced by *A. oryzae* with a 48-fold increase (Uchima et al. 2011). Moreover, the bovine chymosin (CHY), human lysozyme (HLY), and recombinant antibodies (such as adalimumab) were also produced by *A. oryzae* to decrease their production costs (Daba et al. 2021).

Aspergillus vadensis is a new species of black aspergilli which does not acidify the growth medium and produces very low levels of extracellular protease activity, both of which contribute to an increased stability of the protein of choice in the culture broth of this fungal species (de Vries et al. 2005). Additionally, it has a very high transformation frequency, which is convenient for the high-throughput screening of transformations (De Vries et al. 2004). These characteristics make *A. vadensis* a very promising candidate as a host for protein production. For example, the feruloyl esterase B from *A. niger* was synthesized in *A. vadensis* at a 12-fold higher level than the *A. niger* overproducing strain (Alberto et al. 2009). In addition, 11 different promoters for heterologous protein-encoding gene expression in *A. vadensis* have been tested and their efficacy has been addressed (Culleton et al. 2014).

There are also other *Aspergillus* species that have been developed into heterologous protein production platforms. For instance, the suitability of *A. unguis* as a host for heterologous protein production through the expression of the pharmaceutically important human interferon gene has been demonstrated (Madhavan et al. 2017). A gene coding for an α-galactosidase enzyme from *A. fumigatus* has been expressed in *A. sojae*, resulting in approximately 3-fold higher production (Gürkök et al. 2010). The extracellular endo-β-1,4-mannanase gene from *A. fumigatus* was also successfully expressed in *A. sojae* with a 12-fold increase (Duruksu et al. 2009).

On the other hand, *Aspergillus nidulans*, which is not a species commonly used in industry but a model organism for eukaryotic research, has been used for the heterologous production of Carbohydrate Active enZymes (CAZymes) with promising results (Kumar 2020). Using mass spectrometry-based proteomic approaches, Zubieta et al. demonstrated that *A. nidulans* is indeed suitable for high expression and production of heterologous proteins by analyzing its intracellular proteome (Zubieta et al. 2018). Endoxylanase- and arabinofuranosidase-encoding genes from *Penicillium funiculosum* and *A. niger*, respectively, were introduced into *A. nidulans*, yielding 301.2 U/mg and 115.55 U/mg, respectively (Gonçalves et al. 2012).

Trichoderma

Trichoderma spp. are good hosts for heterologous gene expression because of their saprotrophic and mycoparasitic lifestyles, which allow them to grow on a diverse range of nutrients (Tomico-Cuenca et al. 2021). The majority of the cellulolytic enzymes utilized today in the biomass to biofuels or bioproducts industry are produced by *T. reesei* (*Hypocrea jecorina*) (Singh et al. 2015). Production of heterologous proteins in *T. reesei* has been carried out for decades, dating back to 1989, when calf chymosin was produced in this species (Harkki et al. 1989). With the genetic engineering of the strains and optimized culture conditions, up to 1 g/l of BGL from *A. terreus* was produced by *T. reesei* in a shake-flask culture (Wei et al. 2013). Also, 4.5 g/l human interferon alpha-2b could be produced by *T. reesei* in a bioreactor (Landowski et al. 2016). The extremely efficient protein synthesis machinery of *T.* reesei allows homologous protein yields higher than 100 g/l (Rantasalo et al. 2019). However, the yields for heterologous protein production remain modest (Jørgensen et al. 2014). Methods for improving heterologous protein production in this fungus are thus extremely desirable.

Penicillium

Penicillium species are predominantly saprobic in nature, and several species have been used in commercial food production (e.g., dairy products) and the manufacturing of the antibiotic penicillin, among others (Toghueo and Boyom 2020).

P. chrysogenum (*Penicillium rubens*) has significant industrial importance and is a well-studied model for protein secretion with GRAS status. The first heterologous expression system developed in *P. chrysogenum* was to express a recombinant fungal xylanase gene and the cDNA for human tear lipocalin (Graessle et al. 1997). Since then, *P.*

chrysogenum has been successfully used as expression system for protein production applying inducible, repressible, or constitutive promoters (Díez et al. 1999; Huber et al. 2019) and efforts were undertaken to define new promoters for strain engineering in this species (Polli et al. 2016). Recently two expression cassettes for homologous and heterologous gene expression were established in this fungus, resulting in an increase in the production of small, cysteine-rich, and cationic antifungal proteins with purification yields up to 80 mg/l (Sonderegger et al. 2016; Garrigues et al. 2018; Gandía et al. 2022).

Penicillium oxalicum has powerful protein secretion capability and has been applied for commercial cellulase production for many years (Fang et al. 2010). This fungus has been found to have relatively high homologous recombination frequencies in targeted gene manipulations (Li et al. 2010). After genetic engineering of the *P. oxalicum* 114-2, for instance, cellulose productivity of 158.38 U/l/h was reached (Han et al. 2017). Furthermore, an *A. niger* β-glucosidase-encoding gene was heterologously overexpressed in *P. oxalicum*. The resulted strain *P. oxalicum* C3-1 showed 156.6- and 245.2-fold increased production of the corresponding enzyme in the parent strain *P. oxalicum* PT3-1 and the wild-type strain *P. oxalicum* 114-2, respectively (Wang et al. 2019).

Penicillium funiculosum is a non-model hypercellulolytic fungus that produces high-quality protein mixtures for lignocellulosic biomass saccharification (Ogunmolu et al. 2015). This fungus is non-pathogenic, contains a diversity of extracellular enzyme-encoding genes, and is genetically manipulatable (Ogunmolu et al. 2018; Randhawa et al. 2021). To broaden the commercial potential of *P. funiculosum* as a host for the production of heterologous proteins, the promoter of the histone H4.1 gene was effectively exploited to drive the production of an intracellular bacterial enzyme, β-glucuronidase (GUS) (Belshaw et al. 2002).

Penicillium verruculosum is an efficient cellulase producer, making this species a good host for the production of heterologous proteins (Dotsenko et al. 2015). The expression of *A. niger* β-glucosidase gene under the control of either a strong cellobiohydrolase I (*cbh1*) gene promoter or a weaker histone (*hist4*) gene promoter provided notable boosting effect in *P. verruculosum* (Dotsenko et al. 2015). Subsequently, the BGL and lytic polysaccharide monooxygenases (LPMOs) from *T. reesei* were produced by *P. verruculosum* B1-537 strain (Bulakhov et al. 2017). *P. verruculosum* was also used to produce heterologous endo-xanthanase (Denisenko et al. 2021).

Penicillium canescens exhibits an increased capacity for synthesizing secreted *β*-galactosidase and xylanase (Vavilova et al. 2003), and has also been established as a host for the production of heterologous proteins (Sinitsyn and Rozhkova 2015). As examples to illustrate the possibilities of *P. canescens* for the production of high value heterologous proteins, the *cbh1*, *cbh2*, and *egl2* genes encoding cellobiohydrolase I, cellobiohydrolase II, and endo-1,4-β-glucanase from *P. verruculosum* and *bglA* gene encoding the BGL from *A. niger* have been expressed in *P. canescens*, as well as the *inu1* and *inuA* genes from *Aspergillus* sp. encoding exo- and endo-inulinases (Sinitsyn and Rozhkova 2015).

Finally, *Penicillium subrubescens* is such a species that has a specific expansion of certain enzyme families related to hemicellulose, pectin, and inulin degradation (Mansouri et al. 2013; Peng et al. 2017). It has previously been demonstrated to be a potential industrial species and authors have established genome editing methodologies (Salazar-Cerezo et al. 2020). Thus, it is a promising new fungal cell factory for the production of heterologous proteins.

Myceliophthora

Myceliophthora thermophila (synonym: *Sporotrichum thermophile*, previously known as *Chrysosporium lucknowense*) was isolated from the alkaline soil of eastern Russia and has excellent acid-base and temperature tolerance (Visser et al. 2011). With the ability to degrade plant biomass, *M. thermophila* provides a potentially rich reservoir of new enzymes for industrial uses, including numerous thermostable enzymes for biomass degradation (Gu et al. 2018). This thermophilic fungus has been developed into a mature system for carbohydrate hydrolase production at the industrial level, and the cellulase product from this fungus has also been granted GRAS status (Visser et al. 2011). The properties of *M. thermophila* provide an alternative for traditional fungal protein production hosts. For instance, a human IgG antibody against tumor necrosis factor alpha was expressed under control of the *cbh1* promoter in *M. thermophila*. Heterologous overexpression of fumarase gene from *Candida krusei* increased fumarate synthesis by up to 3-fold in *M. thermophila* (Gu et al. 2018). Disruption of protease genes in *M. thermophila* could at least double enhance the cellulase production, and the lower-protease producer could be used to further improve heterologous protein production (Li et al. 2020). *M. thermophila* is used to heterologously overproduce two different proteins, glucoamylase from *Myceliophthora heterothallica* and green fluorescent protein (Li et al. 2020).

M. heterothallica is a mostly unknown host organism with the ability to use sexual mating in strain development. In appearance, physiology, and phylogeny, the thermophile *M. heterothallica* is extremely similar to *M. thermophila* (Visser et al. 2011). The most notable distinction between these two species is the functional mating cycle of *M. heterothallica* (Aguilar-Pontes et al. 2016). In research, heterologous xylanase from *A. niger* was generated in *M. heterothallica*, resulting in an 80-fold increase in xylanase activity 24 h

after sucrose induction. Therefore, *M. heterothallica* has potential as a host organism for heterologous protein production (Corinne 2016).

Alternative fungal cell factories

Although many fungal hosts have already been used to produce proteins, the optimal host is largely unpredictable. Other filamentous fungi different from the aforementioned genera are also being studied in the laboratory to be identified as acceptable hosts for heterologous protein production. Several promising examples are listed in Table 1.

Strategies for heterologous protein production in filamentous fungi

In order to improve protein production in filamentous fungi, strategies such as using strong regulatory sequences (mainly promoters), codon optimization, replacing native signal peptides with more efficient ones, using carrier proteins, engineering glycosylation sites, regulation of unfolded protein response (UPR) and endoplasmic reticulum associated protein degradation (ERAD), optimization of the intracellular transport process, regulation of unconventional protein secretion (UPS), and construction of protease-deficient strains have been developed. These strategies are reviewed below.

Use of different promoter sequences

Promoters are generally defined as the DNA sequence that is located upstream of the transcription start site, controlling the transcription of genes. A robust promoter element is essential for the production of heterologous proteins. Typically, promoters are divided into two types: constitutive and inducible (Jeennor et al. 2022). Constitutive promoters are always expressed regardless of environmental/culture conditions, whereas inducible promoters are activated in response to external biotic or abiotic triggers. Constitutive or inducible promoters may be preferable depending on the goal of gene expression.

Well-known constitutive promoters available for heterologous protein production in filamentous fungi include, e.g., promoters of the alcohol dehydrogenase gene (*adhA*) (Upshall et al. 1987), the small basic protein of unknown function and high expression gene (*cDNA1*) (Wohlschlager et al. 2021), the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene (Long et al. 2018), the pyruvate decarboxylase-encoding gene (*pdc1*) (Wang et al. 2013), and the manganese superoxide dismutase-encoding gene (*sodM*) (Wakai et al. 2014). Examples of enzymes produced with these and other constitutive promoters as well as the production yields achieved are shown in Table 2.

The main disadvantage of utilizing constitutively active gene promoters is that they are active during growth, leading to production of heterologous proteins that may be harmful to the host cells from the very beginning. As alternative, well-known inducible/repressible promoters have been used in many applications, including those from the Taka-amylase gene (*amyA*) (Okazaki et al. 2012), the cellobiohydrolase I (*cbh1*) (Long et al. 2018), the glucoamylase A gene (*glaA*) (Uchima et al. 2011), the thiamine biosynthesis-related gene (*thiA*) (Yoon et al. 2013), and the xylanase III gene (*xyn3*) (Rahman et al. 2009), as well as several others (Kluge et al. 2018; Sakekar et al. 2021). Examples of enzymes produced with these and other inducible promoters as well as the production yields achieved are shown in Table 2.

Codon optimization of the heterologous genes

Codon optimization of heterologous genes is one of the most valuable tools for improving the production level of their corresponding proteins in filamentous fungi. Several studies have found that optimizing codons increases the steady-state mRNA level of heterologous genes (Tanaka et al. 2014; Fu et al. 2020). Most of the codon optimization strategies rely on using more frequently observed codons instead of rarely observed ones, which can increase the efficiency of the translation and the level of expression (Sen et al. 2020). The protein and mRNA production level of Der f7, a secreted protein of the house dust mite *Dermatophagoides farinae*, was increased by codon optimization in *A. oryzae* (Tokuoka et al. 2008). Tanaka et al. further demonstrated

Table 1 Heterologous protein production in alternative potential fungal hosts

Expression host	Protein source	Protein	Yield	Reference
Ashbya gossypii	*Trichoderma reesei*	Endoglucanase I	440 g l^{-1}	(Ribeiro et al. 2013)
Acremonium chrysogenum	Human	Thrombomodulin mutant protein	10 mg l^{-1}	(Honda et al. 1997)
Phanerochaete chrysosporium	*Dichomitus squalens*	Thermostable manganese peroxidase	1.5 mg l^{-1}	(Li et al. 2001)
Phanerochaete sordida	*Pleurocybella porrigens*	Lectin	0.2 mg l^{-1}	(Suzuki et al. 2014)
Talaromyces cellulolyticus	*Pyrococcus* sp.	Endo-type cellulase EGPh and EGPf	0.63 g l^{-1} and 0.80 g l^{-1}	(Kishishita et al. 2015)

Table 2 Summary of constitutive and inducible promoters used in filamentous fungi for heterologous protein production

Protein (source)	Promoter* (origin)	Inducer/repressor	Host	Yield	Reference
Strong/constitutive promoters					
Antifungal protein PAF (*P. chrysogenum*)	*paf* (*P. chrysogenum)*	–/–	*P. digitatum*	83 mg l^{-1}	(Sonderegger et al. 2016)
Antifungal protein NFAP (*Neosartorya fischeri*)	*paf* (*P. chrysogenum)*	–/–	*P. chrysogenum*	3 mg l^{-1}	(Sonderegger et al. 2016)
Anti-MUC1 scFv antibody (human)	*ccg1nr* (*N. crassa*)	–/–	*N. crassa*	3 mg l^{-1}	(Havlik et al. 2017)
Cellobiose dehydrogenase (*Corynascus thermophilus*)	*cDNA1* (*T. reesei*)	–/–	*T. reesei*	29 mg l^{-1}	(Ma et al. 2017)
Class I hydrophobin DewA (*A. nidulans*)	*hfb2* (*T. reesei*)	–/–	*T. reesei*	33 mg l^{-1}	(Schmoll et al. 2010)
Endoxylanase (*P. oxalicum*)	*pdc1* (*T. reesei*)	–/–	*T. reesei*	2 g l^{-1}	(Wang et al. 2013)
Erythropoietin (human)	*gpdA* (*A. nidulans*)	–/–	*A. niger*	73.9 mg l^{-1}	(Rojas-Sánchez et al. 2020)
Glyoxal oxidase (*Phanerochaete chrysosporium*)	*cDNA1* (*T. reesei*)	–/–	*T. reesei*	0.44 g l^{-1}	(Wohlschlager et al. 2021)
Mannanase (*Trichoderma harzianum*)	*pdc1* (*T. reesei*)	–/–	*T. reesei*	1.6 g l^{-1}	(Wang et al. 2014)
RNase A (bovine)	*cfp* (*N. crassa*)	–/–	*N. crassa*	356 mg l^{-1}	(Allgaier et al. 2010)
Thaumatin (*Thaumatococcus daniellii*)	*gpdA* (*S. cerevisiae*)	–/–	*A. oryzae*	50 µg l^{-1}	(Masuda and Kitabatake 2006)
Thaumatin (*T. daniellii*)	*gpdA* (*A. nidulans*)	–/–	*P. roqueforti*	2 mg l^{-1}	(Masuda and Kitabatake 2006)
Tissue plasminogen activator (human)	*adhA* (*A. nidulans*)	–/–	*A. nidulans*	1 mg l^{-1}	(Upshall et al. 1987)
Tissue plasminogen activator (human)	*tpiA* (*A. nidulans*)	–/–	*A. nidulans*	100 µg l^{-1}	(Upshall et al. 1987)
Variable heavy-chain antibody (llama)	*sodM* (*A. oryzae*)	–/–	*A. oryzae*	73.8 mg l^{-1}	(Okazaki et al. 2012)
Inducible promoters					
BGL I (*Aspergillus aculeatus*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	15.1 g l^{-1}	(Nakazawa et al. 2016)
BGL I (*A. aculeatus*)	*eg1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	17.4 g l^{-1}	(Shibata et al. 2021)
BGL I (*A. aculeatus*)	*xyn3* (*T. reesei*)	Xylose	*T. reesei*	14.7 g l^{-1}	(Shibata et al. 2021)
CBH I (*Melanocarpus albomyces*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	4.9 g l^{-1}	(Haakana et al. 2004)
CBH II (*M. albomyces*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	4.3 g l^{-1}	(Haakana et al. 2004)
CHY (bovine)	*amyB* (*A. oryzae*)	Starch or maltose/glucose	*A. oryzae*	109.4 mg l^{-1}	(Yoon et al. 2011)
CHY (bovine)	*thiA* (*A. oryzae*)	Thiamine	*A. oryzae*	80 mg l^{-1}	(Yoon et al. 2013)
Endoglucanase I (*M. albomyces*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	7.4 g l^{-1}	(Haakana et al. 2004)
HLY (human)	*amyB* (*A. oryzae*)	Starch or maltose/glucose	*A. oryzae*	35.8 mg l^{-1}	(Yoon et al. 2011)

Table 2 (continued)

Protein (source)	Promoter* (origin)	Inducer/repressor	Host	Yield	Reference
Interferon alpha-2b (human)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	4.5 g l^{-1}	(Landowski et al. 2016)
Lactoferrin (human)	*amyA* (*A. oryzae*)	Starch or maltose/glucose	*A. oryzae*	25 mg l^{-1}	(Ward et al. 1992)
Thaumatin (*T. daniellii*)	*glaA* (*A. niger*)	Glucose/xylose	*P. roqueforti*	2 mg l^{-1}	(Masuda and Kitabatake 2006)
Tissue plasminogen activator (human)	*glaA* (*A. niger*)	Glucose/xylose	*A. niger*	25 mg l^{-1}	(Wiebe et al. 2001)
Xylanase XYN2 (*Humicola grisea*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	0.5 g l^{-1}	(De Faria et al. 2002)
Xylanase XYN6 (*Acrophialophora nainiana*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	172 mg l^{-1}	(Salles et al. 2007)
Xylanase XYN11A (*Nonomuraea flexuosa*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	820 mg l^{-1}	(Paloheimo et al. 2007)
Xylanase XYNB (*Dictyoglomus thermophilum*)	*cbh1* (*T. reesei*)	Various saccharides/glucose	*T. reesei*	1 g l^{-1}	(Nevalainen et al. 2018)
Xylanase XYNE (*P. canescens*)	*gla1* (*P. verruculosum*)	Glucose/xylose	*P. verruculosum*	29.3 g l^{-1}	(Sinitsyn et al. 2018)

*Abbreviations: *adhA*, alcohol dehydrogenase; *amyA*, Taka-amylase; *amyB*, α-amylase; *cbh1*, cellobiohydrolase I; *ccg1nr*, a variant of glucose-repressible gene 1; *cDNA1*, small basic protein of unknown function and high expression; *cfp*, pyruvate decarboxylase; *egl1*, endoglucanases I; *gla1*, glucoamylase; *glaA*, glucoamylase A; *gpdA*, glyceraldehyde-3-phosphate dehydrogenase; *hfb2*, class II hydrophobin; *paf*, antifungal protein; *pdc1*, pyruvate decarboxylase; *sodM*, manganese superoxide dismutase; *tpiA*, triosephosphate isomerase; *thiA*, thiamine; *xyn3*, xylanase III

that a codon-optimized mite allergen gene *Der f7* increased the level of transcription and translation, as well as mRNA stability (Tanaka et al. 2012). Codon optimization of xylanase gene *xynB* from the thermophilic bacterium *Dictyoglomus thermophilum* allowed its expression in the filamentous fungus *T. reesei* by changing 20 codons (Te'o et al. 2000). In addition, it has been hypothesized that codon usage bias improves translation efficiency by accelerating elongation in highly expressed genes (Qian et al. 2012). Overall, optimal codons enhance the elongation rate, whereas non-optimal codons reduce it (Yu et al. 2015).

Insertion of other signal peptides

Signal peptides are short peptides located in the N-terminal of proteins and are responsible for transport of proteins to and through the endoplasmic reticulum (ER) and the secretory pathway. Consequently, signal peptides are very important for the secretion of the recombinant proteins, as they facilitate the purification processes (Owji et al. 2018). Replacing the original signal peptide with a more efficient one tends to increase the secretion efficiency of heterologous proteins. In *A. niger*, by replacing the native signal peptide of an α-galactosidase with the GlaA signal peptide, the extracellular α-galactosidase activity increased by more than 22 times (Xu et al. 2018). Similarly, by using this approach, the thermostable trehalase gene from *M. thermophila* has also been successfully expressed in *A. niger* (Dong et al. 2020). Also, the use of heterologous signal peptides can result in high levels of recombinant protein secretion. For example, in the phytopathogenic fungus *Penicillium digitatum*, the signal peptide from the *P. chrysogenum* antifungal protein PAF allowed the production and secretion of antifungal proteins in this phytopathogen for the first time in high yields (Garrigues et al. 2017).

Insertion of introns in heterologous genes

Introns as regulatory elements can boost gene expression without functioning as binding sites for transcription factors. This phenomenon was called "intron-mediated enhancement" (Gallegos and Rose 2015). Introns can enhance transcript levels by affecting transcription rate, nuclear export, and transcript stability. Furthermore, introns can improve mRNA translation efficiency (Shaul 2017). There are several reports about the enhancement of gene expression in mammals, plants, yeasts, and insects (Shaul 2017; Baier et al. 2018; Emami et al. 2013), but knowledge in filamentous fungi is sparse. A strategy was tried to improve the heterologous protein production by incorporating introns into the open reading frame of a given gene. Xu and Gong demonstrated that introns play crucial roles in the antifungal protein

gene expression from *Aspergillus giganteus* in *T. viride* by affecting mRNA accumulation (Xu and Gong 2003). The level of expression of human erythropoietin was enhanced in *A. niger* by incorporating the introns from the D-fructose-1,6-bisphosphatase encoding gene of *A. niger* into the erythropoietin sequence (Rojas-Sánchez et al. 2020).

Development of protein production systems

Carrier proteins to enhance production of heterologous proteins

The genetic fusion of a target protein with a native, well-secreted protein known as "carrier" is a commonly used strategy to increase heterologous protein production in filamentous fungi (Hoang et al. 2015). This approach appears to improve mRNA stability, promote translocation in the secretory pathway, and prevent protein from degradation (Ntana et al. 2020). The GlaA, α-amylase, and CbhI proteins from *A. niger*, *A. oryzae*, and *T. reesei*, respectively, have been used as carriers to produce heterologous proteins. With this approach, the production of heterologous proteins was increased by 5- to 1000-fold, depending on the strain and protein, resulting in yields ranging from 1 to 2000 mg/l (James et al. 2012). For instance, the in-frame fusion of human protein granulocyte colony stimulating factor with the native GlaA allowed production of 5–10 mg/l of this growth factor in *A. niger* (Wang et al. 2020). When CHY was fused to the native α-amylase in *A. oryzae*, the fused CHY produced twice as much as the non-fused CHY (Ohno et al. 2011).

However, releasing the heterologous protein from the carrier protein may be a bottleneck. When the production of human interferon alpha-2b in *T. reesei* was fused toe CbhI as a carrier protein, 44% of the fusion protein could not be cleaved by the protease Kex2 (Landowski et al. 2016).

Influence of glycosylation

Protein glycosylation is the most common form of post-translational modification on excreted and extracellular membrane-associated proteins (Spiro 2002). It involves the covalent attachment of many different types of glycans to a protein. Modifying protein glycosylation can be another possible strategy for increasing the stability and production of heterologous proteins in fungi. For example, N-glycosylation of *A. terreus* BGL, which was heterologously produced in *T. reesei*, positively affected stability of the resulting protein (Wei et al. 2013). In addition, it has been reported that removing the N384 glycosylation site of the CbhI during its production in *A. niger* resulted in a mutant strain with 70% higher cellulase activity (Adney et al. 2009). van den Brink et al. (2006) improved a poorly used N-glycosylation site within the prochymosin molecule, and the resulting highly glycosylated chymosin was produced in *A. niger* with a yield increase of more than 100% compared to the native molecule. In conclusion, even though some examples of protein glycosylation modification have shown improved protein production, in some cases, adding or removing glycosylation may change the enzyme properties, and therefore, experiments to further address enzyme functionality are required.

Regulation of the UPR and ERAD to promote protein secretion

Protein secretion, and in particular protein folding and maturation, takes place through the endoplasmic reticulum (ER). Overproduction of homologous or heterologous proteins could disturb the balance of ER and further cause the UPR and ERAD processes to relieve the stress in filamentous fungi (Heimel 2015). Therefore, attempts to improve heterologous protein production in filamentous fungi mostly focus on the secretory pathway and the folding of proteins in the ER (Sun and Su 2019). For example, overexpression of the *hac1* and *bip1* genes separately, which are involved in UPR, was found to enhance secretion of the *A. niger* glucose oxidase in *T. reesei* by 1.5- and 1.8-fold (Wu et al. 2017). In another study, deletion of the *derA* and *hrdC* genes, which are involved in the ERAD pathway, resulted in 6-fold and 2-fold increase in the heterologous β-glucuronidase in *A. niger*, respectively (Carvalho et al. 2011). The beneficial impact of downregulating ERAD components might be attributable to the increased time for proteins to be (re)folded in the ER before being targeted for degradation. However, loss of ERAD components increases UPR activation, which is most likely caused by an accumulation of un- or misfolded proteins in the ER, and can negatively influence growth behavior (Carvalho et al. 2011; Krishnan et al. 2013).

Optimization of the intracellular transport process

Optimization of the intracellular transport process results in enhanced secretion of heterologous proteins (Wang et al. 2020). By genetically deleting the putative ER-Golgi cargo receptors AoVip36 and AoEmp47, which can hinder the secretion of heterologous proteins by promoting their retention in the ER, the production of the α-amylase-fused form of bovine prochymosin was increased by approximately 2-fold in *A. oryzae* (Hoang et al. 2015). Overexpression of the *snc1* gene, which is involved in the fusion of vesicles and plasma membrane, was reported to increase by 2.2-fold the production of an *A. niger* glucose oxidase in *T. reesei* (Wu et al. 2017).

In addition, vacuolar protein sorting (VPS) also affects the secretion of heterologous proteins in filamentous fungi.

An interesting observation was that disruption of the VPS receptor gene *Aovps10* in *A. oryzae* enhanced the production of CHY and HLY by 3- and 2.2-fold (Yoon et al. 2010). Furthermore, autophagy was reported to deliver misfolded secretory proteins accumulated in the ER to vacuoles, which is an important process that adversely affects heterologous protein production (Kimura et al. 2011), since heterologous proteins are often recognized as misfolded proteins. The authors described that disrupting several autophagy genes in *A. oryzae* enhanced the production levels of CHY up to 3-fold compared to the parental strain (Yoon et al. 2013).

Unconventional secretion for production of heterologous proteins

Most of the extracellular proteins are secreted by the classical ER/Golgi-dependent pathway. However, some extracellular proteins can be secreted without passing through the conventional secretion pathway and are transported to the cell membrane via alternative routes, which are known as UPS pathways (Krombach et al. 2018). In filamentous fungi, unconventional secretion has been observed for endochitinase Cst1 from *Ustilago maydis* (Stock et al. 2012) as well as for the aspartic protease from *A. niger* (Burggraaf et al. 2016). It has been reported that the use of this pathway for the production of heterologous proteins could result in improved production yields and activities, as the proteins produced via UPS most likely do not undergo unwanted post-translational modifications. Exploiting the bacterial enzyme β-glucuronidase (GUS) as a reporter for unconventional secretion in *U. maydis*, the yields of Gus-Cst1 fusion protein were increased by a combination of culture buffering and deletion of harmful proteases (Terfrüchte et al. 2018). Furthermore, using the kinase Don3 as a gatekeeper to control the UPS was successfully established in *U. maydis* (Hussnaetter et al. 2021).

Construction of a protease-deficient strain

Proteolytic degradation of the products has been thought to be one of the bottlenecks limiting yields of heterologous protein production in filamentous fungi (Qian et al. 2019). Discovering that extracellular proteases can decrease the amounts of heterologous proteins has led to the development of protease-deficient strains. Deletion of protease-encoding genes is one of the methods to establish protease-deficient strains. In *A. oryzae*, several protease-encoding genes were disrupted to improve the production of CHY and HLY (Jin et al. 2007; Maruyama and Kitamoto 2008; Yoon et al. 2011; Kitamoto et al. 2015). In *T. reesei*, the production levels of human interferon alpha-2b were increased upon deletion of the subtilisin protease gene *slp7* and the metalloprotease gene *amp2*, with yields of 2.1 g/l and 2.4 g/l, respectively (Landowski et al. 2016). To improve the laccase yield, two genes encoding major proteolytic activities, dipeptidyl-peptidase and aspartic protease, were successfully disrupted in *A. nidulans*, and the activity of the *Pycnoporus sanguineus* laccase was increased approximately 13-fold (Li et al. 2018).

Disruption of protease regulatory genes has also been effective in substantially reducing protease activity in many filamentous fungi. For instance, the deletion of the transcription factor PrtT resulted in the reduction of major extracellular proteases in *A. niger*, and the production of *Glomerella cingulata* cutinase was increased 36-fold compared to that of the parental strain (Kamaruddin et al. 2017). Deletion of the p53-like transcriptional factor Vib1 in *T. reesei* exhibited a drastic decrease in cellulase and protease secretion, and the *A. niger* BGL encoding gene *bglA* was expressed at higher levels in Δ*vib1* strain (Sun et al. 2022). In another study, the *T. reesei* engineered platform was constructed by deleting eleven major lignocellulose-degrading enzymes and/or the putative protease transcription factor Pea1. Subsequently, the levels of production of three heterologous proteins, bacterial xylanase, fungal immunomodulatory proteins, and human serum albumin, were increased in *T. reesei* under the control of *cbh1* promoter (Chai et al. 2022).

Conclusions and future prospects

Owing to the high efficiency of protein secretion and excellent capability of posttranslational modification, filamentous fungi are considered as promising cell factories for heterologous protein expression and secretion (Sakekar et al. 2021). However, the expression systems for heterologous genes in filamentous fungi are still underdeveloped due to a lack of profound comprehension of their regulation of gene expression and protein secretory pathways (Ntana et al. 2020). The different approaches that could improve heterologous gene expression in filamentous fungi are elaborated in this review. While the complexity of these multiple methods seems challenging, they provide opportunities to conduct optimization experiments for heterologous protein production and to expand the knowledge on protein secretory pathways and processes in filamentous fungi. The further development of transcriptomics, proteomics, and metabolomics will provide more in depth understanding of protein production in filamentous fungi and will enable the development of more efficient fungal cell factories.

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