

Approaches for refining heterologous protein production in filamentous fungi

Ruchika Sharma · Meenu Katoch · P. S. Srivastava ·
G. N. Qazi

Received: 10 September 2008 / Accepted: 21 July 2009
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Abstract Fungi combine the advantages of a microbial system such as a simple fermentability with the capability of secreting proteins that are modified according to a general eukaryotic scheme. Filamentous fungi such as *Aspergillus niger* efficiently secrete genuine proteins but the secretion of recombinant proteins turned out to be a difficult task. *Aspergillus niger* is an attractive organism because of its high secretion capacity and is frequently used as a model organism. Whereas high production yields can be obtained when homologous proteins are expressed, much lower amounts are obtained with the production of heterologous proteins. To fully exploit the potential of filamentous fungi, understanding of the molecular genetics, their physiology, and the glycosylation metabolism has to be investigated and clarified in more detail. This review summarizes recent developments in heterologous protein production by filamentous fungi and also generalizes the possibilities of improving the protein production by various genetic and bioprocessing approaches, thereby easing recognition of filamentous fungi as a relevant and reliable expression platform.

Keywords Filamentous fungi · Heterologous proteins · Expression system

Introduction

Filamentous fungi are capable of producing large amounts of specific proteins. Their wealth of genetic diversity has opened new doors for exploiting them as a source of new genes and as expression hosts (Punt et al. 2002). The advantages of using filamentous fungi as hosts include their natural abilities to secrete a variety of proteins in large quantities and their intensive use in bioprocessing, which implies much industrial experience of these cultures and their behaviour at a variety of scales (Archer et al. 1994). The world market for industrial enzymes has been estimated to be worth over \$1.6 billion, with almost half of these enzymes produced in filamentous fungal fermentations (Muller 2001). This is because many industrially important strains of fungi such as *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Acremonium chrysogenum* and *Penicillium chrysogenum*, have been classified GRAS (generally regarded as safe), can grow on relatively inexpensive substrates, and can both produce and secrete tremendous amounts of recombinant protein (van den Hondel et al. 1992; Ward et al. 1992). Features such as low cost and high productivity have also attracted many research efforts in both molecular-genetic techniques and bioprocess improvements (Finkelstein and Ball 1992; Banerjee et al. 2003). Many researchers have already summarized their progress in the development of filamentous fungi as hosts for homologous (Durand et al. 1988; Finkelstein et al. 1989) and heterologous (Jeenes et al. 1991; van den Hondel et al. 1991) protein production. A range of different types of recombinant proteins has also been successfully expressed in filamentous fungi (Nevalainen et al. 2005). Heterologous products are approximately one-third of all the industrial applications, with *Aspergillus* and prokaryotic strains being

R. Sharma · M. Katoch (✉) · G. N. Qazi
Biotechnology Division, Indian Institute of Integrative Medicine
(CSIR), Canal Road, Jammu 180001, India
e-mail: meenusamiksha@rediffmail.com

P. S. Srivastava
Department of Biotechnology, Jamia Hamdard, Hamdard Nagar,
New Delhi 110062, India

Table 1 Distribution of industrial enzymes and production strains according to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) list in 2004

Total enzymes	186
Homologous product	64.5%
Heterologous product	35.5%
<i>Aspergillus</i> strains	36.6%
<i>Trichoderma</i> strains	10.8%
<i>Penicillium</i> strains	8.1%
<i>Kluyveromyces</i> strains	1.6%
<i>Saccharomyces</i> strains	1.1%
Prokaryotic strains	30.6%

the largest group of production organisms. *Saccharomyces* only accounts for 1% of the products in this list (Table 1).

The industrial enzyme market reached \$1.6 billion in 1998 for the following application areas: food, 45%; detergents, 34%; textiles, 11%; leather, 3%; pulp and paper 1.2% (Stroh 1998). This does not include diagnostic and therapeutic enzymes. The market for these non-pharmaceutical proteins reached \$2 billion in 2000. Over 60% of the enzymes used in the detergent, food, and starch-processing industries are recombinant products (Cowan 1996); although the number of heterologous fungal enzymes approved for food applications is not very large (Table 2). Due to the low yields achieved with non-fungal proteins, many recombinant food-grade proteins are of fungal origin (Archer 2000). There is one exception in which the donor strain is not another fungus, i.e., calf rennin (chymosin), which is used for cheese making. Despite the apparent

benefit of working with filamentous fungi, it appears difficult to obtain significant heterologous protein expression levels (Broekhuijsen et al. 1993; Wiebe et al. 2001). The problem encountered is that the production levels by fungi of most non-fungal proteins of mammalian, bacterial or plant origin are much lower than those of homologous proteins, usually only reaching a few tens of milligrams per liter of culture medium (Gouka et al. 1997a). To improve the expression of heterologous proteins has become a significant issue in fungal molecular biology. This review will focus on the use of filamentous fungi for the production of heterologous, especially non-fungal, proteins and will deal with the limitations and improvement strategies for over-production of these proteins.

Filamentous fungi as expression host

Filamentous fungi have been used for expression of both native and foreign proteins. The fact that fungi secrete enzymes into the culture medium has a clear advantage in producing proteins that might be toxic if they accumulate inside the cells. Secretion is also beneficial for the purification of the desired products, since there is no need to break the cells and to get rid of all the intracellular proteins, a process that is often tedious and expensive. Moreover, as fermentation techniques are also well developed, fungi can be grown in large quantities in inexpensive media. The secreted levels of native proteins are high in optimal production conditions, for example, *T. reesei* can produce hydrolases at grams per litre quantities (Durand et al. 1988)

Table 2 List of heterologous industrial enzymes. From AMFEP (2004)

Enzyme	Host organism	Donor organism
Aminopeptidase	<i>Trichoderma reesei</i>	<i>Aspergillus</i> sp.
Arabinofuranosidase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.
Catalase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.
Cellulase	<i>Aspergillus oryzae</i>	<i>Humicola</i> sp.
Galactosidase (alpha)	<i>Saccharomyces cerevisiae</i>	Guar plant
Glucanase (beta)	<i>Trichoderma reesei</i>	<i>Trichoderma</i> sp.
Glucoamylase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.
Glucose oxidase	<i>Aspergillus niger</i>	<i>Aspergillus</i> sp.
Laccase	<i>Aspergillus oryzae</i>	<i>Myceliophthora</i> sp.
Lactase or Galactosidase (beta)	<i>Aspergillus oryzae</i>	<i>Aspergillus</i> sp.
Lipase	<i>Aspergillus oryzae</i>	<i>Candida</i> sp.
Mannanase (endo-1, 4-beta)	<i>Trichoderma reesei</i>	<i>Trichoderma</i> sp.
Pectin lyase	<i>Aspergillus niger</i> var. <i>awamori</i>	<i>Aspergillus niger</i>
Phytase	<i>Aspergillus oryzae</i>	<i>Peniophora</i> sp.
Protease	<i>Aspergillus oryzae</i>	<i>Rhizomucor</i> sp.
Pullulanase	<i>Trichoderma longibrachiatum</i>	<i>Hormoconis</i> sp.
Xylanase	<i>Aspergillus niger</i> var. <i>awamori</i>	<i>Aspergillus</i> sp.
Chymosin	<i>A. niger</i> var. <i>awamori</i>	Calf

Table 3 Advantages of fungal expression systems

High yield
Stable production strains
Cost effectiveness
High density growth
Easy scale-up
Safety
High expression levels
Rapid growth in chemically defined media
Product processing similar to mammalian cells
Can handle S-S rich proteins
Can assist protein folding
Can glycosylate protein

and *A. niger* has been shown to excrete 25 g of glucoamylase per litre (Ward et al. 2006). Productivity and secretion potential of filamentous fungi, which is in the range of 30–40 g/l for homologous enzymes like cellulases and amylases, is considered to be superior to any other system, but unfortunately could not be converted into corresponding yields of recombinant products, even when these were fused to such homologous enzyme (Schmidt 2004). Plasmid architecture is an important parameter which influences levels of heterologous proteins production. Significantly higher levels of extracellular enzyme were attained through *LAC4*-directed XynA expression on full pKD1 sequence vectors as compared to expression with other pKD1ori plasmid derivatives (Walsh et al. 1998).

A series of vectors have been constructed by (Mantyla et al. 1998; Penttila 1998; Teo et al. 2000) using the strong main cellobiohydrolase *cbh1* promoter with provision for in-frame fusions of heterologous genes to either the *cbh1* signal sequence or downstream of the *cbh1* catalytic core-linker region. The first attempts to produce foreign proteins in filamentous fungi were made with proteins of fungal origin (Fincham 1989). Since then, many industrially important fungal proteins have been expressed in various production hosts. The major advantages of fungal expression systems are listed in Table 3.

The highest yields that are obtained with heterologous fungal enzymes are shown in Table 4. In contrast to the production of fungal proteins, the yields of non-fungal proteins in filamentous fungi is very low (e.g., in the low milligrams per litre range) which is unattractive for industrial production (van den Hondel et al. 1991).

It is still difficult, however, to obtain the high levels of expression required for commercial purposes (Aifa et al. 1999; Ruiz-Duenas et al. 1999; Stewart et al. 1996).

Bottlenecks of low yield of heterologous protein production

Several factors that negatively affect the production levels of non-fungal proteins have been reported (Punt et al. 1994; Archer and Peberby 1997). Factors that can limit protein production are low transcriptional levels, mRNA instability, inefficient translation, post-transcriptional bottlenecks, and finally degradation of the protein of interest (reviewed by Gouka et al. 1997a). At the post-translational level, glycosylation and protein folding processes predominantly influence structure and function of heterologous proteins. For some heterologous proteins, glycosylation is essential to obtain the proper conformation and high secretion levels and in such cases low yield of heterologous protein produced could be due to ineffective glycosylation (Maras et al. 1999). Unfolded protein response (UPR) is a general reaction in eukaryotes to protein-folding stress in the ER, and this kind of stress is often generated in the expression of heterologous secreted proteins. As overexpression of heterologous proteins often induces a UPR, it seems likely that the folding capacity of the cell cannot cope with the large amount of unfolded proteins in the ER (reviewed by Cudna and Dickson 2003). This may be the main reason for the low levels of heterologous proteins secreted by filamentous fungi (reviewed by Conesa et al. 2001). Goosen et al. (2005) investigated expression of heterologous secreted proteins, which often results in such stress and thus UPR, also in filamentous fungi like *Aspergillus niger* which are renowned for their high secretion capacity. As a

Table 4 Examples of some important heterologous proteins expressed in *Aspergillus*

Protein	Yield	Expression system	Reference
Glucoamylase from <i>A. niger</i>	4.6 g/l	<i>A. awamori</i>	Radzio and Kück (1997)
Human interleukin 6	150 mg/l	<i>A. niger</i>	Punt et al. (2002)
Alkaline protease from <i>Fusarium</i>	4 g/l	<i>A. chrysogenum</i>	Morita et al. (1994)
Chymosin (calf)	0.16 mg/l	<i>A. oryzae</i>	Dunn-Coleman et al. (1991)
Interferon- α 2 (human)	0.2 mg/l	<i>A. nidulans</i>	Macrae et al. (1993)
Lactoferrin (human)	2.0 g/l	<i>A. oryzae</i>	Ward et al. (1995)
Lysozyme (hen egg-white)	1.0 mg/l	<i>A. niger</i>	Archer et al. (1990); Gyamerah et al. (2002)

consequence, the production yield of secreted heterologous proteins usually is very low (milligrams/l at best). Most papers reported the failure of improvement of heterologous protein secretion (Punt et al. 1998; Wang and Ward 2000; Ngiam et al. 2000) in filamentous fungi. In addition to problems like improper posttranslational modifications and unfolded protein response stress, native fungal proteases present in the medium may adversely affect heterologous protein yields (Archer et al. 1992; Van den Hombergh et al. 1997b). Heterologous protein production by filamentous fungi is sometimes severely hampered by fungal proteases. *Aspergillus nidulans* contains about 80 protease genes (Machida 2002). One more factor limiting the development of strains suitable for protein production is that few plasmid vectors are available for *A. niger* relative to the large assortment available for studies with model systems like *Escherichia coli* (Sambrook et al. 1989) and *Saccharomyces cerevisiae* (Elledge and Davis 1988; Fields and Song 1989; Schneider and Guarente 1991; Sikorski and Hieter 1989).

Approaches for refining heterologous protein expression in filamentous fungi

For improving heterologous protein expression in filamentous fungi several strategies have been suggested, both genetic and bioprocessing in nature.

Genetic strategies

The genetic strategies include: gene fusion with a gene encoding all or part of a well-expressed and secreted protein; the introduction of multicopies of the protein gene; engineering of the glycosylation pathway; induction of the unfolded protein response; construction and use of protease-deficient host strains; use of strong promoters and introducing multiple copies of protein-binding sequence (CCAAT) to the promoter.

Gene fusion strategy

For the secretion of foreign proteins, fusion strategies are used to facilitate translocation in the secretion pathway and to protect the heterologous protein from degrading. It is generally believed that a gene fusion has its beneficial effect mainly on the (post) translational level, e.g., by enhanced stabilization, increased efficiency of secretion, or prevention of proteolytic degradation. The gene fusion strategy has been very useful in different expression systems. The best-characterized fusions are those with the *T. reesei* CBH1 and with *A. niger* or *A. awamori* glucoamylase (*glaA*). In all these cases the fusion has been made

to the N-terminus of the foreign protein. Gouka et al. (1997b) showed that *glaA* fusion to the 5' end of the human interleukin 6 gene resulted in an increase of the *hil6* production, where as *glaA* fusion to the 3' end greatly reduced the production of the fusion protein. Fusion has resulted in higher levels of secreted bovine prochymosin (Ward et al. 1990), porcine pancreatic phospholipase A2 (Roberts et al. 1992), human interleukin-6 (Contreras et al. 1991; Broekhuijsen et al. 1993), hen egg-white lysozyme (Jeenes et al. 1993), and human lactoferrin (Ward et al. 1995). The increase varies from 5 to 1000-fold, depending on the protein, resulting in protein levels varying from 5 to 250 mg l⁻¹. Even higher levels, up to 1–2 g l⁻¹ were obtained for bovine chymosin (Dunn-Coleman et al. 1991) and lactoferrin (Ward et al. 1995) when high-level-production strains were subjected to several rounds of mutagenesis. The production of antibody fragments in *T. reesei* as a CBH1 fusion, resulted in more than 150-fold increase in the yield (Nysönen and Keränen 1995).

Introducing multicopies of protein gene for higher yields

To obtain high-level expression of a particular gene, a well-established procedure is targeting multiple copies of the recombinant gene constructs to the locus of a highly expressed endogenous gene. An advantage of such a gene replacement is knowing that the integration occurs in a region of the genome that is actively transcribed. When the target locus is that of a highly expressed secreted protein, the reduced load on the secretion pathway may facilitate expression and secretion of the recombinant gene product. Introduction of multiple gene copies of the genes *amyB*, *glaA*, and *agdA*, encoding industrial Taka-amylase, glucoamylase, and alpha-glucosidase production, respectively, in *A. oryzae* resulted in enhanced expression of these enzymes (Hata et al. 1991; Minetoki et al. 1995; Tada et al. 1991). However, Verdoes et al. (1993) illustrated the limitations of a high copy number approach. High *glaA* copy numbers do not necessarily result in high production levels. As Verdoes et al. (1993) suggested, a kind of linear relationship between copy number and yield is only found for the first 5–6 copies.

Using protease deficient host strain

Proteases have been thought to be one of the limiting steps in the industrial production of enzymes (Van den Hombergh et al. 1997a). The findings that extracellular proteases can decrease the amount of foreign protein have led to the construction of protease-deficient strains. A strain of *A. niger* that lacks *pepA*, *pepB* and *pepE* has been constructed (Van den Hombergh et al. 1997a) and at least in vitro the protease activities were lower than in the

control strain. Four new aspartic protease genes *pepAa*, *pepAb*, *pepAc* and *pepAd* from *Aspergillus niger* were identified using a comparative genomic approach. The secretion level of heterologous laccase in the *pepAa*, *pepAb* and *pepAd* disruption mutants were increased by about 21, 42 and 30%, respectively. And their total glucogenic enzyme secretion was also increased by about 18.7, 37.0 and 5.20%, respectively (Wang et al. 2008). The production of chymosin as well as porcine phospholipase 2 was increased in *A. awamori* by deletion of the *pepA* gene encoding the aspartic proteinase aspergillopepsin A (Berka et al. 1990; Roberts et al. 1992). The applicability of this strategy varies due to different sensitivities of foreign proteins to proteases produced by the host. For example, hiL6 is very sensitive to protease activity (Broekhuijsen et al. 1993), where as hen egg-white lysozyme and porcine pancreatic phospholipase A₂ have different sensitivities to aspergillopepsin A in *A. niger* (Archer et al. 1992). The fungus *Chrysosporium lucknowense* has been genetically converted into a low protease-producing strain that is capable of producing very high yields of heterologous proteins (Verdoes et al. 2007).

Mattern et al. (1992) isolated a mutant strain of *A. niger* deficient in production of extracellular proteases by in vivo UV mutagenesis. Such strains have largely alleviated the problem of extracellular digestion, thereby increasing the yield of heterologous proteins. Inhibition of protease activity and increased heterologous protein production can also be accomplished by applying various bioprocessing approaches that are discussed in the later part of this review.

Engineering of the glycosylation pathway

Glycosylation is an important step in the secretion of heterologous proteins in fungi (Gasser and Mattanovich 2007). In some cases the yield of heterologous protein produced is low due to ineffective glycosylation. Since compared to higher eukaryotes filamentous fungi are very easy to modify genetically, it is feasible to construct “lack of function” mutants that are disturbed in a specific unwanted glycosylation step, provided that the glycosylation pathway and the genes encoding the enzymes involved are known. There are some indications that glycosylation has a role in the production of heterologous proteins. Glycosylation is also important for biological activity of heterologous proteins used in therapeutics (Gemgross 2004). N-Glycosylation has been shown to increase the amount of secreted chymosin in *A. awamori*, although the specific activity of the chymosin was reduced (Ward 1989). In *T. reesei*, it has been shown that the overexpression of the mannosylphosphodolichol synthase-encoding gene from *S. cerevisiae*, which is required for O-glycan

precursor synthesis, increased the production of CBH1 (Kruszewska et al. 1999). As for foreign proteins, it has been shown that *Candida antarctica* lipase produced in *A. oryzae* (Heogh et al. 1995) and *Hormoconis resinae* glucoamylase P produced in *T. reesei* (Joutsjoki et al. 1993) are overglycosylated.

Van den Brink et al. (2006) improved a poorly used N-glycosylation site within the prochymosin molecule. The resulting highly glycosylated chymosin molecule was expressed in *Aspergillus niger*. It was shown that production of the glycosylated protein was much more efficient, giving a yield increase of more than 100% compared to production of the native chymosin molecule. This strategy might offer a powerful tool for increasing production levels of other heterologous proteins as well.

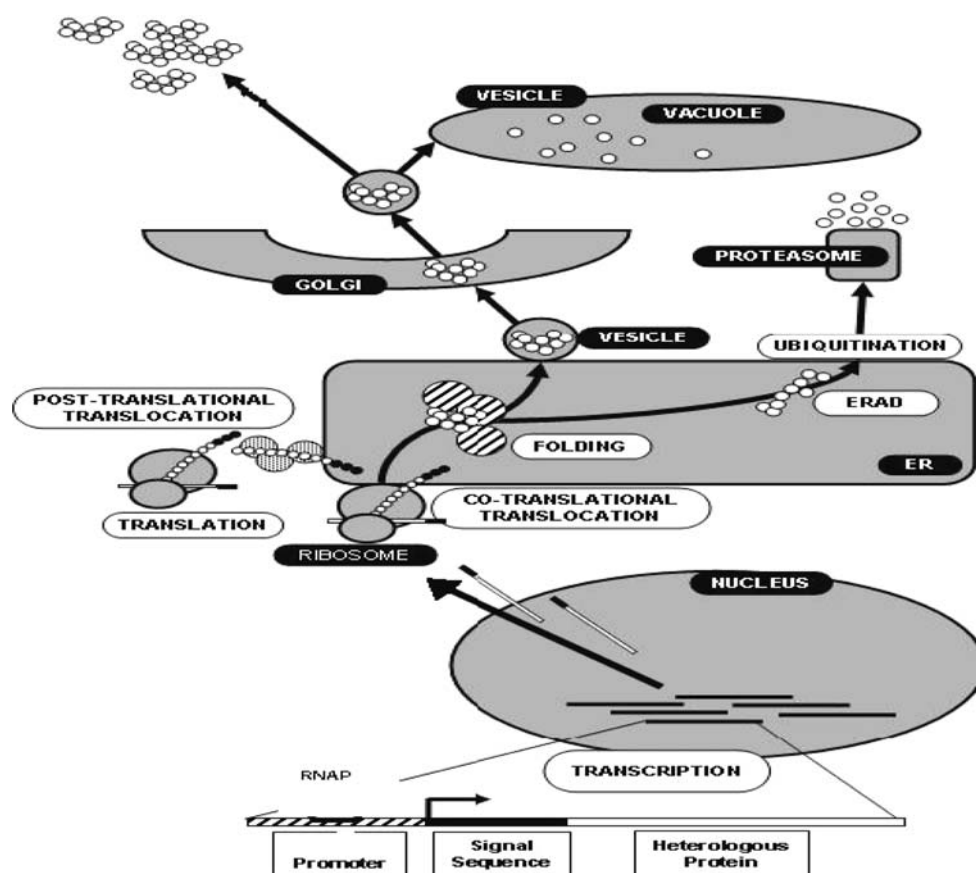
Induction of the unfolded protein response

Proteins that are destined to be secreted enter the endoplasmic reticulum (ER) through a pore in the ER membrane and are subjected to a series of post-translational modifications, including folding and maturation. In the lumen of the ER, several folding assistants are present, like folding enzymes and molecular chaperones (e.g., the molecular chaperone BiP; Pedrazzini and Vitale 1996; Gething 1999). When the nascent protein is properly modified it can leave the ER by means of vesicle budding and is transported to the Golgi apparatus for further modifications and subsequently secreted through vesicle fusion with the plasma membrane. The folding assistants are also involved in other processes such as translocation into the ER (reviewed by Sitia and Braakman 2003) and ER-associated degradation (ERAD) pathway (reviewed by Brodsky and McCracken 1999). Figure 1 represents the general secretion pathway of heterologous proteins in the endoplasmic reticulum.

Accumulation of unfolded or misfolded proteins (due to inefficient folding capacity of the cell or stress-causing agents), an increased load of proteins destined for secretion, altered metabolic conditions and depletion of Ca²⁺ can activate an intracellular signaling pathway referred to as the Unfolded Protein Response (UPR) (Kaufman 1999; Rutkowski and Kaufman 2004).

As a consequence, expression of genes encoding chaperones and foldases is induced to increase the folding capacity (reviewed by Chapman et al. 1998; Conesa et al. 2001; Welihinda et al. 1999) and thereby limiting the accumulation of unfolded or misfolded proteins in the ER. The genes for several ER chaperones and foldases have been isolated from filamentous fungi, such as *bipA* (from a family of binding proteins, bip) (Van Gemeren et al. 1997); *pdiA* (from a family of protein disulfide isomerase) (Jeenes et al. 1997; Ngiam et al. 2000); and a family of calnexins

Fig. 1 General scheme of secretion pathway for heterologous protein production



(Conesa et al. 2001), which have been overexpressed to improve the heterologous protein production. Previous studies showed that BiP overexpression does not result in increased secreted levels of hIL-6 in *Aspergillus* (Gouka et al. 1997b). Filamentous fungi overproducing specific homologous or heterologous proteins had increased levels of *bipA* transcription, whereas forced *bipA* overexpression had no significant effect on secreted protein yields in these strains (Punt et al. 1998). Punt et al. (1998) have analysed the role of the BiP-encoding gene in protein overproduction from *A. niger* and *A. awamori*. Overproduction of fungal proteins increased *bipA* mRNA levels in *A. niger*. Similarly, a 2-fold induction in *bipA* mRNA levels was measured in two *A. niger* strains producing the hen egg-white lysozyme (HEWL) (Ngiam et al. 2000). However, BiP levels remained unchanged when hIL-6 was produced in *A. niger* (Punt et al. 1998). Although no clear relationship between BiP induction and a particular characteristic of the overproduced protein can be deduced, protein overproduction may lead to increased levels of unfolded proteins and thus result in *bipA* overexpression (Conesa et al. 2001). While the amount of secreted recombinant protein did not change with increased BiP A levels, increased levels of unprocessed fusion protein were detected in the total protein extracts of these strains (Punt et al. 1998). Functional analysis of *pdiA* showed that it

catalyses the refolding of denatured and reduced RNase (Ngiam et al. 2000). An increased level of *pdiA* transcripts was also observed in two *A. niger* strains overproducing a heterologous protein, HEWL. Overexpression of *pdiA* did not increase secreted yields of HEWL in *A. niger*, suggesting that *pdiA* itself is not limiting for secretion of this protein (Ngiam et al. 2000). Also *A. niger clxA*, encoding calnexin, was induced upon heterologous overexpression of bovine prochymosin (Wang et al. 2003) and MnP (Conesa et al. 2002). The lectin-like proteins calnexin and calreticulin are chaperones that interact with nascent and newly synthesised glycoproteins in the ER and function in Quality Control by aiding folding (Parodi 1999). The unfolded protein response (UPR) involves a complex signaling pathway in which the transcription factor HACA plays a central role. Mulder et al. (2004) showed that *A. niger* HACA was able to bind to the UPRE (Unfolded Protein Response Element) in the promoter sequence of several ER chaperones and foldases. Furthermore, overexpression of the processed form of *hacA* in *A. niger* resulted in an induction of the genes encoding the ER chaperone BiP, the foldases peptidyl-prolyl *cis*-trans isomerase CYPB and protein disulfide isomerase PDIA, and HACA itself. Valkonen et al. (2003) evaluated that the constitutive induction of the UPR pathway in *Aspergillus niger* var. *awamori* can be achieved by expressing the

activated form of the transcription factor *hacA*. This induction enhances the production of *Trametes versicolor* laccase by up to 7-fold and of bovine preprochymosin by up to 2.8-fold in this biotechnically important fungus.

Using strong promoters and efficient secretion signals

When producing the target protein by genetic engineering, the production efficiency is regulated by various factors but the most important factor is how to increase the transcriptional level. For the efficient production of heterologous proteins, it is essential to have a strong and high gene transcription and an efficient secretion mechanism. Therefore the heterologous gene is placed in a production/expression vector linked to a signal sequence, which directs the transcript to the secretory pathway and behind a strong inducible promoter (Fig. 1). Fungal promoters that are used for the expression of heterologous genes, can be divided into two groups (Saunders et al. 1989): promoters from housekeeping genes with a high level of expression; and inducible promoters. The most frequently used constitutive promoter is the *gpdA* promoter from *Aspergillus nidulans*, which is part of a gene encoding glyceraldehyde-3-phosphatedehydrogenase. This promoter is functional in several species, including *P. chrysogenum* (Kolar et al. 1988), *A. nidulans* (Punt et al. 1991) and *A. niger* (Archer et al. 1990). A disadvantage in using constitutively expressed housekeeping gene promoters is that the promoters are functional during growth and therefore unsuitable for expression of foreign proteins that might be toxic to the host cells. Well-known inducible promoters used in many applications are those of the cellobiohydrolase I (*cbh1*) from *T. reesei* (Harkki et al. 1991), glucoamylase A (*glaA*) from *A. niger* (Smith et al. 1990), Taka-amylase (*amyA*) from *A. oryzae* (Tsuchiya et al. 1992) and xylanase (*exlA*) from *A. awamori* (Gouka et al. 1996). In most cases promoters that are homologous with respect to the host lead to more efficient expression than heterologous promoters (Jeenes et al. 1991). Rate-limiting steps and ways of improving the production in *Trichoderma reesei* especially using antibody Fab fragments have also been studied, but foreign protein production has been increased considerably by fusing the protein to the fungal cellulase cellobiohydrolase I (*cbh1*) promoter (Keranen and Penttilä 1995). A novel metabolically independent expression system that can be regulated by oestrogenic compounds has been presented for *Aspergillus* (Pachlinger et al. 2005).

Introducing multiple copies of protein-binding sequence (CCAAT) to the promoter

The CCAAT motif has been considered as an essential and functional element for high-level expression of a large

number of genes in filamentous fungi (Bucher 1990) and higher eukaryotes. It binds protein activators specifically and then initiates a series of interactions that result in high-level gene expression. The activity of the *cbh2* promoter in *T. reesei* depends on the Hap protein complex binding to the CCAAT box (Tsukagoshi et al. 2001).

Liu et al. (2003) provided evidence that the CCAAT-containing region of the *glaA* promoter of *A. niger* is a binding site for a positive transcriptional factor and demonstrated the possibility for improving the expression of both heterologous and endogenous genes in *Aspergillus* sp. The promoter activity was increased by the introduction of the CCAAT-containing sequence resulting in increased gene expression.

Bioprocessing strategies

Improvement in protein yield by fermentation processes

Wang et al. (2005) and Grimm et al. (2005) discussed the bioprocessing strategies to improve heterologous protein production, which includes fungal morphology, fungal mycelium immobilization, fermentation pH effects, cultivation medium, agitation intensity and dissolved oxygen tension. The dependence of protein production on fungal morphology has been investigated by Xu et al. (2000) in shake flasks cultures by employing the recombinant *A. niger* strain, which carries a gene for the glucoamylase-green fluorescent protein (GFP) fusion protein.

There is an enormous influence of the chemical and physical environment on culture morphology (Gibbs et al. 2000). Li et al. (2000) assessed morphology and fragmentation behavior in large-scale, fed-batch, fungal fermentations used for the production of protein and found that increased impeller power had little effect on biomass concentration, fungal morphology, or fragmentation behavior. Cell immobilization lowers the apparent broth viscosity and makes the rheological features more favorable for oxygen supply and mass transfer (Thongchul and Yang 2003). Moreover, cells are protected from shear damage by immobilization, resulting in higher cell loading, and higher volumetric productivities. Agitation intensity may affect both fungal morphology (Amanullah et al. 2002) and mass transfer in the bioreactor (Li et al. 2002). The effect of agitation on fungal morphology has been widely discussed by Cui et al. (1997). Increasing agitation intensity enhanced the mechanical forces on the fungal cells as well as the gas-liquid mass transfer. Pellet size and hair length in a submerged fermentation were controlled by mechanical forces. A higher agitation rate leads to good mixing and a higher mass transfer rate in the reactor. Dissolved oxygen tension affects productivity, cell autolysis, the rigidity of the cell wall, and many other features

of fungal fermentation. The effect of dissolved oxygen on protein production is profound. Oxygen can potentially impact fungal cultures in a number of ways. It may enhance growth and protein secretion directly. It may also indirectly influence these processes by altering the morphology of the culture, either positively or negatively. In the culture of aerobic microorganisms, oxygen serves as a substrate for energy generation. It may enhance the cell growth and protein production directly or through changes in morphology. Bai et al. (2004) found that the enrichment of oxygen could result in a change in the respiratory pathway, and thus affect protein synthesis greatly. The negative effect can be caused by oxygen toxicity or undesirable morphological changes, which is correlated with both the main hyphal length and total hyphal length. Some studies have indicated that the primary effect of morphology on product formation is due to viscosity. When the fungal morphology is better controlled, the productivity can be increased. Bhargava et al. (2003) found that when a fed-batch, process strategy along with the pulsed addition of a limiting-carbon source is used, the fungal broth viscosity was reduced which ultimately increased the productivity of recombinant glucoamylase.

In the pH range 2.5–6.0, the specific gluco-amylase productivity and the specific growth rate of the *A. niger* strain BO-1 were independent of pH when grown in batch cultivations. The glucoamylase production increased linearly with the specific growth rate in the range 0–0.1 h⁻¹ and was constant in the range 0.1–0.2 h⁻¹ (Pedersen et al. 2000). Heterologous protein production processes should also avoid high temperatures, which hamper the proper folding of the heterologous protein and hence, negatively affect the yield (Fox et al. 2004).

Reducing proteolytic degradation by various bioprocessing strategies

Heterologous protein production by filamentous fungi is often limited by the expression of proteases at high levels. By eliminating specific protease activities, protein production in *Aspergillus niger* can be improved considerably. Reduced protease activities can be achieved by combining different approaches. One involves selecting or screening for fungal host strains with low protease activity. This can be accomplished either by direct selection, using “suicide” selection substrates, or by mutagenesis and screening for reduced protease activity on indicator plates. Other approaches are optimizing medium composition and fermentation protocols (Van den Hombergh et al. 1997b). Bioprocess strategies have also been shown to be of great help (O'Donnell et al. 2001; Xu et al. 2000) in reducing proteolytic degradation. In *A. niger* cultures, extracellular protease secretion was related to the morphological state.

A dramatic decrease in protease activity was found when the fungal cells grow as pellets instead of free mycelia. The inoculum level was found to be directly related to morphology. Researchers have seen a dramatic effect of morphology on protein production. Xu et al. (2000) investigated the dependence of protein production on fungal morphology in shake flasks cultures by employing the recombinant *A. niger* strain which carries a gene for the glucoamylase-green fluorescent protein (GFP) fusion protein. Different inoculum levels were used to obtain different sizes of pellet or free mycelia. The extracellular protease activity of the cultures varied with the pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets, resulting in a dramatic 3.4-fold increase in the GFP yield. An optimal inoculum level of 4×10^6 spores/ml resulted in a culture consisting of a pellet size of 1.6 mm, which produced a specific protease activity of 158 U/l and a specific GFP yield of 0.98 mg/g, much higher than the 0.29 mg/g produced in filamentous growth. The results indicated that a morphology control strategy could be effective in inhibiting protease activity in filamentous fungal fermentation, thereby enhancing heterologous protein production. Most extracellular proteases are repressed under the conditions of high glucose and ammonium levels in the medium. For example, the extracellular proteases of *A. nidulans* are subjected to carbon, nitrogen and sulfur metabolite reprocessing. The depletion of low molecular weight sources of nitrogen, carbon, phosphorus, and sulfur elevates protease activity (Van den Hombergh et al. 1997b; Katz et al. 1996). Since proteases were found to be responsible for the loss of many heterologous proteins, the regulation of proteases by carbon and nitrogen sources would benefit the heterologous protein production. Li et al. (2008) investigated and characterized extracellular proteases produced by a recombinant filamentous fungus, *Aspergillus niger* B1-D. It was found that the increased extracellular protease activity associated with elevated temperatures may also contribute to the negative effects of high temperature on heterologous protein yields. Their activity could be partially inhibited by protease inhibitors, indicating the existence of at least four kinds of proteases in the culture fluids, aspartic, serine, cysteine, and metallo-proteases. Denison (2000) proposed that production of extracellular proteases is regulated by ambient pH in *A. niger*. Similarly, O'Donnell et al. (2001) investigated a pH control strategy to inhibit extracellular protease activity and to enhance the heterologous protein production from the recombinant *A. niger*. Swift et al. (1998) reported that in the pH auxostat culture of *A. niger* B1, pH values had effects on morphological mutants formation and the recombinant glucoamylase production.

Liu et al. (1998) showed that cell immobilization drastically reduced the secretion of extracellular proteases of

A. niger. A metal-coated pad of polyester latex felt was found to be the best among the six types of immobilization material used for the *A. niger* culture in shake flasks. It was found that compared with the free suspension culture, the maximum specific activity of the extracellular proteases from immobilized cells was reduced from 129 to 28 units/g cell dry weight. A similar reduction in protease activity was also achieved with immobilized culture in a 3–l (working volume) airlift reactor (10 cm ID, 50 cm length) using a multi-layer rolled stainless steel screen in the center as both a draft tube and the immobilization substrate.

Conclusion

The filamentous fungi provide a seemingly inexhaustible supply of a variety of enzymes and potential for innovation, and the application of genetic transformation to their study and manipulation promises a healthy future for fungal biotechnology. In this review, the basic heterologous protein production system is analysed on both biological and technological factors to see where optimizations can be done for improving the product yield. It has been concluded that there seems to be no general way to improve the production of all proteins in a fungal system. Rather it seems that the improvement is more dependent on the properties of proteins being produced. To enhance the production of a given protein, the step causing the problem in the production should be assessed for each individual protein in order to get maximal yields. With the increasing information about the machinery of gene expression regulation and developments in the field of biotechnology, the currently available gene expression systems will be improved functionally, and one or more novel systems will emerge.

Moreover, the growing knowledge of fungal metabolism and the unique capabilities of filamentous fungi assure them a continued place among the most biotechnologically useful organisms known to humanity.

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