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Review

Aspergillus: A Powerful Protein Production Platform

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Abstract: Aspergilli have been widely used in the production of organic acids, enzymes, and secondary metabolites for almost a century. Today, several GRAS (generally recognized as safe) *Aspergillus* species hold a central role in the field of industrial biotechnology with multiple profitable applications. Since the 1990s, research has focused on the use of *Aspergillus* species in the development of cell factories for the production of recombinant proteins mainly due to their natively high secretion capacity. Advances in the *Aspergillus*-specific molecular toolkit and combination of several engineering strategies (e.g., protease-deficient strains and fusions to carrier proteins) resulted in strains able to generate high titers of recombinant fungal proteins. However, the production of non-fungal proteins appears to still be inefficient due to bottlenecks in fungal expression and secretion machinery. After a brief overview of the different heterologous expression systems currently available, this review focuses on the filamentous fungi belonging to the genus *Aspergillus* and their use in recombinant protein production. We describe key steps in protein synthesis and secretion that may limit production efficiency in *Aspergillus* systems and present genetic engineering approaches and bioprocessing strategies that have been adopted in order to improve recombinant protein titers and expand the potential of *Aspergilli* as competitive production platforms.

Keywords: *Aspergillus*; fermentation; filamentous fungi; genetic engineering; heterologous expression; recombinant protein; secretion; transcriptional regulation

1. Introduction

Proteins are functionally versatile biomolecules (e.g., enzymes, structural proteins, and hormones) involved in multiple biological processes in the cell. Despite their role in supporting biological systems, proteins have been extensively studied for their potential in the formulation of commercial products. They often find applicability in the production of pharmaceuticals, food, beverages, biofuels, cosmetics, detergents, etc. [1,2].

Market demand for industrially relevant proteins has guided research into exploring practices that can lead to large-scale production levels [3]. The development of recombinant DNA technology has opened up the possibility of producing recombinant proteins in heterologous expression systems that can support high production yields. In that respect, any gene can now be transferred into a production host able to generate large quantities of the corresponding protein of interest, avoiding limitations related to the conventional extraction of the protein from its native host [4]. Human insulin produced in *E. coli* cells was the first recombinant protein that was actually approved by the FDA for clinical use. The recombinant insulin Humulin[®], originally developed by Genentech, was eventually commercialized in 1982 [3]. Since then, a plethora of other proteins with pharmaceutical and industrial applications have successfully been synthesized in heterologous expression systems and have made their way into the market [1].

Today, recombinant proteins can be synthesized using a wide range of production platforms, including bacteria, yeasts and filamentous fungi, mammalian or insect cells, and transgenic plants, to name a few. Every heterologous production system though comes with certain advantages and drawbacks (Table 1). In most cases, the structure and function of the protein of interest determines which production system is the most appropriate to be used. For example, when it comes to manufacturing therapeutic proteins of high quality, mammalian cell lines are predominantly used, as they can produce complex, human-like glycosylated proteins that are safe for patients. In fact, almost 84% of the biopharmaceutical proteins are currently produced by Chinese Hamster Ovarian (CHO) cell lines [5].

For the production of non-medicinal proteins, a more economical approach is usually followed, using either bacterial or fungal production hosts [1,6,7]. While bacteria are often suitable for smaller proteins that do not require complex post-translational modifications, production of larger and more complex proteins is usually performed in yeast, e.g., *Pichia pastoris* [8]. However, yeasts have the tendency to hyperglycosylate secreted proteins, and thus reduce their in vivo half-life and affect their efficacy [9]. Additional limitations including low expression levels and plasmid instability have restricted the use of some yeasts (e.g., *S. cerevisiae*) in the production of industrial enzymes [10]. An alternative production platform that can support low-cost synthesis of large proteins with complex modifications, but with a lesser degree of hypermannosylation during glycosylation compared to yeast is filamentous fungi. In addition, due to their saprophytic lifestyle, most filamentous fungi have already developed the ability to produce and secrete a vast amount of enzymes in order to break down and feed on organic matter [11]. Strains belonging to the genera *Aspergillus*, *Trichoderma*, and *Neurospora* are in fact widely used for production of recombinant proteins with industrial applications [12–15]. Several reviews have described the potentials of filamentous fungi in the production of pharmaceutical and other industrial proteins, as well as the genetic engineering approaches followed to maximize production levels [7,16,17]. In this review, we specifically focus on the use of *Aspergillus* species in the manufacturing of recombinant proteins. Bottlenecks in protein synthesis and secretion are discussed, while our comprehensive literature search provides a general overview of the most important genetic engineering projects and bioprocessing strategies applied over the past 30 years to improve recombinant protein yields in *Aspergillus*.

Table 1. Comparison of the most commonly used heterologous expression systems in the field of recombinant protein production.

Expression Platform	Genetic Manipulation	Growth Rate	Product Titers	Product Quality	Product Purification	Contamination Risk	Production Cost	Relevant Literature
Bacteria (<i>Escherichia coli</i>)	Simple	Fast	High	Products can be non-functional (codon bias, no adequate post-translation modifications)	Can be problematic (e.g., inclusion bodies)	Medium (endotoxins)	Low	[18,19]
Yeasts (<i>Saccharomyces cerevisiae</i> , <i>Pichia pastoris</i> , etc.)	Simple	Fast	<i>S. cerevisiae</i> limited <i>P. pastoris</i> higher	Hypermannosylation of glycoproteins often occurs (shortens half-life of the protein in vivo, leads to immunogenic reactions)	Feasible	Low	Low	[9,10]
Filamentous fungi (<i>Aspergillus niger</i> , <i>Trichoderma reesei</i> , <i>Neurospora crassa</i>)	Feasible	Medium	High	Less hypermannosylation compared to yeasts, but still differences from mammalian glycosylation patterns	Simple	Medium (mycotoxins)	Low	[7]
Insect cells (<i>Spodoptera frugiperda</i> , <i>Drosophila melanogaster</i>)	Laborious	Fast	High	Not able to carry out N-glycosylation	Feasible	Very low	High	[20]
Mammalian cells (CHO cells, Human cell lines)	Laborious	Slow	Low	High quality therapeutic proteins, human-like glycosylation pattern	Simple	High (viruses and prions)	High	[21]
Transgenic animals (goats, chickens)	Laborious	Very slow	High	High quality therapeutic proteins	Simple	High (viruses and prions)	High, ethically questionable	[22]
Transgenic plants (rice, bananas, carrots, potatoes)	Feasible	Slow	High	Some differences in glycan structures from human-like pattern	Complex and expensive downstream processing	Very low	Medium	[23,24]

2. Industrial Application of Aspergilli

2.1. Traditional Uses of Aspergillus Species

The use of *Aspergillus* species in biotechnology begun approximately a century ago, when James Currie, a food chemist, discovered that the filamentous mold *A. niger* was able to produce citric acid, a food and beverage additive that was conventionally extracted from citrus fruits [25]. Since then, production of citric acid, now performed in *A. niger* cultures that grow on inexpensive sugar-based minimal media, has turned into a multibillion dollar business [26].

Nonetheless, industrial applications of Aspergilli are not limited to the production of citric acid. Several species have been used as prolific producers of other organic acids (e.g., itaconic), secondary metabolites, and enzymes of biotechnological significance [11]. For example, *A. niger* produces several enzymes used in food and feed production such as glucoamylases, proteases, and phytases [26]. *A. oryzae*, traditionally used in Asian cuisine, has been exploited as a cell factory for producing malate, which is used in the development of food and pharmaceutical products [27]. *A. terreus* has attracted interest due to its ability to produce a group of secondary metabolites called statins that are used in the production of cholesterol-lowering drugs [28]. In fact, AB Enzymes, BASF, Chr. Hansen, DuPont, and Novozymes are only a few examples of companies that have been or are still using *Aspergillus* species in large-scale manufacturing of commercial products such as organic acids, enzymes, proteins, and secondary metabolites [29].

2.2. The Use of Aspergillus Species in Heterologous Protein Production

Filamentous fungi are generally considered promising hosts for production of recombinant proteins, mainly due to their secretory capacity and metabolic versatility. However, only a few species appear to be able to produce competitive recombinant protein levels and even fewer have been developed into industrial production platforms. This can be attributed mainly to our incomplete knowledge of fungal physiology. For example, the mechanisms behind protein production and secretion in fungal cells are not yet fully understood for most of the species. In addition, the presence of unwanted metabolites (e.g., mycotoxins) has excluded several fungi from industrial production [29].

Aspergillus is a genus that has been studied extensively due to its value as a model organism in fungal research (*A. nidulans*) and its industrial importance in citric acid and enzyme production (*A. niger*, *A. oryzae*) [26]. Several molecular tools (e.g., synthetic promoters and terminators, selection markers, RNA interference-RNAi, and CRISPR-Clusters of Regularly Interspaced Short Palindromic Repeats-associated technologies), suitable for *Aspergillus* species, have also been developed, facilitating efficient and targeted manipulation of their genomes [30,31]. CRISPR/Cas, for example, a system developed to create site-specific double strand DNA breaks, has been successfully applied in editing the genome of *A. niger* [32–35], *A. nidulans* [35], *A. oryzae* [36], *A. fumigatus* [37], and other aspergilli [35]. With a relatively well-understood physiology (growth and development, gene expression, and secretion machinery) and several molecular tools available, the GRAS *A. niger* has already been used in industrial production of recombinant proteins, such as calf chymosin [38], human lactoferrin [39], and the plant-derived sweetener neoculin [40]. Nevertheless, heterologous protein production in *Aspergillus* species is not always efficient, leading to low production titers. In such cases, strategies that are usually applied to improve titers involve genetic engineering of the production strains and establishing the appropriate fermentation conditions.

3. Genetic Engineering Approaches for Aspergillus Strain Improvement

Due to their capacity to secrete large quantities of proteins into the culture medium, *Aspergillus* species, and especially *A. niger*, are considered promising candidates for the development of large-scale heterologous protein production platforms. However, production yields for heterologous proteins are usually much lower compared to the ones detected for the native proteins. Failure to achieve the desired protein amounts in *Aspergillus* cultures can be attributed to limitations related to

transcription, translation, and the post-translation processing and modifications during protein production. Additionally, bottlenecks in the fungal secretion machinery and the problem of extracellular degradation by fungal proteases further hinder the efficient production of foreign proteins in *Aspergillus* species [41]. These limitations during protein production in aspergilli will be discussed in detail in the following paragraphs.

3.1. Transcriptional Regulation

3.1.1. Promoters

Regulation of protein synthesis begins on the level of transcription. The first step for achieving high protein yields in heterologous production systems is the use of strong promoters that can drive high gene expression. A variety of constitutively active (e.g., PgpdA, glyceraldehyde-3-phosphate dehydrogenase promoter; PadhA, aldehyde dehydrogenase promoter; Ptef1, translation elongation factor 1 promoter; and Ph4h3, histones H4.1 and H3 bidirectional promoter) and inducible promoters (e.g., PglA, glucoamylase promoter; PalcC, alcohol dehydrogenase promoter; and PamyA, amylase promoter) are currently available for *Aspergillus* species [42,43].

Native inducible promoters are commonly used as being more efficient in achieving high protein titers, as they allow separation of protein synthesis from biomass formation. This separation can also be extremely useful when the protein to be produced is toxic for the fungus [42]. The inducible promoter of the *A. niger* glucoamylase gene (PglA) is frequently used in many *Aspergillus* expression systems. Expression of *glaA* is highly induced when maltose or starch are used as carbon sources, but repressed in the presence of xylose. High *glaA* expression levels have been correlated with a 5' cis-regulatory element, and specifically the region within 500 bp upstream of the translational start codon. This region (−464 to −426) contains a protein-binding CCAAT motif, crucial for the high activity of PglA [44]. Insertion of eight copies of this region into the PglA sequence significantly increased expression levels of a heterologous gene (*Vitreoscilla* haemoglobin), multiplying protein production by almost 20-fold [45] (Table 2).

Table 2. Approaches for improving recombinant protein production through promoter engineering.

Process	Modification	Performance	Improvement Factor	Reference
Promoters	Use of several promoters (P) in <i>A. awamori</i>	PB2 from <i>Acremonium chrysogenum</i> : 0.25–2 mg/L thaumatin	-	[46]
		PpcbC from <i>Penicillium chrysogenum</i> : 0.25–2 mg/L thaumatin		
		PgdhA from <i>A. awamori</i> : 1–9 mg/L thaumatin		
		PgpdA from <i>A. nidulans</i> : 0.75–11 mg/L thaumatin		
	Insertion of multiple copies of an activator protein-binding site from the cis-regulatory region of <i>A. niger glaA</i> to the new promoter in <i>A. niger</i>	396.0 ± 51.5 mg/L of <i>Vitreoscilla</i> hemoglobin compared to 19.7 ± 4.8 mg/L from the strain with 1 copy	20	[45]
Use of hybrid promoters (combination of a human hERa-activated promoter (pERE), <i>S. cerevisiae</i> URA3 promoter and <i>A. nidulans nirA</i> promoter) in <i>A. nidulans</i>		pERE-URA-nirA + lacZ: 25 U of β-galactosidase activity/mg of protein	-	[47]
		pERE-URA-RS (random stuffer-link) + lacZ: 100 U of β-galactosidase activity/mg of protein	4	
		pERE-RS-nirA + lacZ: 1400 U of β-galactosidase activity/mg of protein [1 pM inducer (DES)]	56	

Table 2. Cont.

Process	Modification	Performance	Improvement Factor	Reference
Use of a hemolysin-like protein promoter (Phyl) for heterologous production in <i>A. oryzae</i>		Reporter gene: Endoglucanase Cel B Pamy: 24.1 ± 5.5 U/mL, Phyl: 57.9 ± 17.4 U/mL	2.4	[48]
		Reporter gene: <i>Trichoderma</i> endoglucanase I Pamy: 7.7 ± 3.9 U/mL, Phyl: 27.8 ± 1.3 U/mL	3.6	
		Reporter gene: <i>Trichoderma</i> endoglucanase III Pamy: 4.0 ± 0.6 U/mL, hyl: 31.7 ± 3.3 U/mL	7.9	
Regulatory elements (TerR and PterA) from <i>A. terreus</i> terrain gene cluster for <i>E. coli lacZ</i> expression in <i>A. niger</i>		Promoter activity ~5000 mU/mg when TerR under PgpdA (No activity when TerR under the native promoter)	-	[49]
		Promoter activity ~10,000 mU/mg (when TerR under PgpdA in 2 copies)	2	
		Promoter activity ~15,000 mU/mg (when TerR under PamyB)	3	
<i>A. niger</i> α -glucosyltransferase produced under the <i>A. niger</i> pyruvate kinase promoter		2000 U/mL total activity of α -glucosyltransferase compared to 600 U/mL in the wild type	3.3	[50]
Overexpression of the transcription factor RsmA, while the aflR promoter was inserted in front of the <i>pslcc</i> in <i>A. nidulans</i>		60,000 U/mL of <i>Pycnoporus</i> <i>sanguineus</i> laccase compared to 4000 U/mL in the control strain	15	[51,52]
A novel promoter from <i>Talaromyces emersonii</i> (Pglucan1200) for expressing <i>glaA</i> in <i>A. niger</i>		6000 U/mL of <i>GlaA</i> , enzyme activity increased by about 25% compared to 5000 U/mL in the strain with the <i>PglaA</i>	1.2	[53]
The constitutive promoter of <i>ecm33</i> (Pecm33) from <i>A. niger</i> in <i>A. niger</i>	Maltose:	Pecm33 activity induced by 1.7 compared to <i>PglaA</i> activity that induced by 2.7	-	[54]
	Glucose:	Pecm33 activity induced by 1.1 compared to <i>PglaA</i> activity that induced by 1.8		
	Xylose:	Pecm33 activity induced by 2 compared to <i>PglaA</i> activity that induced by 1.3 Increased Pecm33 activity at 37 °C		

Although the majority of the promoters used are derived from the primary metabolism, there have also been attempts to develop expression cassettes using regulatory elements from fungal secondary metabolite pathways (Table 2) [49,51,52]. In such cases, the strain carries the heterologous gene under the control of an inducible promoter and it is engineered to overexpress the gene that encodes the transcription factor, which activates the specific promoter, thus achieving high expression levels.

Apart from the endogenous promoters discussed above, non-endogenous, or synthetic, tunable promoter systems have also been developed for *Aspergillus* species [47,55–57]. One of them, the Tet On/Off system, was adapted from the mechanism regulating the tetracycline resistance operon in *E. coli*. It is also based on a dual player system, where the production strain is co-transformed with a plasmid carrying the heterologous gene regulated by the activity of a tetracycline-responsive promoter and a plasmid encoding a tetracycline transactivator (tTA). In the absence of the tetracycline or its derivative, doxycycline (DOX), the tTA binds to the promoter, inducing heterologous gene expression. However, where DOX is added, tTA disassociates from the binding sites and expression shuts down [56].

3.1.2. Gene Copy Number and Integration Site

In general, it has been suggested that integration of multiple gene copies can result in increased protein production levels. This has been observed frequently in *Aspergillus* systems expressing native proteins, such as glucoamylase or amylase (Table 3) [58–60]. In fact, *A. niger* strains containing multiple *glaA* copies (20 and 80) were able to secrete five to eight times more glucoamylase [59]. Similarly, introducing additional copies of heterologous genes also leads to higher amounts of protein produced (Table 3) [47,61]. However, this is not always the case. While studying the effect of copy number on heterologous expression in *A. nidulans*, Lubertozzi and Keasling (2006) observed that β -galactosidase activity of the transformants did not consistently correlate with the *lacZ* dosage. They suggested that this could be due to a gene silencing mechanism, previously observed in filamentous fungi, or due to pleiotropic effects of random integration [62]. In addition, Verdoes et al. (1995) suggests that the reasons for this limitation in protein production by strains harboring multiple copies is both the site of integration and the availability of trans-regulatory factors involved in transcription [63].

Table 3. Approaches for improving recombinant protein production through integration of multiple gene copies.

Process	Modification	Performance	Improvement Factor	Reference
Copy number	Integration of up to 200 additional copies of the <i>glaA</i> in <i>A. niger</i>	355 mg/L of glucoamylase compared to parental strain (50 mg/L)	7.1	[58]
	Integration of 80 additional copies of the <i>glaA</i> in <i>A. niger</i>	1268 mU of glucoamylase/mL culture filtrate compared to 280 mU/mL in the parental strain	4.5	[59]
	Integration of multiple copies of cassettes with thaumatin gene in <i>A. awamori</i> (two types of cassettes, 1 with PB2 and 1 with PgdhA)	8 copies: 10 \pm 0.4 mg/L thaumatin	-	[46]
		11 copies: 14 \pm 1.1 mg/L thaumatin	1.4	
		14 copies: 11 \pm 0.8 mg/L thaumatin	1.1	
		10 copies: 14 \pm 1.3 mg/L thaumatin	1.4	
	Insertion of multiple copies of the cassette pERE-URA-RS + <i>lacZ</i> in <i>A. nidulans</i>	1 copy of pERE-URA-RS + <i>lacZ</i> : 9500 U of β -galactosidase/mg of protein	5.4	[47]
		Multiple copies of pERE-URA-RS + <i>lacZ</i> : 51,000U of β -galactosidase/mg of protein [1 nM inducer (DES)]		
	Integration of an additional copy of the <i>glaA</i> -RFP (2 in total) in <i>A. nidulans</i>	A 70% increase in maximum fluorescence level (quantification data not available)	1.7	[61]

The genomic site, where the expression cassette is integrated during transformation, is indeed an important factor that influences transcription of the heterologous gene and consequently protein production [62,63]. In order to tackle unpredicted limitations related to random insertion, site-specific integration systems are usually applied [32,64]. Moreover, identification of genomic loci with high transcriptional activity, followed by targeted integration of expression cassettes in the specific sites, provides an additional approach for boosting transgene expression and recombinant protein synthesis [65]. A promising integration site for expression and characterization of heterologous genes was identified in the genome of *A. nidulans*. Insertion of heterologous genes in the specific locus (Integration Site 1-IS1) did not interfere with the fitness of the strain, while it allowed for high and stable expression levels in a tissue-independent manner [66].

3.2. Translational Regulation

Codon Usage and mRNA Stability

Most of the amino acids found in nature are encoded by more than one codon (synonymous codons). The preference for one codon over another, known as codon usage, varies among organisms and can be a limitation when heterologous genes are expressed in a host with different codon usage compared

to the codon usage of the organism from which the genes has been isolated [67]. Codon optimization is commonly used in such cases, where rare codons found in heterologous genes are replaced by synonymous codons that encode the same amino acid but are more frequently used in the expression host. Codon optimization has been proposed as a successful practice for improving mRNA stability and translational efficiency, leading to higher levels of heterologous protein production [68,69].

Several studies in recombinant protein production in *Aspergillus* species have reported that codon optimized genes are expressed more efficiently, resulting in improved heterologous protein yields (Table 4) [69–72]. For example, *A. oryzae* strains carrying a codon optimized *der f7*, a gene that encodes *Dermatophagoides farina* mite allergen 7, showed increased gene expression levels and it produced almost three to five more protein than the strains with the non-optimized gene [70]. Tanaka et al. (2012) showed later that the increase in transcriptional and translational efficiency was clearly assigned to improved mRNA stability due to codon optimization of *der f7* [73].

Table 4. Approaches for improving recombinant protein production through codon optimization of the expressed sequences.

Process	Modification	Performance	Improvement Factor	Reference
Codon usage	<i>A. Cyamopsis tetragonoloba</i> α -galactosidase gene optimized based on <i>Saccharomyces cerevisiae</i> codon usage and expressed in <i>A. awamori</i>	Synthetic gene: 0.4 mg/L α -galactosidase Wild type gene: Undetectable levels	-	[72]
	<i>A. Solanum tuberosum</i> α -glucan phosphorylase synthetic gene optimized based on <i>A. niger</i> -preferred codon usage for production in <i>A. niger</i>	Synthetic gene: 39.6–94.6 mg/L α -glucan phosphorylase Wild type gene: <0.1 mg/L	-	[71]
	A codon optimized <i>Dermatophagoides farina der f7</i> gene based on <i>A. oryzae</i> codon usage and expressed in <i>A. oryzae</i>	Non-fused: undetectable level to a detectable level Fused to GlaA: 3 to 5 fold increase (Signal intensity quantification of the bands from the SDS-PAGE)	3–5	[70]

However, the codon usage of the native gene “donor” hosts is not trivial. It has been shown that codon usage influences local rates of translation elongation (preferred codons speed up elongation and rare codons slow it down), assisting in proper co-translational protein folding. This means that codon optimization of a genetic sequence that results in increased translation velocity, can also disrupt protein folding, secretion and activity [74].

In general, post-transcriptional events, such as mRNA processing (addition of the 3' polyA-tail or the 5' cap) and base modifications, the presence of rare codons or destabilizing sequences can have an impact on the length and stability of mRNA, thus limiting efficient translation and protein synthesis [75]. Limitations related to mRNA stability can also be addressed by using gene fusions, which contain heterologous proteins fused to a well-secreted carrier protein [75], a strategy that will be discussed later in the review.

3.3. Glycosylation

Glycosylation is a post-translational modification of eukaryotic proteins, during which glycans are added on specific amino acid residues. Two main types of glycosylation have been described: the O-linked glycosylation, where glycans are added on the side-chain hydroxyl groups of serine or threonine residues [76], and the N-linked glycosylation, which takes place on the side-chain amino groups of asparagine residues (at a N-X-S/T motif, where X is any amino acid except proline) [77].

Glycosylation is crucial for glycoproteins, as it influences their stability, activity, but also their passage through the secretory pathway [78,79] (see also below Section 3.4.1. The fungal Secretory Pathway—Glycosylation).

Filamentous fungi, and thus aspergilli, have the ability to perform post-translational modifications, including glycosylation, making them appropriate hosts for production of eukaryotic proteins. However, when it comes to production of mammalian proteins, fungal glycosylation is a bottleneck [78]. Filamentous fungi typically produce *N*-glycans that differ in composition and structure from mammalian ones. This can create problems during heterologous production of mammalian proteins in fungal expression systems, such as incorrect folding and subsequent elimination of the aberrant proteins by the cell quality control mechanisms. Moreover, addition of unusual fungal glycan structures on the heterologous protein may affect its stability and activity, or in the case of therapeutic proteins, increase their immunogenicity [80].

During *N*-linked glycosylation, the glycan (Glc3Man9GlcNAc2), consisting of three glucose (Glc3), nine mannose (Man9), and two *N*-acetylglucosamine (GlcNAc2) residues, is transferred to the conserved consensus sequence Asn-X-Ser/Thr. Shortly after, the removal of three Glc and one Man sugar results in the Man8GlcNAc2 glucan, a process conserved among all eukaryotes. However, further modifications on Man8GlcNAc2 differ between mammalian and fungal cells. Filamentous fungi and yeasts usually produce small, high-mannose *N*-glycans, while the mammalian glycans are more complex, containing *N*-acetylglucosamine, galactose, fucose, and sialic acid.

Several attempts have been made to engineer the glycosylation pathway in aspergilli towards the synthesis of complex mammalian-like glycans [81–84]. Kainz et al. (2008) focused on two crucial steps in the pathway for obtaining glycoproteins of mammalian type: First, the trimming of terminal mannose residues from Man8GlcNAc2 to Man5GlcNAc2 structures, a process catalyzed by a mannosidase, and second, the subsequent transfer of *N*-acetylglucosamine to yield GlcNAcMan5GlcNAc2, catalyzed by a glycosyltransferase. Insertion of genes encoding the α -1,2-mannosidase and β -1,2-*N*-acetylglucosaminyltransferase I in *A. niger* and *A. nidulans* resulted in the synthesis of glycans with the desirable structure described before. In addition, deleting a gene (*algC*) involved in the early steps of fungal glycosylation, further contributed to the synthesis of glycan structures resembling those of humanized glycoproteins [81].

3.4. Secretion

Another advantage of using *Aspergillus* species as protein production systems is their natural capacity to secrete high amounts of protein in the extracellular environment. Nevertheless, heterologous proteins often lack specific features of the native secreted proteins, leading to low secretion efficiency and low yields. Therefore, several studies have looked into unraveling the secretion processes in filamentous fungi [85,86]. In fact, multiple bottlenecks of the fungal secretory pathway have been identified and key factors of secretion have been engineered, improving production of heterologous proteins. Alternatively, fusion of these proteins to native, secreted proteins (carrier proteins) has also been able to enhance secretion, bypassing the complexity of engineering steps of fungal secretion [42].

3.4.1. The fungal Secretory Pathway

Proteins moving through the fungal secretory pathway are subjected to several quality control tests until they are finally secreted. Attempts to engineer fungal secretion have been made in all the possible rate-limiting steps, starting from the processing that occurs in the endoplasmic reticulum (ER), to protein transport and degradation pathways (Figure 1).

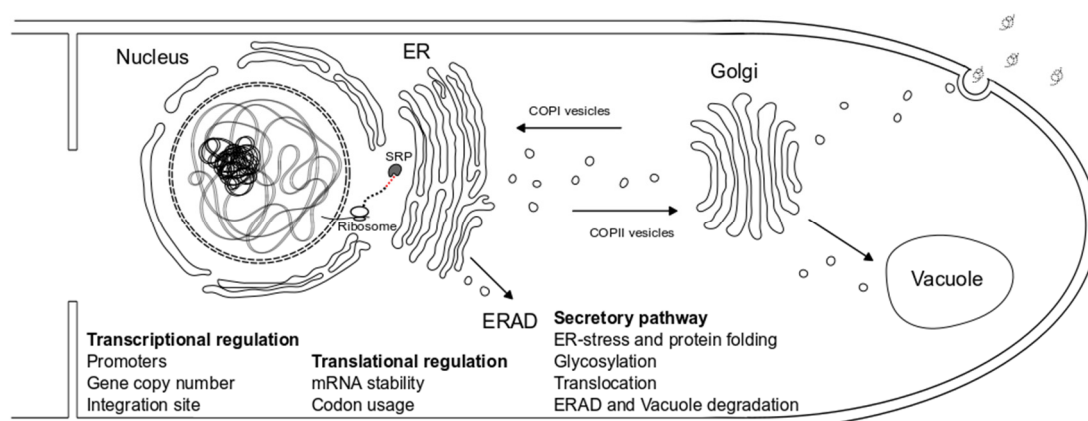


Figure 1. Protein synthesis and secretion in a fungal (e.g., *A. niger*) cell are schematically described. The figure also presents key steps that can be bottlenecks in the production of recombinant proteins in filamentous fungi. At transcription level, high expression of the gene of interest can be achieved by using strong promoters, integrating multiple gene copies and choosing integration sites that positively influences recombinant gene expression. Translation can also be a limiting step in recombinant protein production and can be improved by increasing mRNA stability and adjusting codon usage of the heterologous coding sequence to the fungal host. Following translation, proteins guided by the signal recognition particle (SRP) enter the ER lumen, where they receive post-translation modifications. At this stage, accumulation of unfolded proteins due to high gene expression can lead to ER-stress. Activation of several ER-resident chaperones and foldases (see text for details) can assist with proper folding of most proteins, relieving the overloaded ER. Proteins that fail to be properly folded are guided to the cytoplasm where they are degraded through the ERAD (ER-associated protein degradation) pathway by the proteasome. Proper folding, and consequently efficient secretion, is also dependent on the glycosylation process in the ER. Properly folded and glycosylated proteins destined to be secreted are packed into Coat Protein complex II (COPII) vesicles and transported to the Golgi complex, where further glycosylation takes place. Through a vesicle-mediated process, proteins finally reach the cell surface, where they are released into the periplasmic region. Additional protein translocation pathways, such as retrograde trafficking and vacuole degradation, can indirectly affect protein secretion efficiency. In retrograde trafficking, ER-resident proteins that have escaped or misfolded Golgi-resident proteins are transported from the Golgi back to the ER packed in COPI vesicles. Finally, misfolded and dysfunctional proteins that fail to pass the quality control of the secretory pathway can be transported to the vacuole by Vacuolar protein sorting (Vps) receptors and are degraded (autophagy-related degradation).

- ER-stress and protein folding

Secretion starts with the protein being transported to the ER, where post-translational modifications, like glycosylation, disulfide bond formation, and folding, take place. Accumulation of misfolded or unfolded proteins in the ER can activate stress response pathways in the cell, including the unfolded-protein response (UPR) and the ER-associated degradation (ERAD) pathway (Figure 1). Activation of the UPR usually increases expression of genes related to post-translation protein processing (e.g., lectins, chaperones, foldases, and protein disulfide isomerases), resulting in proper protein folding, and eventually alleviates ER-stress [87].

ER-overload is often observed in heterologous expression systems, where high transcriptional activity leads to buildup of incompletely folded proteins. As a result, efficient protein secretion is hindered, resulting in low production yields of heterologous proteins. In such cases, engineering the UPR is considered to be a promising practice to improve efficiency of heterologous expression systems [85]. Induction of the UPR by overexpressing the gene encoding the UPR transcription factor HacA in *A. awamori* increased production yields of the *Trametes versicolor* laccase by sevenfold

and these of bovine preprochymosin by almost threefold [88]. In addition, constitutive expression of *hacA* resulted in 1.5 times higher production levels of neoculin, a plant nonglycemic sweetener, in *A. oryzae* cultures [40]. Many genetic engineering projects have also targeted single UPR components (e.g., ER-resident chaperones and foldases) for enhancing protein secretion in several *Aspergillus* species (Table 5) [88–93]. For example, overexpression of the gene encoding the lectin-like chaperone calnexin resulted in 60–73 mg/L of the *Phanerochaete chrysosporium* manganese peroxidase in *A. niger* mutants, while the parental strain production reached only 14 mg/L [92]. However, a positive correlation between chaperone overexpression and protein secretion appears to depend specifically on the protein to be produced. For example, increased production of the chaperone protein BipA negatively affected production of the *P. chrysosporium* manganese peroxidase in *A. niger* [92], but improved production titers of the plant sweet protein thaumatin in *A. awamori* [93].

Table 5. Approaches for improving recombinant protein production through engineering unfolded-protein response (UPR) response.

Process	Modification	Performance	Improvement Factor	Reference
ER-stress and protein folding	Overexpression of <i>prpA</i> (multicopy integrated vector) in <i>A. niger</i> var. <i>awamori</i>	The level of chymosin by the control transformants was similar to the transformants with overexpression of the <i>prpA</i>	-	[90]
	Deletion of <i>prpA</i> in <i>A. niger</i> var. <i>awamori</i>	The production level of bovine prochymosin was lower than expected for randomly isolated transformants (3/19 transformants)	-	[90]
	Overexpression of <i>cypB</i> in <i>A. niger</i>	Twofold increase in glucoamylase production	2	[89]
	Insertion of multiple copies of <i>pdiA</i> in <i>A. awamori</i>	19 mg/L thaumatin compared to 5 mg/L in the parental strain	3.8	[91]
		Optimal bioreactor conditions: 150 mg/L thaumatin compared to 40 mg/L in the parental strain	3.8	
	Overexpression of <i>clxA</i> in <i>A. niger</i>	60–73 mg/L <i>P. chrysosporium</i> manganese peroxidase compared to 14 mg/L in the parental strain	4–5	[92]
	Overexpression of <i>bipA</i> in <i>A. niger</i>	<i>P. chrysosporium</i> manganese peroxidase was severely reduced-almost undetectable levels	-	[92]

Table 5. Cont.

Process	Modification	Performance	Improvement Factor	Reference
	Overexpression of <i>hacA</i> in <i>A. niger</i> var. <i>awamori</i>	13–34 mg/L chymosin compared to 12.5 mg/L in parental strain	1.3–2.8	[88]
		3.9–8.5 nkat/mL laccase compared to 0.9 nkat/mL in parental strain	3–7.6	
	Overexpression of <i>bipA</i> in <i>A. awamori</i>	20 mg/L thaumatin compared to 9 mg/L in the parental strain	2–2.5	[93]
	Constitutive expression of the active form of <i>hacA</i> cDNA in <i>A. oryzae</i>	2 mg/L neoculin compared to 1.5 mg/L in the control strain	1.5	[40]

- Glycosylation

As mentioned before, glycosylation affects protein activity and stability and in many cases it also influences protein folding and secretion efficiency [78,79]. *N*-glycosylation has been linked to an ER-quality control system of glycoprotein folding in filamentous fungi [94,95]. In fact, inhibiting the specific process in *A. nidulans* appeared to hinder secretion of α -galactosidase, resulting in the accumulation of the under-glycosylated protein to the cell wall [96]. Engineering *N*-glycosylation has been applied as a strategy to increase levels of heterologous protein secretion (Table 6) [97–99]. For example, improving a poorly used *N*-glycosylation site or adding a new one in a chymosin gene almost doubled the levels of secreted protein in *A. niger* strains [98].

Table 6. Approaches for improving recombinant protein production through engineering glycosylation.

Process	Modification	Performance	Improvement Factor	Reference
Glycosylation	Overexpression of <i>S. cerevisiae</i> DPM1 in <i>A. nidulans</i> strains impaired in DPMS activity	No significant increase in protein secretion observed Production of invertase and glucoamylase was higher but the proteins were trapped in the periplasmic space	28	[97]
	Bovine prochymosin synthetic gene with a single mutation (S335T)—This mutation resulted in a potentially better <i>N</i> -glycosylation site (NHT) in <i>A. niger</i>	207 IMCU/mL (0.9 g/L) chymosin compared to 90 IMCU/mL in the parental strain 90% of the chymosin molecules were glycosylated compared to 10% in the parental strain	3	[98]
	Bovine prochymosin synthetic gene with a N–S–T glycosylation site (TDNST) in the short peptide linker in <i>A. niger</i>	141 International Milk Clotting Unit/mL (0.6 g/L) chymosin compared to 90 IMCU/mL in the parental strain Same glycosylation pattern	1.5	[98]
	Deletion of <i>mnn9</i> , <i>mnn10</i> , <i>ochA</i> in <i>A. niger</i>	Δ mnn9: 14.6% increase in <i>Tramete</i> laccase production	1.1	[99]
		Δ mnn10: 12.7% increase	1.1	
		Δ ochA: 7.2% increase	1.1	
		Δ mnn9/ochA: 16.8% increase	1.2	

Strangely enough, it appears that glycosylation is not always essential for the secretion of proteins. For example, secretion of α -amylase was not affected at all when the antibiotic tunicamycin was used to block *N*-glycosylation in *A. oryzae* cultures [79]. In another attempt, overexpressing the yeast DPM1 gene, a key gene of *O*-glycosylation, improved the generation of native proteins in *O*-glycosylation-deficient *A. nidulans* strains, but the proteins produced were mostly localized in the periplasmic space [97]. In addition, deleting *algC*, a gene involved in early steps of *N*-glycosylation, resulted in an increase of the overall protein secretion in *A. niger* strains [100]. It is apparent that the role of glycosylation in protein production is not yet clearly understood and requires more research in order to be employed for improving heterologous protein production.

- Protein translocation

Following glycosylation and quality control in the ER, secreted proteins are packed in ER-derived vesicles (COPII-coated vesicles) and are transported to the Golgi apparatus. Proteins are further glycosylated there and are then guided to the plasma membrane in Golgi-derived vesicles. Additionally, other types of vesicles starting from Golgi carry ER-residents back to the ER or recycle important Golgi-enzymes and trafficking components (COPI-coated vesicles) [101] (Figure 1).

Regulating expression of key factors involved in vesicle trafficking (e.g., cargo receptors recruiting proteins into the vesicles) has been applied lately in order to assist protein transport through the secretory pathway and has improved production yields in aspergilli (Table 7) [61,102–104]. Indicatively, overproduction of the Rab GTPase RabD, a protein involved in cargo transport from the Golgi apparatus to the plasma membrane, increased the secretion yields of a fluorescent reporter protein (mRFP) in *A. nidulans* cultures by approximately 25% [61]. Similarly, deleting genes encoding the receptors AoVip36 and AoEmp47, which retain proteins in the ER, almost doubled the level of chymosin secreted in *A. oryzae* strains [104].

Table 7. Projects for improving recombinant protein production through engineering protein trafficking.

Process	Modification	Performance	Improvement Factor	Reference
Protein translocation	Deletion of <i>Aovip36</i> in <i>A. oryzae</i>	50 mg/L chymosin compared to 27 mg/L in the parental strain	1.9	[104]
		300% EGFP in culture supernatant compared to 100% in the parental strain	3	
	Deletion of <i>AoEmp47</i> in <i>A. oryzae</i>	The α -amylase activity (native protein) was reduced by approximately 30% compared with the activity in the control strain	3	
		50 mg/L chymosin compared to 27 mg/L in the parental strain	1.9	
Overexpression of <i>rabD</i> in <i>A. nidulans</i>		210% EGFP in culture supernatant compared to 100% in the parental strain	2.1	[104]
		No difference in the α -amylase activity (native protein)	2.1	
		25% increase in mRFP secretion in submerged cultivations in shake flasks	1.3	[61]
		40% increase in RFP secretion in 2l bioreactor	1.4	

Table 7. Cont.

Process	Modification	Performance	Improvement Factor	Reference
	Deletion of <i>racA</i> in <i>A. niger</i>	Native GlaA secreted into the culture medium is four times more compared to its parental strain, when ensuring continuous high-level expression of <i>glaA</i> . Quantification was done by dot blot analysis using a monoclonal antibody (Arbitrary units)	4	[102]
	Overexpression of <i>arfA</i> in <i>A. niger</i>	Quantitative abundance of GlaA-dtomato reporter protein was 397.4 absolute fluorescence compared to 298.7 in control strain	1.3	[103]

- Protein degradation pathways—ERAD and Vacuole

Misfolded proteins that fail to be refolded properly through the UPR enter the ERAD pathway (Figure 1). They are transported from the ER to the cytoplasm, where they are ubiquitinated and degraded by the proteasome [105]. Disrupting key genes of the ERAD has been applied in order to study intracellular degradation of heterologous proteins and improve production yields in *Aspergillus* species (Table 8) [99,106,107]. Inactivation of *doaA*, which encodes a factor required for ubiquitin-mediated proteolysis, combined with induction of UPR-related genes (*sttC*) contributed to improved heterologous protein expression in *A. niger* [106].

Table 8. Approaches for improving recombinant protein production through engineering protein degradation pathways.

Process	Modification	Performance	Improvement Factor	Reference
Protein degradation pathways—ERAD and Vacuole	Deletion of <i>derA</i> and <i>derB</i> in <i>A. niger</i>	Δ derA: 80% decrease in <i>Tramete</i> laccase production	0.2	[99]
	-	Δ derB: 15.7% increase in <i>Tramete</i> laccase	1.15	
	Deletion of <i>doaA</i> and overexpression of <i>sttC</i> in <i>A. niger</i>	Higher GUS activity compared to parental strain (no quantitative data available)	-	[106]
	Disruption of <i>Aoops10</i> in <i>A. oryzae</i>	83.1 and 70.3 mg/L chymosin compared to 28.7 mg/L in parental strain	3–2.5	[108]
		22.6 and 24.6 mg/L human lysozyme compared to 11.1 mg/L in parental strain	2–2.2	
	Deletion of ERAD key genes (<i>derA</i> , <i>doaA</i> , <i>hrdC</i> , <i>mifA</i> and <i>mnsA</i>) in <i>A. niger</i>	Δ derA and Δ hrdC: 2-fold increase compared to parental strain (single-copy)	2	[107]
		Δ derA: 6-fold increase compared to parental strain (multi-copy) Relative amount of intracellular GlaGus (β -glucuronidase levels) fusion protein detected in total protein extracts of strains with impaired ERAD and respective parental strain	6	

Table 8. Cont.

Process	Modification	Performance	Improvement Factor	Reference
		Δ Aoatg1: 60 mg/L chymosin	2.3	
		Δ Aoatg13: 37 mg/L chymosin	1.4	
	Disruption of genes involved in autophagy in <i>A. oryzae</i>	Δ Aoatg4: 80 mg/L chymosin	3.1	[109]
		Δ Aoatg8: 66 mg/L chymosin	2.5	
		Δ Aoatg15: Not detectable	-	
		Control: 26 mg/L chymosin	-	

During the last steps of secretion additional protein quality control mechanisms can target aberrant proteins to degradation. During autophagy proteins are guided through vesicle trafficking to vacuoles, where they get degraded (Figure 1). Disruption of the autophagic process has been proposed as a way to enhance production of recombinant heterologous proteins (Table 8) [108,109]. For example, deleting the vacuolar protein sorting receptor gene *AoVPS* in *A. oryzae* resulted in increased extracellular production levels of chymosin and human lysozyme in *A. oryzae* by 3- and 2-fold, respectively [108].

Collectively, these studies suggest that the fungal secretory pathway offers multiple engineering targets and opens up a new perspective on developing hypersecreting *Aspergillus* strains for industrial applications.

3.4.2. Carrier Proteins

A commonly applied approach for successful secretion of foreign proteins in *Aspergillus* cultures is the use of native, well-secreted carrier proteins. Fusion of a carrier protein to heterologously produced proteins appears to improve mRNA stability [75] and facilitate proper folding and translocation through the fungal secretory pathway [110]. Multiple proteins of industrial and pharmaceutical relevance have been produced and efficiently secreted following this strategy. Chymosin, a protease used as a milk clotting agent in cheese manufacturing, was one of the first examples of a heterologous protein to be produced in *Aspergillus* cultures using the natively secreted glucoamylase A (GlaA) as a carrier protein [111,112] or just the GlaA signal peptide [113]. Since then, the advantages of using entire or parts of carrier proteins were exploited further for the production of human interleukin-6 [114], antibodies [115,116], and other commercially relevant proteins [40,117] in several *Aspergillus* species improving production yields (Table 9).

As GlaA is the most abundant and highly secreted enzyme in most *Aspergillus* species, it is a popular carrier choice for heterologous protein production [113,114,116,117]. However, other naturally secreted proteins, or their signal peptides, have also been used successfully for this purpose, e.g., the *A. oryzae* α -amylase [40,118,119] and signal peptides of an *A. niger* endoxylanase [120] (Table 9).

Table 9. Approaches for improving recombinant protein production through the use of carrier proteins.

Process	Modification	Performance	Improvement Factor	Reference
Carriers	Prochymosin sequence fused to <i>A. niger</i> GlaA signal peptide in <i>A. nidulans</i>	146 μ g/g dry weight chymosin compared to 93 μ g/g dry weight in the control strain	1.56	[113]
	Prochymosin sequence fused to codons for the GlaA signal peptide, propeptide, and 11 amino acids of mature glucoamylase in <i>A. nidulans</i>	119 μ g/g dry weight chymosin compared to 93 μ g/g dry weight in the control strain	1.27	[113]

Table 9. Cont.

Process	Modification	Performance	Improvement Factor	Reference
	Prochymosin sequence fused to GlaA signal peptide and propeptide in <i>A. nidulans</i>	23 µg/g dry weight compared to 93 µg/g dry weight in the control strain	0.24	[113]
	Prochymosin sequence fused after the last codon of <i>A. awamori</i> GlaA in <i>A. awamori</i>	140 µg/mL secreted chymosin compared to 8 µg/mL in the control strain (prochymosin + GlaA signal peptide)	17.5	[111,112]
	hLL6 fused to 1-514 nt of <i>A. niger</i> GlaA in <i>A. niger</i>	15 mg/L hLL6 compared to less than 1 µg/l in the control strain (hLL6 fused to the GlaA signal peptide)	>15	[114]
	<i>E. coli uidA</i> (13-glucuronidase) and the <i>T. lanuginosa</i> lipase fused to <i>A. niger</i> var. <i>awamori</i> endoxylanase II secretion signals	798 Arbitrary units of glucuronidase activity/mg of total protein (the control did not carry uidA)	-	[120]
	ScFv-LYS encoding fragments fused to <i>A. niger</i> propeptide + GlaA (514nt)	47.5 Arbitrary units of lipase activity compared to 47 Arbitrary units when the native lipase signal is used	1	
	ScFv-LYS encoding fragments fused to <i>A. niger</i> propeptide + GlaA (514nt)	90 mg/L ScFv-LYS compared to 2–22 mg/L in the control strains (18 aa GlaA signal sequence + ScFv-LYS)	4–45	[115]
	Human antibodies (κ- and γ-chain of IgG1) fused to GlaA in <i>A. niger</i>	0.9 g/l of trastuzumab IgG1 and 0.2 g/l of Hu1D10	-	[116]
	Neoculin gene fused to α-amylase in <i>A. oryzae</i>	1.3 mg/L of NCL	-	[40]
	Bovine chymosin gene fused to α-amylase in <i>A. oryzae</i>	42 mg/L chymosin compared to 20 mg/L in strains with non-fused gene	2.1	[119]
	Hemicellulose degrading enzymes sequences fused to GlaA secretion peptide in <i>A. nidulans</i> strains	50–100 mg/L xylanase B, xylanase C, xylosidase D, arabinofuranosidase B, ferulic acid esterase and arabinase	-	[117]

Regardless of the carrier protein or the signal peptide used, the heterologous protein needs to be detached from the protein fusion after secretion in order to gain full activity. An alternative practice to downstream in vitro treatment with proteases is to incorporate a protease cleavage site (e.g., Lys-Arg) between the native and the foreign protein, which can be proteolytically cleaved by fungal endoproteases (e.g., KEXB endoprotease in *A. niger*) during secretion [114,121,122]. Optimization of the sequence upstream the KEXB cleavage site appeared to increase Trastuzumab light chain production in *A. niger* [122].

3.5. Proteases

Proteolytic degradation by extracellular fungal proteases is one of the main reasons why secreted yields of heterologous proteins fail to reach the gram-per-liter production level of native proteins in *Aspergillus* species. Several bioprocessing strategies, such as maintaining high pH during fermentation [123] or low temperatures during downstream processing, separating the product from the protease-containing medium and using protease inhibitors, are often used to decrease protein degradation. However, proteolysis still occurs, making production of foreign proteins inefficient [124].

An alternative and more efficient approach is the use of protease-deficient strains [124]. Conventional mutagenesis and genetic engineering were applied to several *Aspergillus* species

in order to disrupt genes that encode extracellular proteases, e.g., aspergillopepsin A (pepA) [125,126] or protease regulatory genes [126,127] (Table 10). The specific mutants exhibit reduced extracellular proteolytic activity and often appear to be more efficient producers of heterologous proteins than the wild types [124]. Deletion of *pepA* in *A. awamori* resulted in an aspergillopepsin A-deficient mutant, with decreased proteolytic activity [125] and able to produce higher levels of bovine chymosin (~430 mg/L), when compared to the control strain (*A. awamori* strain GC12- Δ argB3, Δ pyrG5: ~180 mg/L) [128]. *A. niger* mutants lacking the transcription factor PrtT, which regulates expression of both aspergillopepsin A and B genes (*pepA* and *pepB*) [129], showed only 1–2% of the parental strain extracellular protease activity [126] and were used to produce highly stable heterologous cutinase with 1.7-fold increased activity [127].

Table 10. Approaches for improving recombinant protein production through disruption of protease genes.

Process	Modification	Performance	Improvement Factor	Reference
Proteases	Deletion of <i>pepA</i> in <i>A. awamori</i> strains	Decreased extracellular proteolytic activity compared to the wild type (immunoassay using antibodies specific for PepA, but absolute values for PepA concentration were not determined)	-	[125]
	Deletion of <i>pepA</i> in <i>A. awamori</i>	430 mg/L of chymosin compared to 180 mg/L in the parental strain	2.4	[128]
	Deletion of <i>pepA</i> in <i>A. niger</i> (AB1.1)	15–20% proteolytic activity compared to the parent strain AB4.1	-	[126]
	Mutation on <i>prtT</i> (UV irradiation) in <i>A. niger</i> (AB1.13)	1–2% proteolytic activity compared to the parent strain AB4.1	-	[126]
Deletion of <i>prtR</i> , <i>pepA</i> , <i>cpl</i> , <i>tppA</i> in <i>A. oryzae</i>	Δ prtR/ <i>pepA</i> / <i>cpl</i> : 24.23 mg/L of <i>Acremonium cellulolyticus</i> cellobiohydrolase		1.2	[133]
	Δ prtR/ <i>pepA</i> / <i>tppA</i> : 21.30 mg/L		1.1	
	Δ prtR/ <i>cpl</i> / <i>tppA</i> : 22.08 mg/L		1.1	
	Δ prtR/ <i>pepA</i> / <i>cpl</i> / <i>tppA</i> : 19.93 mg/L compared to 19.54 mg/L in the control strains		1.02	
Deletion of <i>alp</i> and <i>Npl</i> in <i>A. oryzae</i>	1041 U/g of <i>Candida antarctica</i> lipase B compared to 575 U/g in the parental strains		1.8	[132]
Deletion of various proteases in <i>A. niger</i>	Δ dpp4: 6% increase in <i>Tramete</i> laccase		1.1	[99]
	Δ dpp5: 15.4% increase		1.2	
	Δ pepB: 8.6% increase		1.1	
	Δ pepD: 4.8% increase		1.0	
	Δ pepF: 5.3% increase		1.1	
	Δ pepAa: 0.5% increase		1.1	
	Δ pepAb: 13.4% increase		1.1	
	Δ pepAd: 2.7% increase		1.0	
	Δ dpp4/ <i>dpp5</i> : 26.6% increase		1.3	
Disruption of <i>tppA</i> and <i>pepE</i> in <i>A. oryzae</i> strains	25.4 mg/L of human lysozyme compared to 15 mg/L in the parental strains		1.7	[118]
Disruption of <i>tppA</i> , <i>pepE</i> , <i>nptB</i> , <i>dppIV</i> and <i>dppV</i> in <i>A. oryzae</i>	84.4 mg/L of chymosin compared to the 63.1 mg/L in the double protease gene disruptant (Δ tppA/ <i>pepE</i>)		1.3	[130]
Disruption of <i>tppA</i> , <i>pepE</i> , <i>nptB</i> , <i>dppIV</i> , and <i>dppV</i> , <i>alpA</i> , <i>pepA</i> , <i>AoepAa</i> , <i>AoepAd</i> and <i>cpl</i> in <i>A. oryzae</i>	109.4 mg/L of chymosin and 35.8 mg/L of human lysozyme compared to the quintuple protease gene disruptant (Δ tppA/ <i>pepE</i> / <i>nptB</i> / <i>dppIV</i> / <i>dppV</i> ; 84.4 mg/L and 26.5 mg/L, respectively)		1.3 and 1.35	[131]

Table 10. Cont.

Process	Modification	Performance	Improvement Factor	Reference
		36.3–36.7 U/mL of mL <i>G. cingulate</i> cutinase compared to 21.2–20.4 U/mL in the parental strain	1.7	
	Deletion of <i>prtT</i> in <i>A. niger</i>	Stability: Cutinase activity retained at 80% over the entire 14-day incubation period, while the parental lost more than 50% of their initial activities after six days of incubation and retained negligible activity after 14 days	-	[127]
	Deletion of <i>dppV</i> and <i>pepA</i> in <i>A. nidulans</i>	<i>P. sanguineus</i> laccase activity 500,000 U/mL compared to 40,000 U/mL in the control strain	12.5	[51]
	Deletion of <i>mmn9</i> and <i>pepA</i> in <i>A. nidulans</i>	<i>P. sanguineus</i> laccase activity 300,000 U/mL compared to 40,000 U/mL in the control strain	7.5	[51]

Research on *Aspergillus* protease repertoire and development of molecular tools for multiple gene targeting allowed the disruption of multiple protease-related genes in a single production host, a successful tactic to further decrease proteolytic degradation and therefore improve protein production titers (Table 10) [51,118,130,131]. Disruption of two protease genes in *A. oryzae* (*tppA* and *pepE*) resulted in production of 25.4 mg/L of human lysozyme (HLY), which represents a 63% increase in production yields compared to the control strain [118]. Subsequently, *A. oryzae* quintuple and decuple protease gene disruptants produced even higher HLY amounts (26.5 mg/L and 35.8 mg/L, respectively) [130,131]. Although multiple protease-related gene disruptions appear to be a time-consuming and tedious procedure [127], it is a strategy commonly used for the optimization of heterologous protein production in several *Aspergillus* systems of industrial interest [99,132,133].

3.6. Altering Fungal Morphology Using Genetic Engineering

Protein secretion in filamentous fungi has been shown to happen mostly at the tip of growing hypha, thus hyperbranched phenotypes are more desirable when developing a protein production platform. Additionally, combining a hyperbranched phenotype with shortened mycelia may result in reduced culture viscosity, which is beneficial for high density submerged fermentation [134,135].

Multiple studies have focused on the effect of morphology on protein production in aspergilli. Genetic engineering attempts [102,103] (Table 11) or variation of fermentation parameters (see also Section 4.2.: Fungal morphology and bioprocessing) have been employed to obtain hyperbranching strains that can secrete large amounts of proteins. In *A. niger* deleting the gene that encodes the Rho-GTPase *RacA*, which mediates actin polymerization and depolymerisation at the hyphal apex, generated a strain producing 20% more hyphal tips. Under continuous high-level expression, this hyperbranching strain produced four times more glucoamylase compared to its parental strain [102].

Table 11. Approaches for improving recombinant protein production through engineering genes involved in fungal morphology.

Process	Modification	Performance	Improvement Factor	Reference
Fungal morphology	Deletion of <i>racA</i> in <i>A. niger</i>	<i>GlaA</i> secreted into the culture medium is four times more compared to its parental strain, when ensuring continuous high-level expression of <i>glaA</i> . Quantification was done by dot blot analysis using a monoclonal antibody (Arbitrary units)	4	[102]
	Overexpression of <i>arfA</i> in <i>A. niger</i>	Quantitative abundance of <i>GlaA</i> -dtomato reporter protein was 397.4 absolute fluorescence compared to 298.7 in control strain	1.3	[103]

4. Fermentation Conditions for Improved Heterologous Production in *Aspergillus*

Development of most heterologous expression platforms begins with strain improvement, which hopefully results in obtaining strains able to produce large quantities of a specific protein. Once strain improvement is complete, the fermentation process for production of the desirable protein in large-scale has to be established [7,136]. Designing and setting up fungal fermentations is a complex process that has to be repeated every time a newly engineered strain is used or a new protein is to be produced. This process requires several optimization steps, starting from finding the optimal growth medium and fermentation parameters (temperature, pH, and oxygenation) to choosing the appropriate type of fermentation and the fungal morphology that favors high production yields of the specific protein [137–139].

4.1. Fermentation Conditions

Multiple strategies have been applied to optimize fermentation conditions for improving recombinant protein production in *Aspergillus* cultures (Table 12). Several studies have focused on the effect of growth medium and culture conditions on protein production [140–145]. MacKenzie et al. (1994) studied the effect that temperature and growth medium have on the production of hen eggwhite lysozyme (HEWL) in *A. niger* cultures. When a standard expression medium (1% *w/v* soluble starch and 50 mM sodium phosphate buffer) was used, 20–25 °C was the optimal temperature range to obtain lysozyme in previously observed levels (8–10 mg/L). In addition, as the HEWL gene was under the control of the *glaA* promoter, production of lysozyme was highly induced when soluble starch was used as carbon source. Using a richer medium with soy milk led to even higher yields of up to 30–60 mg/L lysozyme, but interestingly growth temperature was adjusted to 37 °C to achieve these levels [141]. In another attempt, the impact of the nitrogen source was evaluated [143]. Swift et al. (2000) showed that glucoamylase production was increased by 115% with the addition of casamino acids, yeast extract, peptone, and gelatin in cultures of a recombinant *A. niger* strain, compared to non-supplemented cultures [143].

Table 12. Approaches for improving recombinant protein production through bioprocessing modifications.

Process	Modification	Performance	Improvement Factor	Reference
Fermentation conditions	Effect of growth medium and temperature on hen egg white lysozyme (HEWL) production in <i>A. niger</i>	20–25 °C 8–10 mg/L HEWL while 30–37 °C 3–5 mg/L HEWL	Temperature: 2–2.6	[141]
		soluble starch: 8.0 mg/L HEWL	Carbon source: 1.7–2	
		maltose: 4.5 mg/L HEWL	-	
		glucose: 4.0 mg/L HEWL	-	
		xylose: 0.2 mg/L HEWL	-	
	soy milk medium: 30–60 mg/L HEWL	Rich medium: 3.8–7.5		
	Effect of organic nitrogen sources on recombinant glucoamylase production in <i>A. niger</i>	Unsupplemented: 44 mg glucoamylase/g biomass	-	[143]
		L-alanine: 32 mg glucoamylase/g biomass	0.7	
		L-methionine: 26 mg glucoamylase/g	0.6	
		casamino acids, yeast extract, peptone, and gelatin: 100 mg glucoamylase/g	2.2	
	Effect of agitation intensity on recombinant amyloglucosidase (AMG) production in <i>A. oryzae</i>	Titer at the end of the batch phase	-	[146]
		525 rpm: 110 U/L AMG		
		675 rpm: 230 U/L AMG	1.6	
		825 rpm: 370 U/L AMG	3.3	

Table 12. Cont.

Process	Modification	Performance	Improvement Factor	Reference
	Effects of bioprocess parameters—agitation intensity, initial glucose concentration, initial yeast extract concentration, and dissolved oxygen tension (DO)—on heterologous protein production in <i>A. oryzae</i>	Highest GFP yields were achieved under these conditions: agitation 400 rpm, glucose 25 g/L, yeast extract 0 g/dm ³ , DO 15%	-	[142]
	Effect of agitation intensity on recombinant glucose oxidase production in <i>A. niger</i>	200 rpm: 300 mkat/L of glucose oxidase	-	[144]
		500 rpm: 800 mkat/L of glucose oxidase	2.6	
		800 rpm: 600 mkat/L of glucose oxidase	1.3	
	Effect of temperature on <i>Pleurotus eryngii</i> versatile peroxidase production in <i>A. nidulans</i> and <i>A. niger</i>	- <i>A. nidulans</i> 31 °C: 24 U/L peroxidase activity	-	[145]
		28 °C: 80 U/L peroxidase activity	3.3	
		19 °C: 466 U/L peroxidase activity	19.4	
		- <i>A. niger</i> 28 °C: 107 U/L peroxidase activity	-	
		19 °C: 412 U/L peroxidase activity	3.8	
Fungal morphology	Effect of raising the viscosity of the medium by addition of polyvinylpyrrolidone-PVP (transition from aggregated mycelia (pellets) to dispersed mycelia) on hen egg white lysozyme (HEWL) in <i>A. niger</i>	Medium with no PVP: 110 mg/L fresh and 8 mg/g dry weight of HEWL	1.7	[147]
		Medium with PVP: 190 mg/L fresh and 14 mg/g dry weight of HEWL		
	Effect of addition of microparticles (linked to the formation of freely dispersed mycelium) on titers of native glucoamylase (GlaA) and recombinant fructofuranosidase (FF) produced in <i>A. niger</i>	No microparticles: 17 U/mL GlaA and 42 U/mL FF	3.5 GlaA 2–3.8 FF	[148]
		Talc microparticles: 61 U/mL GlaA and 92 U/mL FF FF production can reach up to 160 U/mL (10 g/L talc microparticles of size 6 mm)		
	Effect of addition of titanate microparticles (TiSiO ₄ , 8 mm) on titers of native glucoamylase (GlaA) and recombinant fructofuranosidase (FF) produced in <i>A. niger</i>	No microparticles: 19 U/mL GlaA and 40 U/mL FF	9.5 GlaA 3.7 FF	[149]
		Microparticles: 190 U/mL glucoamylase and 150 U/mL fructofuranosidase		
	Effect of growth type on hen egg white lysozyme (HEWL) production and protease activity in <i>A. niger</i>	Free suspension: 5.8 mg/g HEWL 95.3 U/g Protease activity	1.5	[140]
		Mycelial pellets: 5.0 mg/g HEWL 58.6 U/g Protease activity	1.2	
		Celite-560-immobilized cultures: 4.1 mg/g HEWL 56.3 U/g Protease activity	-	

Additional bioprocess parameters such as agitation intensity, initial nutrient concentration and dissolved oxygen levels were also studied with regard to heterologous protein production in different *Aspergillus* fermentation types [142,146].

4.2. Altering Fungal Morphology Using Bioprocessing

Filamentous fungi that grow in submerged cultures, including aspergilli, exhibit variable morphologies, which can affect the overall performance of the microorganism during fermentation. The commonly observed filamentous growth results in undesirable viscous cultures, which require

high consumption of energy for agitation and usually complicate downstream processing. On the contrary, a pelleted morphology (growth in pellets) decreases viscosity of the culture, improves mixing, and facilitates downstream processing. However, when large pellets are formed in the culture, diffusion of oxygen and nutrients to the inner pellet core is hindered, resulting in reduced productivity [135].

Many studies have attempted to correlate high protein production yields to a specific type of fungal morphology. For *A. niger*, dispersed mycelial suspensions led to higher protein yields over the pelleted form [140] (Table 12). In fact, microparticles are often added into *Aspergillus* cultures in order to prevent formation of large pellets and to favor formation of disperse mycelium, and consequently recombinant protein production [144,147–149]. However, Gyamerah et al. (2002) showed that the free suspension culture presented a higher protease activity, compared to the cultures with immobilized fungal biomass (mycelial pellet or by entrapment in Celite beads), and this could be an additional limitation for recombinant protein production [140].

5. Conclusions and Future Perspectives

Filamentous fungi hold unlimited potential for industrial applications, from the development of meat-like products and biomaterials, to bioremediation and biofuel production. One of their best qualities, largely exploited by the industry, is their innate capacity for the secretion of enzymes, which facilitate downstream processing and product recovery. Moreover, their ability to produce complex proteins with post-translational modifications and the fact that they can be cultivated on inexpensive media makes them a promising alternative for production of eukaryotic proteins. Despite their undeniable potential though, filamentous fungi have not yet been exploited to the fullest in the industrial production of recombinant proteins.

Advances in the molecular toolkit available for genetic manipulation of several *Aspergillus* species opened up the path for developing them into production systems for recombinant proteins. Nevertheless, due to a number of factors described in the review, aspergilli have not yet met the expected production levels. Many studies that focused on engineering different steps of protein synthesis and secretion, or generating protease-deficient strains, have resulted in a significant increase of protein yields. Additionally, optimization of the fungal fermentation process has further improved protein production. However, there are aspects of the fungal physiology that limit protein production and remain unclear. Continuous data input from “omics” studies sheds light on the complex fungal mechanisms related to protein quality control and secretion stress, as well as their impact on protein productivity. The knowledge generated from these studies combined with advances in the field of synthetic biology will soon place *Aspergillus*, and possibly other filamentous fungi, in the race for the most efficient recombinant protein production system. Its potential as a large-scale production platform not only for recombinant proteins, but also for organic acids, bioactive compounds, enzymes, and peptides, as well as new perspectives related to the use of *Aspergillus* in waste treatment and bioremediation processes, prove that this fungus can provide sustainable solutions for multiple and diverse markets and industries.

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