Aspergillus

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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

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

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].

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	Relev Litera
Bacteria (*Escherichia coli*)	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	[6][7]

Yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*, etc.)	Simple	Fast	*S. cerevisiae* limited *P. pastoris* higher	Hypermannosylation of glycoproteins often occurs (shortens half-life of the protein in vivo, leads to immunogenic reactions)	Feasible	Low	Low	[8][9]
Filamentous fungi (*Aspergillus niger*, *Trichoderma reesei*, *Neurospora crassa*)	Feasible	Medium	High	Less hypermannosylation compared to yeasts, but still differences from mammalian glycosylation patterns	Simple	Medium (mycotoxins)	Low	[10]
Insect cells (*Spodoptera frugiperda*, *Drosophila melanogaster*)	Laborious	Fast	High	Not able to carry out *N*-glycosylation	Feasible	Very low	High	[11]
Mammalian cells (CHO cells, Human cell lines)	Laborious	Slow	Low	High quality therapeutic proteins, human-like glycosylation pattern	Simple	High (viruses and prions)	High	[12]
Transgenic animals (goats, chickens)	Laborious	Very slow	High	High quality therapeutic proteins	Simple	High (viruses and prions)	High, ethically questionable	[13]
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	[14][15]

For the production of non-medicinal proteins, a more economical approach is usually followed, using either bacterial or fungal production hosts [1][16][10][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* [17]. However, yeasts have the tendency to hyperglycosylate secreted proteins, and thus reduce their in vivo half-life and affect their efficacy [8]. 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 [9]. 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 [18]. Strains belonging to the genera Aspergillus, Trichoderma, and

Neurospora are in fact widely used for production of recombinant proteins with industrial applications [19][20][21][22]. 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 [10][23][24]. 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.

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 [18]. 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][33][34][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].

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 [10][42]. 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 [43][44][45].

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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