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Review

Anticancer Asparaginases: Perspectives in Using Filamentous Fungi as Cell Factories

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Abstract: The enzyme L-asparaginase (L-asparagine amidohydrolase) catalyzes the breakdown of L-asparagine into aspartate and ammonia, which leads to an anti-neoplastic activity stemming from its capacity to deplete L-asparagine concentrations in the bloodstream, and it is therefore used in cases of acute lymphoblastic leukemia (ALL) to inhibit malignant cell growth. Nowadays, this anti-cancer enzyme, largely produced by *Escherichia coli*, is well established on the market. However, *E. coli* L-asparaginase therapy has side effects such as anaphylaxis, coagulation abnormality, low plasma half-life, hepatotoxicity, pancreatitis, protease action, hyperglycemia, and cerebral dysfunction. This review provides a perspective on the use of filamentous fungi as alternative cell factories for L-asparaginase production. Filamentous fungi, such as various Aspergillus species, have superior protein secretion capacity compared to yeast and bacteria and studies show their potential for the future production of proteins with humanized N-linked glycans. This article explores the past and present applications of this important enzyme and discusses the prospects for using filamentous fungi to produce safe eukaryotic asparaginases with high production yields.

Keywords: cell factory; L-asparaginase; filamentous fungi; anti-neoplastic; biopharmaceutical

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1. Introduction

L-asparagine amidohydrolase (E.C. 3.5.1.1) catalyzes the breakdown of L-asparagine into L-aspartate and ammonia and has been found in microorganisms, plants, and vertebrates. Interestingly, L-asparaginases have been identified as monomeric, dimeric, and hexameric proteins, although the tetrameric protein is the most common [1]. The hydrolysis of L-asparagine is catalyzed in two steps via a covalent β-acyl-enzyme intermediate, formed by nucleophilic attack on the amino acid. In terms of L-asparaginase production for anticancer purposes, most studies focus on bacteria, which makes them a reference point for further investigations into other sources of the enzyme and production platforms. Asparaginases originating from Gram-negative bacteria are divided into two types: Type I, which uses both L-asparagine and L-glutamine as substrates, and Type II, which presents higher substrate specificity towards L-asparagine and is the one used in anticancer therapy [2].

The anti-neoplastic activity of L-asparaginase stems from its capacity to deplete L-asparagine concentrations in the bloodstream, and it is therefore used in cases of acute lymphoblastic leukemia for inhibiting malignant cell growth. Acute lymphoblastic leukemia, which is mostly found in children between the ages of two and ten, affected more than 64 thousand people globally in 2017 [3]. While healthy cells produce L-asparagine from aspartate by the action of asparagine synthetase, malignant cells either lack or have small amounts of this enzyme, which makes them dependent on external sources of L-asparagine. When exogenous L-asparagine is removed from the bloodstream, lymphoblastic cell growth

is impaired due to protein synthesis complications, while normal cells have higher chances of survival [4]. Currently, there are four commercially available types of L-asparaginase for ALL treatment, namely, a native L-asparaginase from *Escherichia coli*, a PEGylated version from *E. coli*, a native L-asparaginase from *Dickeya dadantii* (previously known as *Erwinia chrysanthemi*), and a *D. dadantii* L-asparaginase produced from Pseudomonas fluorescens [5,6]. However, *E. coli* L-asparaginase therapy has side effects such as anaphylaxis, coagulation abnormality, low plasma half-life, hepatotoxicity, pancreatitis, protease action, hyperglycemia, and cerebral dysfunction. These side effects are due to either the production of anti-asparaginase antibodies in the body or the L-glutaminase activity of L-asparaginase [7,8].

L-asparaginases have also been used in the food industry for acrylamide mitigation in fried foods such as potatoes, as acrylamide is a potential human carcinogen [9,10]. Some commercial L-asparaginases used in the food industry are Acrylaway® (Novozymes, Gladsaxe, Denmark) and PreventASe® (DSM, Heerlen, The Netherlands). Interestingly, some of these L-asparaginases used in the food sector are produced in Aspergilli, which is the case for Acrylaway, a preparation from *Aspergillus oryzae*. This is a relevant piece of information in the context of the promise of using fungal L-asparaginases for cancer treatment. More specifically, far from implying that the current commercial fungal preparations are ideal candidates, the fact that these organisms have large-scale processes implemented for L-asparaginase production supports the possibility of using filamentous fungi as cell factories for this purpose. However, the screening of a good L-asparaginase candidate with the possibility of protein engineering is very encouraging.

In this article, we explore the possibility of using filamentous fungi as an alternative and superior secretion chassis for anticancer L-asparaginases by reviewing the most recent advances in fungal strains and protein engineering. We expect to shed light on the use of filamentous fungi as future hosts for biopharmaceutical production.

2. Filamentous Fungi as L-Asparaginase Production Sources

L-asparaginase is largely found in microorganisms [11], primarily in the bacterial kingdom. It is well stabilized on the market and largely produced from *Escherichia coli* for its anti-tumor activity [12]. However, *E. coli* L-asparaginase therapy has side effects due to the production of anti-asparaginase antibodies or L-glutaminase activity of L-asparaginases (a secondary enzyme activity) [7,8]. The second biggest group of deposited protein sequences is those of yeasts and filamentous fungi, according to the National Center for Biotechnology Information [6], permitting a new horizon of choices among different enzyme sources.

The main potential benefit of using eukaryotic microorganisms, such as filamentous fungi, is not only the possibility of producing humanized L-asparaginase, but also their superior protein secretion capacity, protein stability, and productivity [5,9]. In fact, L-asparaginase production is allocated in two phases: upstream and downstream processing. In this context, the choice of the cell line is imperative once it can affect the bioprocess robustness, mainly the initial recovery, purification, and final polishing steps. Using *Escherichia coli* and *Erwinia chrysanthemi* as intracellular sources of L-asparaginase in the downstream process will be harder due to the use of disruption cell procedures and the co-release of vast intracellular components (HCP: host cell protein, nucleic acids, and aggregates, among others) together with the intended biomolecule. Costa-Silva et al. evaluated several disruption cell methods to release intracellular L-asparaginase from bacteria and yeast [13]. In all cases, the best techniques were mechanical disintegration methods: sonication and glass bead stirring. These methods are not suitable for industrial scale and can cause temperature increase and therefore require an efficient cooling system. Depending on the commercial L-asparaginases source, the purification process is carried out through at least five steps: homogenization, ammonium sulfate precipitation, anion exchange chromatography, cation exchange chromatography, hydrophobic interaction chromatography, and ultrafiltration [14]. The downstream processing is typically associated with the high production costs (60–80%) of enzymes used as therapeutic drugs [15]. Therefore, the best scenario is

the extracellular production system since the enzyme is easier to purify and less costly to obtain in industrial-scale production. Other important features of filamentous fungi are the ease of their genetic engineering and of structural modeling applied to protein folding. However, it is important to highlight that the choice of the correct genus among L-asparaginase producing fungi is important as some may produce mycotoxins in their secondary metabolism with toxic effects for humans [16].

In addition to the microbial system applied, enzyme production is related to cultivation parameters such as pH, temperature, oxygen level, culture media (carbon and nitrogen sources), and others. In the case of L-asparaginase production using filamentous fungi, both submerged and solid-state cultivation has been used. However, the modified Czapek–Dox Modified Medium supplemented with glucose and one amino acid (L-asparagine or L-proline) is the most used media composition. It is recommended that special attention be paid to the fact that the metabolism of L-asparaginase production from filamentous fungi is regulated by nitrogen sources. In fact, the influence of nitrogen on filamentous fungi metabolism regulation has been deeply evaluated. It has been shown that the GATA factor switches the structural gene expression since the activators ARE-A and NIT-2 control the gene transcription according to the presence/absence of nitrogen [17]. In this context, the cultural conditions of L-asparaginase production using *Aspergillus terreus* were studied [2]. The authors showed the importance of separating the phases of biomass production and enzyme synthesis. For the biomass production, a modified Czapek–Dox medium containing glucose (14 $g \cdot L^{-1}$), L-proline (10 $g \cdot L^{-1}$), and ammonium nitrate (2 $g \cdot L^{-1}$) was used, while for the L-asparaginase production, the microbial cells were recovered and inoculated in the new media composed of a modified Czapek–Dox medium containing glucose (2 $g \cdot L^{-1}$) and L-proline (10 $g \cdot L^{-1}$). In this new medium, the concentration of glucose was reduced and the nitrogen inorganic source (an easily assimilated nitrogen source) was removed, leading to nitrogen starvation that resulted in ammonium depression [2]. They used nitrogen-free conditions to ensure the activation of secondary nitrogen source-degrading pathways (proline assimilation) and consequently obtained high enzyme yields.

More adapted production methods must be used to achieve the best extraction process, since the construction of the strain for the engineering of N-glycosylation and secretory pathway, including several factors such as pH (hydrogenionic potential), temperature, aeration, water and moisture activity, and quality and type of substrate, will lead to an optimization of L-asparaginase production to achieve an industrial scale.

Among the fungi producing L-asparaginase, the genera *Aspergillus*, *Bipolaris*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma* [1,4,10,18–21] are the object of the leading research efforts and are displayed in Table 1 below.

Table 1. L-asparaginases production by filamentous fungi: cultivation conditions and biochemical parameters.

Fungal Source	Carbon Source	Nitrogen Source	Production Time (h)	Optimal Temperature (°C)	Optimal pH	Km (mM)	Vmax (U/mL)	Enzyme Activity	In Vitro Trial	Authors
Aspergillus niger LBA 02	Passion fruit peel flour	Passion fruit peel flour	24	25	8	-	-	3023.98 U/g	No	[4]
Bipolaris sp. BR438	Glucose	Sodium nitrate	72	27–30	8.5	-	-	6.2 U/mL	No	[18]
Aspergillus candidus IR-A4	Asparagine	Asparagine	120	35–45	8.5± 0.5	-	-	17.26 U/mg	Cytotoxic assay using HL-60 cell lines	[3]
Aspergillus tamarii	Sucrose	Asparagine	168	30	7	-	-	11.01 U/mL	No	[22]
Aspergillus oryzae sp IOC 3999	Lactose	Asparagine/ Hydrolyzed casein	4	30	5	3.28	45.04	742.22 U/mg	No	[23]
Aspergillus terreus CCT7693	Glucose	Proline	7	37.2	8.56	2.42	-	13.81 U/mL	Cytotoxic assay using HL-60 cell lines	[24]
Penicillium sp.	Wheat Bran and sucrose	Wheat bran and Asparagine	72	35	5	-	-	2.33 U/mL	No	[24]
Fusarium equiseti AHMF4	Glucose	Sodium nitrate	16	30	7	-	-	488.1 U/mg	Cytotoxic assay using Hela, Hep-2, HepG-2 and HCT-116 cell lines	[19]
Cladosporium tenuissimum	Glucose	Asparagine	72	37	6.2	-	-	2.6471 U/mL	No	[10]
Mucor hiemalis	Maltose	Peptone	72	37	7	4.3	625	825.4 U/mL	No	[21]
Aspergillus tamarii	Glucose	Proline	72	30	-	-	-	38 U/L	No	[25]
Aspergillus terreus S-18	Glucose	Proline	120	30	8.5	-	-	3.3 U/g	No	[26]
Aspergillus sydowii	Glucose	Asparagine	120	4	-	-	-	146 U/mL	Cytotoxic assay using murine RAW264.7 leukemia cells line	[27]
Fusarium oxysporum	Glucose	Asparagine	120	40	-	-	-	143 U/mL	Cytotoxic assay using murine RAW264.7 leukemia cells line	[27]
Penicillium cyclopium	Glucose	Proline	72	30	-	0.3	625	39480 U/mg	Cytotoxic assay using Hep-G2, MCF-7 and PC3 cell lines	[28]
Fusarium culmorum	Glucose	Soybean meal	144	30	-		-	18.91 U/gds	No	[29]
Penicillium digitatum	Glucose	Asparagine	96	30	-		-	833.15 U/mg	No	[30]
Talaromyces cf. *cecidicola*	Glucose	Asparagine	96	30	-		-	2.30 U/g	-	[31]

Different types of filamentous fungi were studied and screened in the search for the one with the highest protein-making potential. *Aspergillus sp.* is the main focus of research thanks to its natural high production when used together with correct optimal medium sources for its growth, and the carbon and nitrogen sources are of central importance to extracellular L-asparaginase [21].

Currently, the objective is to transform those fungi by genetic engineering into something near the actual bacterial industrial production scale, not only to have a comparable amount of enzyme production but to create a safer and more humanized medication.

The ideal candidate for the therapeutic drug to be used in the treatment of patients with acute lymphoblastic leukemia needs to show some special features: a low K_M, a high specific activity, low or no glutaminase activity, low immunogenicity and human blood stability (resistance to main human proteases that recognizes and degrades the L-asparaginase: Cathepsin B (CTSB) and asparaginase endopeptidase (AEP)) [14]. Unfortunately, most of the L-asparaginases produced by filamentous fungi are not characterized by these biochemical/kinetics parameters, especially the substrate affinity (Michaelis constant K_M), and therefore, the viability use of these enzymes as biopharmaceutical cannot be evaluated.

Furthermore, the measurement of enzyme activity and the anti-cancer properties must be comparable to those already well established on the market, and a treatment must be created with higher immune acceptance and fewer adverse effects [14]. To achieve these standards, the enzyme tests must go beyond merely quantifying their capability to transform asparagine into ammonia and test with tumorous cells in vitro. There are several reports about new L-asparaginases that are considered promising substitutes of the commercial enzymes currently used in the ALL treatment based just on the results of cytotoxic studies using leukemic cell lines in vitro. In fact, even an L-asparaginase with a high K_M value and low specific enzyme activity can show cytotoxic activity in in vitro conditions (closed system). However, it might be necessary for the screening or engineering of L-asparaginases with high K_M and K_{cat} values to reach similar or superior therapeutic effects to the commercial L-asparaginases. It is important to mention that even with superior kinetic properties, the side effects of fungal L-asparaginases must be deeply investigated, including treatment resistance. Therefore, a careful analysis of the biochemical/kinetic parameters should always be performed before proposing a substitute L-asparaginase for the ones currently used in the market.

3. Fungal Cell Factory Construction for Enzyme Production

The production of industrial enzymes relies on two important points, production yields and how straightforward the downstream processes are. Filamentous fungi are among the most interesting organisms for enzyme production due to their highly efficient secretory pathways and capability to perform post-translational modifications, including protein glycosylation and disulfide bond formation [32]. In addition, species such as *Aspergillus niger* and *Trichoderma reesei* can reach tens of grams of protein per liter [33,34]. This potential, added to the number of L-asparaginase genes in several filamentous fungi genomes, enables them to be engineered and explored for the production of this anticancer enzyme. Below, we will show some strategies to improve protein production without focusing on the enzyme, as there is a lack in the literature on engineering strains of filamentous fungi for recombinant L-asparaginase production.

For a recombinant protein to be highly secreted, the first important feature is high gene expression levels of the desired proteins, followed by the correct protein translation, folding, further processing, and exocytosis [32]. After translation in the ribosome, the signal peptide is recognized by the signal recognition particle (SNP), which targets the protein to the endoplasmic reticulum (ER), releasing the signal peptide by signal peptidases. In the ER lumen, the proteins are folded by the actions of chaperones and foldases, such as BipA and heat shock proteins, following or simultaneous with the addition of N-glycans. The presence of such glycans has the role of signalizing in the ER quality control of the correct protein folding performed by the calnexin cycle. Once the protein is correctly

folded, it is transported through vesicles to the Golgi apparatus, where it passes through further modifications, including N-glycan modifications and protein cleavage. The protein is exported from the Golgi to the cell membrane through exocytosis (Figure 1) [35].

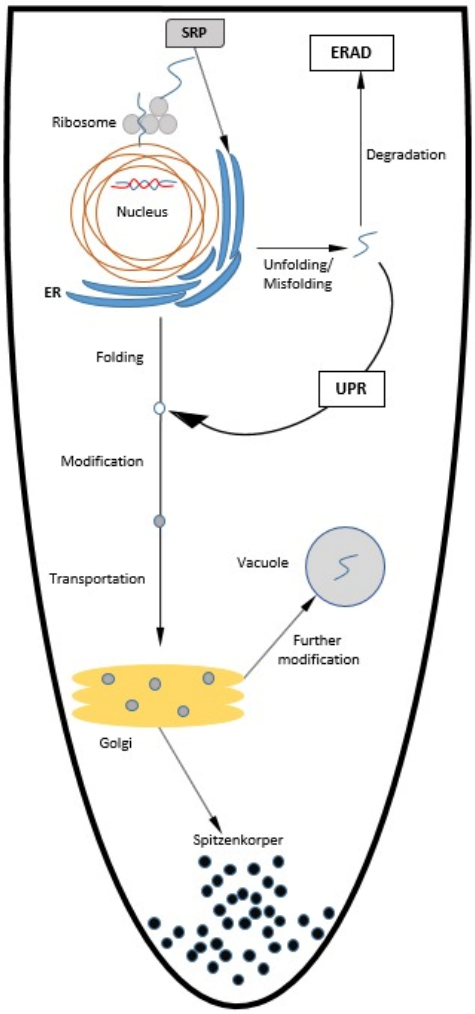

Figure 1. Secretory pathway of filamentous fungi. The main pathways and bottlenecks are illustrated. ER—endoplasmic reticulum; ERAD—endoplasmic reticulum-associated degradation; SRP—signal recognition particle; UPR—unfolded protein response.

An efficient way to rationally engineer strains of filamentous fungi is the disruption of the non-homologous end-joining (NHEJ) pathway to avoid multiple gene integrations in non-desired loci. This has been efficiently achieved by the deletion of *ku70*, a gene involved in NHEJ, resulting in strains more prone to performing homologous recombination [36], which enables precise control of genome modifications. Filamentous fungi can currently be more rapidly engineered due to the implementation of state-of-the-art gene-editing technologies, such as CRISPR/Cas9 [37]. With this technique, gene deletions, insertions, and point mutations can easily be performed [38].

The classic first approach to increase the level of recombinant proteins to be secreted is to increase the recombinant gene expression levels. This can be achieved by the use of strong promoters, e.g., amylase promoters [39]. However, high gene-expression levels can result in high demands in the ER of protein folding [40], often disrupting ER homeostasis an resulting in stress in the organelle where misfolded proteins are accumulated, triggering a feedback mechanism termed unfolded protein response (UPR) [41]. In the ER, in the case of high levels of unfolded proteins, BipA, an ER-resident Hsp70 chaperone, is dissociated from IreA, resulting in IreA oligomerization and trans-autophosphorylation. Consequently,

IreA conformation is changed, resulting in an RNase domain that has *hacA*u (uninduced) as its substrate, removing an unconventional intron and producing the *hacA*i (induced) mRNA. The bZIP transcription factor HacA then produces binding to cis-acting elements present in the promoters of UPR-target genes, known as UPR elements, restoring ER homeostasis [42].

Most of the studies on fungal strain engineering of industrial enzymes are focused on *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*, although the latter is mainly used for cellulase production. There are several general approaches that can be applied to engineer fungal secretory pathways, resulting in improved production, which can be investigated for homologous or heterologous L-asparaginase production by filamentous fungi. A very efficient strategy reported in the literature is the deletion of transcription factors that in different ways can benefit enzyme production and application. Zhang et al., 2016, knocked out *amyR* in *A. niger* and observed a cleaner secretome that can benefit recombinant enzyme production [43]. As *amyR* is involved in amylolytic enzyme gene regulation and is induced when the strains are cultivated in many carbon sources, including glucose and maltose, the deletion of this activator can facilitate downstream processes. Another important approach for recombinant protein production is the deletion of *prtT* in *A. niger*, which was reported by Kamaruddin et al. (2018) [44]. The activator *prtT* is a transcription factor involved in the regulation of extracellular proteases, and its deletion resulted in the retention of a heterologous cutinase activity in 80% after two weeks of incubation compared to 3% retention in the strain where the transcription factor was not deleted.

An efficient approach to increase protein production is the manipulation of genes involved in the morphology of filamentous fungi. The deletion of *racA*, a gene that encodes an Rho GTPase, required for normal colony growth and conidiophore development, resulted in a hyperbranched phenotype, causing a more compact macromorphology in shake flask cultivations and a four-fold increase in the homologous glucoamylase [45].

The manipulation of genes with predicted roles in the secretory pathway in filamentous fungi can result in increased protein production. Zubieta et al. (2020) deleted *pbnA* (glycosyltransferase), *ydjA* (Hsp40 co-chaperone), *trxA* (thioredoxin), and *cypA* (cyclophilin) in *Aspergillus nidulans*, resulting in an increase in homologous xylanase by 78, 171, 105, and 125%, respectively, showing that the knockout of genes predicted to play roles in the predicted secretory pathway is a promising approach for recombinant enzyme production [46].

The strategies described above are some of the examples of efficient strain engineering approaches used to increase enzyme production, and they can be applied in order to construct strains potentially overproducing L-asparaginases, as well as other therapeutic proteins. However, it is important to note that a certain strategy showing efficient results for some proteins will not necessarily result in improved yields for other enzymes. This is because steps including mRNA stability and processing, protein structure, and folding can drastically affect protein production. Therefore, for each enzyme, different approaches should be considered. Finally, the combination of the different strategies can be targeted in order to construct superior cell factories for enzyme production. A good example would be the increase in gene expression levels using strong promoters and high L-asparaginase gene copy numbers, upregulation of (co)-chaperones and foldases, and deletions of amylase and protease genes in order to generate a minimal secretome with a more stable environment for L-asparaginases produced.

4. Perspectives in the Humanization of N-Glycosylation Pathways for L-Asparaginase Production

The glycosylation of therapeutic proteins should always be considered, as it is related to the stability, activity, and localization of the protein of interest. Moreover, the presence of non-humanized N-glycans can have major roles in the pharmacokinetics and immunogenicity of therapeutic proteins [47]. Microbial proteins or mammalian proteins produced in a microbial cell factory will not be naturally glycosylated with human-like glycans, and it has been shown that they can reduce the half-life and quality of the product in addition to

triggering immune responses, which decreases the efficiency of the treatment [48]. The production of these proteins in mammalian cell factories (e.g., Chinese hamster ovary (CHO) cells) also presents some drawbacks, even though these cells contain more human-like glycans. Production is expensive and usually results in lower yields when compared to some fast and efficient microbial production systems [49].

Attempts to alter the glycosylation pathway in a multitude of organisms have been carried out, with a focus on N-glycosylation. In eukaryotes, the biosynthesis of the glycans starts in the cytosolic side of the ER by the addition of an N-acetylglucosamine (GlcNac) to a dolichol phosphate (Dol-P) anchored to the membrane. Subsequent specific enzymes build upon this structure, adding another GlcNac, nine mannoses, and three glucoses, resulting in the core structure Dol-PP-GlcNac2Man9Glc3. This core, which is now located in the ER lumen, is then transferred to the protein in an Asn (Asparagine) residue if located in the sequence motif Asn-X-Ser (Serine)/Thr (Threonine) (where X can be any amino acid except proline). The structure is further processed in the ER, resulting in GlcNac2Man8 and then transferred to the Golgi apparatus. It is in this organelle that the glycosylation differs among eukaryotic organisms. In mammals, residues of galactose, sialic acid, and fucose are added to the final structures, whereas yeast and fungi follow a high mannose pathway, with a final level of mannosylation higher in yeast than fungi [49].

In bacteria, it has been possible to re-engineer *E. coli* to obtain glycosylated proteins. Using a bacterial oligosaccahryltransferase (which transfers the glycan to the protein) together with four glycosyltransferases from Saccharomyces cerevisiae resulted in Man3GlcNac2 structures. Nevertheless, the efficiency of these glycosylations is still very low, with a maximum yield of 3% [50].

With yeasts, even though they normally glycosylate secreted proteins, the profile is extremely different from that of humans, resulting in more drawbacks than benefits. The first significant achievement was the elimination of the hypermannosylated structures, through single-gene deletion of the enzyme responsible for the first step in this pathway. This resulted in Man8GlcNac2 structures in S. cerevisiae, as well as other classic yeasts such as *P. pastoris* [51]. Through the introduction of heterologous enzymes into the Golgi, consisting of two mannosidases and two N-acetylglucosaminyl transferases, the structure was converted to GlcNac2Man3GlcNac2, which is the common intermediate preceding complex human-like glycans [52]. Finally, further introduction of genes encoding sugar transferases made it possible to decorate this final structure, resulting in more advanced human glycans. As a proof of concept, IgG (Immunoglobulin G) with human-like glycans has been successfully produced in a modified *P. pastoris* strain [53].

The L-asparaginase-encoding gene from *E. coli* was used to create a new L-asparaginase with human-like glycosylation through the expression by an engineered Pichia pastoris strain [54]. This new L-asparaginase showed a decrease in enzyme immunogenicity in vitro and increased stability in human serum compared to the wild-type L-asparaginase. In another study, a recombinant Erwinase expressed in the Glycoswitch® Pichia pastoris produced a glycosylated active enzyme. This glycosylated Erwinase showed a significant reduction in antibody recognition, highlighting that the glycans had a hiding effect against antibodies [55].

A comparative study of 12 filamentous fungi, which included the more relevant genera such as Aspergilli and Trichoderma, revealed N-glycosylation to be highly conserved, identifying highly specialized N-glycan structures. The less mannosylated glycans found in these species compared to yeasts were linked to the lack of terminal mannosyltransferases. These findings suggest that knowledge obtained through rational engineering to obtain more human-like structures could be applied to these organisms [48].

A first attempt in these organisms consisted of a similar strategy to that which had been used in yeast. The glycan structure was modified to GlcNacMan5GlcNac2 through the introduction of a Golgi-localized mannosidase and an N-acetylglucosaminyltransferase in both *A. nidulans* and *A. niger*. Lipid-linked oligosaccharides (LLOs) can also be modified through the deletion of mannosyltransferases, which has also been validated in yeasts. The

deletion of alg3, an algC ortholog from S. cerevisiae, resulted in Man3-6GlcNac2 structures, which were further processed to Man3GlcNac2 by native mannosidases. However, a heterogeneous pool of glycan structures was found in both fungi, with the presence of higher mannose forms [56].

A more recent study investigated the nature of glycan structures in *A. nidulans* ΔalgC with the objective of obtaining a strain where the N-linked glycan structures could serve as precursors for more complex humanized glycans. Through several rounds of rational engineering, combining both gene deletions and overexpression, the glycan profile was reshaped towards Man3-5GlcNac2. The final pool of these structures constituted ≥ 70%, with Man3-4GlcNac2, which are not present in the unmodified strain, representing 56% of the structures. Two main bottlenecks in obtaining more of the desirable Man3GlcNac2 structure were identified. Due to the accumulation of Man4-5GlcNac2 in this strain, it is suspected that an optimized expression of α1-2 mannosidase is needed, and since some of the structures were found to be resistant to mannosidases due to galactofuranose capping, an active galactofuranosidase is needed to remove these caps and allow the structures to be further trimmed [49].

These advances in the N-glycosylation pathway in fungi will enable the synthesis of therapeutic proteins, such as L-asparaginase, with a glycan profile that, either in vivo or in vitro, can be further processed into human-like glycans. Further studies validating an improvement in the in vivo treatment are needed, possibly obtaining an increased half-life, a reduced immune response, and a stable or increased activity. Those improvements, together with a reduction in the production cost, will enable more people to be treated and in a more efficient manner. In Figure 2, the native and proposed engineered N-glycosylation pathway in filamentous fungi is illustrated.

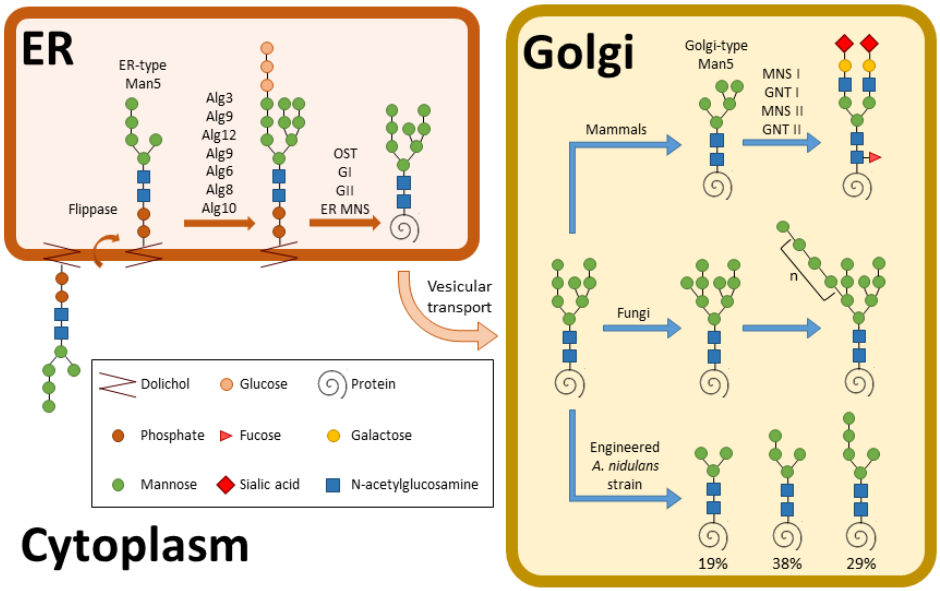

Figure 2. Native and engineered N-glycosylation pathway in Aspergillus.

5. Protein Engineering of L-Asparaginases

Although L-asparaginases have been extensively used in the pharma market, after a vast search in the literature, no works have been found focused on the engineering of the fungal proteins. However, as a therapeutic agent extensively used in the treatment of ALL, L-asparaginases from *E. coli* and *Erwiniae sp.* have been the target of several protein engineering efforts. These studies have approached several of the key issues related to the treatment of ALL with EcA (*E. coli* L-Asparaginase) and ErA (*Erwiniae* L-Asparaginase). They include drug-induced immune response, enzyme half-life in the bloodstream, and L-glutaminase activity.

5.1. L-Glutaminase Activity

The bacterial L-asparaginases originating from *E. coli* and *Erwiniae sp.* are associated with L-glutaminase activity of 2% and 10%, respectively, regarding their asparaginase activity. The depletion of blood glutamine levels due to treatment with bacterial L-asparaginase has been found to lead to several serious side effects, including neurotoxicity and hepatitis [57,58]. Some degree of glutaminase activity has been suggested to be beneficial in the treatment of cancer cells that retain ASNS (Asparagine Synthetase) activity, as these may otherwise survive the depletion of asparagine in the bloodstream by the conversion of glutamine to asparagine. However, due to the side effects caused by glutaminase activity, several studies have employed protein engineering to remove or reduce glutaminase activity in both EcA and ErA. This has proven challenging to achieve without also impacting the asparaginase activity of the enzyme [59,60].

In EcA, a number of different approaches have been employed to reduce glutaminase activity. Researchers have targeted residues located in the active site with varying degrees of success. aa (amino acid) substitutions at position Asn248 have been found to reduce glutaminase activity but additionally have resulted in asparaginase activity less than 25% of that of the Wt (wild-type) enzyme [59,60]. On the other hand, exchanging Q59, also located in the active site, for leucine resulted in undetectable glutaminase activity, while asparaginase activity was not significantly affected [61].

Other targets in EcA include residues located in the dimer interface in the tetramer and the flexible loop, which forms a lid of the active site [62,63]. A combination of aa substitutions of Asn24 in the flexible loop and Arg195 in the dimer interface achieved an approximately 50% reduction in glutaminase activity without significantly affecting the asparaginase activity [62]. Further reductions in glutaminase activity have been achieved by exchanging Y176 for phenylalanine or serine in the dimer interface [63,64].

Similarly to work conducted in EcA, reducing the L-glutaminase activity of ErA without affecting L-asparaginase activity has proven challenging. Based on the ErA crystal structure, Nguyen and co-workers targeted residues in the active site and the flexible loop for aa substitution to increase steric hindrance for the glutamine substrate. The combination of aa substitutions A31L/E63Q/S254Q resulted in a significant decrease in the glutaminase k_{cat} (number of times each enzyme site converts the substrate to product per unit time) and increased K_M (concentration of substrate at which half of the enzyme active sites are saturated with substrate). However, the observed improvements were also accompanied by a two-fold increase in the K_M for asparagine.

Most engineering efforts in ErA and EcA have employed a rational engineering strategy utilizing existing crystal structures [59] and molecular dynamics simulations to select target residues for substitution [60–62]. Recently however, non-rational strategies have also been employed to reduce glutaminase activity. Munhoz Costa and co-workers successfully applied error-prone PCR (Polimerase Chain Reaction) to ErwIIA to generate 1056 mutants, which were subsequently screened for L-asparaginase activity. Mutants with 80% L-asparaginase activity or more, compared to the wild-type enzyme, were further kinetically evaluated and sequenced to determine the introduced mutations. One mutant Q227E/V272M was found to have an increased asparaginase activity relative to the ErA WT. Q227E and V272M are located in the vicinity of the active site and on the protein surface and were found to increase the asparagine turnover rate while decreasing glutamine turnover [65].

5.2. Reducing Immunogenic Reaction

Both EcA and ErA have been found to elicit an immune response in some patients, leading to either a need to halt the treatment with the respective enzyme due to side effects or reduced efficacy of the treatment. Although PEGylation is used to decrease the immunogenicity of L-asparaginases treatment [66], a protein-engineering strategy is the identification and elimination of T and B cell epitopes within L-asparaginases [67,68]. Mappings of B and T cell

epitopes have been achieved by the evaluation of the binding of L-asparaginase antiserum to synthetic L-asparaginase peptides [68,69], as well as by computational predictions [70].

In EcA, epitope elimination has been successfully applied. The substitution of 195-RKH-197, part of an important epitope, for alanine, showed that EcA rabbit IgG binding could be greatly decreased [70]. Kumar Mehta and coworkers found that individual aa substitutions in five previously mapped B-cell epitopes did not reduce antibody binding. However, a combination of one of the epitope disruption L288S with Y176F, located in the dimer interface, resulted in a 40% reduction in antibody binding [64]. Furthermore, the mutant enzyme was found to induce the formation of antibodies to a significantly smaller degree than the wild-type enzyme [64]. Mapping of the main sites contributing to T-cell recognition of EcA allowed for site-directed saturation mutagenesis to generate mutants with reduced antigen response. A preliminary screening for L-asparaginase activity enabled the selection of mutants not impaired in terms of asparaginase activity. Subsequent characterization of a selection of the mutant enzymes identified one candidate containing eight aa substitutions within the three main T-cell epitopes, which led to a significant reduction in T-cell response [70]. Rodrigues et al. reported the creation of mutant L-asparaginase by error-prone polymerase chain reaction from *Escherichia coli* resistant to the main lysosomal human proteases, asparaginyl endopeptidase (AEP) and cathepsin B (CTSB) [71]. The new L-asparaginase showed enzymatic activity and cytotoxicity levels equivalent to or better than the wild-type enzyme. Besides that, in vivo assays showed that the mutant L-asparaginase presented increased serum half-life and alteration of the phenotypic profile of B cells (less immunogenic protein drugs).

5.3. Enzyme Stability

Increased enzyme half-life in the bloodstream represents a highly desirable characteristic as it leads to more efficient and longer-lasting L-asparaginase treatment [57,72]. Furthermore, enzyme degradation leads to the exposure of epitopes within the protein structure, potentially eliciting an immune response from the patient. There is therefore an interest in increasing L-asparaginase proteolytic resistance, as well as thermostability which is used as a measure of the general stability of the enzyme [73].

In EcA, the residue N24, located in the flexible loop, which acts as a loop of the active site, represents the primary cut site for the proteases asparaginase endopeptidase (AEP) and cathepsin. Replacing N24 with glycine resulted in an AEP-resistant mutant but also significantly reduced the L-asparaginase activity [74]. Subsequently, Offman and Maggi et al. demonstrated that alternative aa substitutions N24A and N24S could be implemented without adversely affecting L-asparaginase activity [62,75]. In addition to making EcA AEP-resistant, the N24S also resulted in increased thermal stability [75].

The replacement of underexposed charged residues on the protein surface with hydrophobic has been explored as a method for increasing the stability of EcA. While K139A and K207A did indeed increase thermal stability, the substitutions also substantially reduced L-asparaginase activity [76]. Similarly, in the ErA application of a staggered extension process, using the more stable L-asparaginase gene from Erwinia carotovora as a template resulted in the identification of ErA mutant D133V displaying increased thermal stability. The increased thermal stability observed upon replacement of D133 located on the protein surface was accompanied by a significant decrease in L-asparaginase activity [77].

5.4. Perspectives for Engineering Fungal Asparaginases

The results of protein engineering in EcA and ErA demonstrate the potential for improving enzyme characteristics of fungal L-asparaginases. However, challenges for fungal L-asparaginases may be different from those encountered using EcA and ErA. Human L-asparaginase (hASNase) has been evaluated as a potential ALL treatment. However, high K_M renders it inapplicable for this purpose. To address these shortcomings, efforts have been made to engineer hASNase to a lower K_M and higher kcat. The site saturation of residues in the vicinity of the active site of hASNase was successful in lowering the K_M

but remains too high for ALL treatment [78]. Shuffling the sequence of hASNase and gASNase of guinea pig origin, which has been found to display micromolar K_M, Schalk and coworkers were able to create chimeric enzymes retaining high sequence similarity to hASNase while decreasing K_M as much as 100-fold [78,79]. It is clear that engineering L-asparaginases for ALL treatment is a complicated endeavor due to the multifaceted requirements of a successful ALL drug. Modifying a single aspect of the enzyme such as glutaminase activity or stability without potentially affecting other characteristics is no trivial task.

6. Conclusions and Perspectives

L-asparaginases are highly relevant enzymes in the pharmaceutical market, and although bacterial versions have been used intensively since their approval by the FDA in the 1970s, there is a gigantic interest in finding alternative sources with less side effects. Filamentous fungi stand out as promising sources and chassis for protein secretion, these organisms are far from being one of the selected sources as cell factories for L-asparaginase production. Among different ascomycetes, Aspergilli belong to a highlighted group of candidates not only for their superior secretory pathways when compared to yeast and bacteria but also because they harbor L-asparaginase genes in their genomes, facilitating homologous secretion. In this context, there are at least two main points that should be addressed before commercially using these strains for biopharmaceutics production. On one hand, protein glycosylation plays important roles in protein stability against proteases and thermostability, and folding quality control plays an important role in the endoplasmic reticulum. However, the fungal glycan structures have different compositions compared to the human ones. This, therefore, hampers the use of filamentous fungi for therapeutic protein production. However, the scenario is changing as there are interesting papers showing the humanization of N-glycosylation pathways that could, in the future, enable the use of fungal strains as a chassis for biopharmaceutical production. On the other hand, many Aspergilli fungi produce secondary metabolites that can be highly toxic to humans. One alternative is using species and strains with less capacity for producing such mycotoxins as Aspergillus oryzae or deleting active gene clusters involved in secondary metabolite production. Moreover, with the advances of cutting-edge genetic engineering technologies such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), it is almost possible to rapidly engineer many Aspergilli. Consequently, if not lethal to the fungus, strains deficient in mycotoxin production can be generated. Hence, using filamentous fungi for producing recombinant engineered and non-engineered L-asparaginases and other therapeutic proteins still demands intense engineering. In this case, the development and implementation of medium and high-throughput technologies and robotics are mandatory for the generation of hundreds of mutants, simultaneously increasing the chances for the construction of cell factories for eukaryotic L-asparaginases. However, attention should be given to the development of technologies to analyze several mutants constructed, including the digitalization of data generated.

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