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Review

Genetic manipulation of fungal strains for the improvement of heterologous genes expression (a mini-review)

Mehdi Mehrabi Koushki[1], Hamid Rouhani[1] and Mohammad Farsi[2]

[1]Plant Protection Department, Ferdowsi University of mashhad, Iran.
[2]Department Of Biotechnology And Plant Breeding, Ferdowsi University of mashhad, Iran.

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Fungi are eukaryotic microorganisms that influence our everyday lives in areas as diverse as agriculture, medicine and basic science. With the advent of molecular biology, it has been attempted to improve the expression level of heterologous genes, which encode favorable traits in fungal strains. The expression of heterologous genes can be limited in transcription, post-transcription, translation and post-translation levels. Several genetic strategies have been developed to reduce the expression constrains and to enhance genes functionality. Among these strategies can be pointed to the introduction of multicopies of the desired gene, change of AT-rich sequences, gene fusion with a well-expressed gene, the use of strong promoters and signal sequences, optimization of codon usage, the construction and use of protease-deficient and chaperones/foldases-overproduced strains and the use of native or artificial intron-containing genes. These strategies have often resulted in the expected increase in the expression of heterologus genes. With the isolation of a large number of genes encoding desired traits and the availability of a large collection of wild isolates, the improvement of strains with a better functional performance would be possible.

Key words: Strain improvement, gene, heterologous, intron, promoter, codon.

INTRODUCTION

Fungi are eukaryotic microorganisms that influence our everyday lives in areas as diverse as agriculture, medicine and basic science. With the growth of the biotechnology industry, fungi have been employed for commercial production of biocontrol agents as well as high-level production of enzymes, proteins and different metabolites. For about 70 years, biocontrol fungi, such as *Trichoderma* strains, have been shown to be able to control phytopathogenic fungi through different mechanism (Harman, 2006; Harman et al., 2004). A large number of fungal strains have been used for producing antibiotics, drugs, recombinant proteins, organic acids and other useful compounds (Webster and Weber, 2007).

Most of wild type fungal strains have produced low level of genes products and it has been attempted to enhance the production of desired genes via genetic manipulation. Particularly, the initial level of production of heterologous fungal proteins have not been economical but, after optimizing the production process, applying mutagenesis and screening programs, production level have been increased (van den Hondel et al., 1991). For instance, the cellulase (CBHI) in *Trichoderma reesei* (Durand et al., 1988) and glucoamylase in *Aspergillus* spp. (Finkelstein and Ball, 1992) have been produced in quantities up to 30 gl^{-1}. Similarly, an alkaline protease of *Fusarium* have been produced to 4 gl^{-1} in *Acremonium chrysogenum* (Morita et al., 1994) and an aspartyl protease of *Mucor miehei* have been produced to 3 gl^{-1} in *Aspergillus oryzae* (Christensen et al., 1988). Also, *Aspergillus niger* has been shown to produce glucoamylase

*Corresponding author. E-mail: me_me484@stu-mail.um.ac.ir or mhdmhrb@gmail.com. Tel: +98 511 7621104 or +98 918 3605425. Fax: +98 511 7628330.

more than 20 gl^{-1} (Berka et al., 1991b).

The cumulative knowledge of fungal genetics and their biochemical pathways has been applied for strains improvement. Genetic improvement can be carried out by different methods such as chemical and physical mutation, sexual hybrids, homocaryons, directed mutagenesis, protoplast fusion, recombination and transformation (Nevalainen, 2001; Stasz, 1990; Haggag Wafaa and Mohamed, 2007). Random mutagenesis and screening has been carried out to improve production of antifungal metabolites and antagonistic potential of biocontrol agents in fungal strains such as *Trichoderma* spp. and *Gliocladium* spp. (Haggag Wafaa and Mohamed, 2007). Most filamentous fungi are transformed by plasmids that integrate into the fungal genome, suggesting potentially superior long-term stability of the fungal transformants. For these reasons, filamentous fungi have tremendous potential as hosts of recombinant DNA. The transformed strains of species such as; *Aspergillus* spp., *T. reesei*, *Chrysosporium lucknowense*, *Mortierella alpinis* have been used with success as a host for the production of recombinant proteins of both fungal and non-fungal origins (Mach and Zeilinger, 2003; Gouka et al., 1997; Van den Hombergh et al., 1997; Dunn-Coleman et al., 1991; Harkki et al., 1989; Nyyssonen et al., 1993). Genetic manipulation has successfully been employed to improve the biocontrol ability of biocontrol agents. In order to achieve this goal, the researchers have attempted to enhance antifungal metabolites productivity of fungal strains, improve antagonistic potential of biocontrol agents, control a broad spectrum of phytopathogens, increase their competitiveness potential and develop tolerant strains to stress conditions (Harman et al., 2004; Haggag Wafaa and Mohamed, 2007).

THE IMPROVEMENT OF HETEROLOGOUS GENES EXPRESSION IN FUNGAL STRAINS

The choice of a commercial fungal strain for a high-level expression of the desired genes, which produce high-level amounts of target proteins and metabolites, depends on many factors. They include growth conditions, the level of desired gene expression, post-translational modifications and biological activity of the desired metabolites. Although, the expressed productions of homologous genes by filamentous fungi can reach a considerable level, the production level of heterologous genes can be lower. Several factors that affect the production level of heterologus genes have been reported, showing that the production can be limited at any level that is, transcription, translation, secretion and extracellular degradation (Punt et al., 1994; Archer and Peberdy, 1997). Several strategies have been developed to improve heterologous gene production, both genetic and growth conditions in nature and bioreactors (Gouka et al., 1997). In this review, the main genetic strategies studied in different researches which have been effective in improving the strains, is discussed.

The introduction of multicopies of the desired gene

The simplest approach to improve the transcription level of the introduced gene is to increase its copy number. It seems that a high copy number of the heterologous expression cassette has in some cases been revealed to increase the production of heterologous genes. Analysis of *A. niger* and *A. nidulans* multicopy transformants for glucoamylase and alpha interferon-2 genes has shown a gene dosage dependent expression of glucoamylase and alpha interferon-2 up to about 20 and 10 copies respectively (Davies, 1990; Verdoes et al., 1993). Similarly, the amount of penicillin V and isopenicillin N produced by the high-yielding *Penicillium chrysogenum* DS17690 increased with the penicillin biosynthetic gene cluster number, but was saturated at high copy numbers (Nijland et al., 2010). Accomplished studies with the glucoamylase gene suggested that transcription in multi-copy transformants was limited due to titration of trans-acting regulatory proteins (Verdoes et al., 1994a) and was also suggested to be a limiting factor in the expression of other fungal genes (Margolles- Clark et al., 1996). In some cases, this limitation was overcome by raising the expression level of the regulatory gene as well. The biosynthesis of cell wall degrading enzymes (CWDEs) in *Trichoderma* spp. is controlled mostly at the transcriptional level (De la Cruz et al., 1993, 1995; Garcia et al., 1994) and their encoding genes are present as single-copy genes (Garcia et al., 1994; Lora et al., 1995). To overproduce these enzymes, their genes copy number has been increased by transformation (Herrera-Estrella et al., 1990; Kubicek-Pranz et al., 1991). Improvement in biocontrol effectiveness of *Trichoderma* spp. by transformation with genes *prb1* (basic protease) and *egl1* (β-1, 4-glucanase) has been illustrated by Flores et al. (1997) and Migheli et al. (1998). Transformation studies have demonstrated a relation between some mycoparasitism-related genes (such as *prb1*) and biocontrol potential. Increased biocontrol activity has been attained by integrating multiple copies of the *prb1* gene (proteinase) into the genome of *Trichoderma atroviride* (Flores et al., 1997). Introduction of multiple copies of the endochitinase *chit33* in *Trichoderma harzianum* also resulted in increased biocontrol ability (Dana et al., 2001). In contrast, due to the high copy number (12 to 14 copies) of glucose oxidase gene (*goxA*) in *T. atroviride* SJ3-4, this strain constitutively expressed a low level of glucose oxidase (Mach et al., 1999). Limon et al. (1999) have also showed that there is no relation between the number of integrated copies and the level of expression of the *chit33* gene in the transformants.

However, there seems to be no obvious correlation

between the copy number and the quantity of gene products (Fowler et al., 1990; Punt et al., 1991). The differences seen in the expression of heterlogous genes may partially be caused by the result of the integration place of the expression cassette in the genome (Graessle et al., 1997). The study of gene products level of transformants having a similar number of gene copies suggests that the site of integration also affects the expression of the introduced gene (Verdoes et al., 1993, 1994b, 1995). The effect of random integration can be overcome by targeting the expression vector to site of known high transcriptional activity, like to the loci for glucoamylase and CBHI in *A. niger* and *T. reesei* hosts, respectively. Harkki et al. (1991) and Nyyssonen and Keränen (1995) reported that in transformants giving the best production of endoglucanase I (EGI) and antibody Fab fragments, respectively, the expression constructs were incorporated into the *CBHI* locus. Variable expression in multi-copy integrants may in part be due to a gene silencing effect; several transcriptional and posttranscriptional silencing of duplicated genes copies have been observed in filamentous fungi such as *Neurospora crassa*, *Aspergillus nidulans* and *Aspergillus flavus* (Cogoni, 2001; Clutterbuck, 2004; Schmidt, 2004; Hammond and Keller, 2005).

Change of AT-rich sequences in desired gene

Limitation of heterologous genes expression at the transcriptional level can also be caused by partial processing of pre-mRNA. The recent researches suggested that AT-rich sequences in the coding regions of heterologous genes act as internal polyadenylation sequences and produce short and incomplete transcripts. It was reported, premature termination of transcription can be overcome by replacing an AT-rich sequence in gene with a more GC-rich sequence or changing codon usage (Romanos et al., 1992; Teo et al., 2000). For examples, when wild-type α-galactosidase (*aglA*) was expressed in *A. niger,* a shortened pre-mRNA was observed that contained only 200 bp of the *aglA* gene (Gouka et al., 1996). The truncated pre-mRNAs have been observed in *A. niger, A. nidulans, Pichia pastoris* (Scorer et al., 1993) and *Schizophylum commune* (Schuren and Wessels, 1998). AT-rich sequences have been also impeded expression of heterologous genes in *S. commune* (Schuren and Wessels, 1998). When prokaryotic reporter genes (β-glucuronidase, β-galactosidase) or resistance genes (hygromycin B phosphotransferase) (Schuren and Wessels, 1998) were expressed in this fungus, no full-length pre-mRNAs were detected, because, they were cut in the 5' part of the coding sequence at the point of AT-rich stretches. Similar results have been shown with the expression of the α-galactosidase (*aglA*) gene from *Cyamopsis tetragonoloba* in *A. niger* (Gouka et al., 1996). In all the mentioned cases, the partial processing of transcripts has been overcome by increasing the GC-content in the AT-rich area without changing the amino acid sequences of protein (replacing synonymous codons). When the α-galactosidase and β-galactosidase prokaryotic genes were expressed in *A. niger* (Gouka et al., 1996) and *S. commune* (Schuren and Wessels, 1998), truncation of pre-mRNAs was banned by replacing an AT-rich stretch with GC-rich stretch.

Desired gene fusion with a well-expressed genes

Limitation of heterologous gene expression at the transcriptional level can also be caused by a low mRNA stability. Many of mRNAs in eucaryotic cells are unstable, because they contain specific sequences (AU-rich) in the 3' untranslated region (UTR) that induce their degradation. This AU-rich sequence appears to accelerate mRNA degradation by stimulating the removal of the poly-A tail found at the 3' end of almost all eucaryotic mRNAs. Other unstable mRNAs contain recognition sites in their 3' UTR for specific endonucleases that cleave the mRNA.

The effects of integration place on the mRNA stability and expression of native and heterologous genes have been celebrated (Verdoes et al., 1995) possibly due to more active native transcription in the neighborhood of highly expressed genes (Davis and Hynes, 1991). Low mRNA stability can be partly overcome by fusing the desired gene to the 3' end of a homologous gene. Fusion of the gene to be expressed behind a highly expressed homologous gene does not only increase stability of the mRNAs, but can also resolve limitations at early stages in the secretion pathway (ER) of protein metabolites. For instance, the best-characterized fusions in fungi are those with the *T. reesei* cellobiohydrolase (CBHI) and *A. niger* or *Aspergillus awamori* glucoamylase (*glaA*) genes. This strategy has been used capably in the production of bovine chymosin (Dunn-Coleman et al., 1991), human interleukin 6 (Broekhuijsen et al., 1993), porcine pancreatic phospholipase A2 (Roberts et al., 1992), hen egg-white lysozyme (Jeenes et al., 1993), human lactoferrin (Ward et al., 1995) and the catalytic subunit of bovine enterokinase (Svetina et al., 2000). The production of antibody fragments in *T. reesei* as a CBHI fusion resulted in more than 150-fold increase in the yield (Nyyssonen and Keränen, 1995). Using glucoamylase of *A. niger* or cellobiohydrolase of *T. reesei* as a carrier increased level of secreted desired protein 5-1000 fold (Roberts et al., 1992; Broekhuijsen et al., 1993; Jeenes et al., 1993; Nyyssonen and Keränen, 1995; Ward et al., 1995). By fusing human interleukin-6 (*hil6*) or α-galactosidase (*aglAsyn*) genes behind the glucoamylase gene, transcription level of these genes increased compared with that of non-fused genes (Gouka et al., 1996). In contrast, Conesa et al. (2000) showed that the

expression of two fungal peroxidases, *Phanerochaete chrysosporium* lignin peroxidase H8 (*lipA*) and manganase peroxidase (*mnp1*), was not affected through fusion to the *glaA* gene.

The use of strong promoters in desired gene

A promoter for transgenic expression in fungal strains should have certain characteristics to be selected as a suitable promoter for a high-level expression of the interested gene. It must be strong (30% or more of the total gene products), exhibit a minimal level of basal transcriptional activity, be easily transferable to other vectors genomes and to be induced via simple, natural or cost-effective manner. Strong promoters have a high affinity for RNA polymerase. Promoter strength and specificity are characteristics of regulated endogenous gene expression and are necessary for genetic engineering of heterologous gene expression. Limited numbers of promoters allow high level of transgenic expression, usually by ubiquitous and constitutive expression in all tissue types.

The selection of a suitable promoter may be difficult, since original pathway regulation is interactive and some genes are regulated by several systems in concert, both global and pathway-specific. For example, secondary metabolism and asexual development are coordinately regulated by a complex signaling system (Yu and Keller, 2005); aflatoxin and penicillin pathway expression are influenced by pH, carbon and nitrogen sources (Calvo et al., 2002). However, at a molecular genetic level, It has been attempted to increase the biocontrol ability of fungi such as *Trichoderma* by increasing chitinase or proteinase activity either by increasing the number of copies of the appropriate genes or by fusing them with strong promoters (p*cbh1* and *ech42*) (Limon et al., 1999; Margolles-Clark et al., 1996). In large-scale enzymes production, well-known inducible promoters used in different 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., 1996a). Also, among constitutively expressed genes, the *A. nidulans gpd* promoter has been shown to be more common and functional in many filamentous fungi. To improve the production of *T. reesei* EGI in a hypercellulolytic mutant strain, the *egl1* promoter of *T. reesei* was exchanged with the *T. reesei cbh1* promoter and the copy number of the *egl1* gene was increased (Karhunen et al., 1993). It was also found that the strength of a promoter (*rbcS2*) could be improved by placing the other promoter (*HSP70A*) in front of it (Schroda et al., 2000).

In transformation constructs, the used promoters should be easily induced under natural or simple condition. For example, the availability of a versatile expression system for *T. atroviride* based on application of recently characterized biocontrol-related promoters (Mach et al., 1999; Zeilinger et al., 1999) suggested that it might be possible to improve the biocontrol agents. It have been shown that genes implicated in mycoparasitism by *T. atroviride* contain motifs in the promoter region, nominated MYRE1- MYRE4, that are proposed to act as binding sites for a global inducer of the mycoparasitic response (Steyaert et al., 2004). Carbon catabolite repression through binding of Cre1 to the promoter (Lorito et al., 1996) is considered the major negative regulator of the mycoparasitic response. Nitrogen repression of *prb1* gene (proteinase) more recently was demonstrated (Olmedo_Monfil et al., 2002). Zeilinger et al. (1999) reported that the *nag1* gene is active after *Trichoderma* hyphae contact their host but it is not expressed constitutively. Brunner et al. (2005) determined whether transgenic strain SJ3-4 of *T. atroviride*, which expresses the *A. niger* glucose oxidase-encoding gene, *goxA*, under a homologous chitinase (*nag1*) promoter had increased capabilities as a fungal biocontrol agent. *goxA* expression occurred immediately after contact with the plant pathogen and the glucose oxidase was secreted (Brunner et al., 2005). Seidl et al. (2006) analysed the stimulation of β-N-acetyl-glucosaminidase formation in *Hypocrea atroviridis* influenced by various carbon sources and showed that NAGase activities were enhanced on carbon sources sharing certain structural properties, especially on α-glucans (for example, glycogen, dextrin and maltotriose) and oligosaccharides containing galactose. Gene expression of cell wall-degrading enzymes (CWDEs) in *Trichoderma* spp. frequently has been reported to be induced by fungal cell wall components and repressed by carbon catabolite repressors, such as glucose (Carsolio et al., 1994; Donzelli et al., 2001; Elad et al., 1982; Peterbauer et al., 1996; Tronsmo and Harman, 1992). In some cases, starvation conditions alone could trigger CWDEs production (Ramot et al., 2000), while in others, cell walls or cell wall components were needed (Elad et al., 1982). The transcription factor Cre1 (Ilmen et al., 1996) can bind to the upstream regulatory region of the gene (ech42) encoding the endochitinase CHIT42 (Carsolio et al., 1994; Garcia et al., 1994; Hayes et al., 1994) from *T. atroviride*, suggesting that this factor may be involved in glucose repression in *Trichoderma* (Lorito et al., 1996). Upstream regulatory regions of gluc78 (which encodes GLUC78, a 78-kDa exo-1,3- -glucosidase) (Donzelli et al., 2001), ech42 and nag1 (which encodes CHIT73, a 73- kDa N-acetyl-hexosaminidase) (Peterbauer et al., 1996) from *T. atroviride* contain clustered putative binding sites, such as GATA, stress response elements, BrlA response elements and AbaA response elements, suggesting that they are regulated by a number of stimuli. A study showed that complex response elements existing in the

promoter region of the *Antarctosaurus giganteus* AFP gene (encoded a antifungal protein) are responsible for its efficient transcription in its expression vectors (Meyer et al., 2002).

The use of signal sequences of well-expressed genes in desired gene

The use of signal sequences from well-expressed genes can increase some gene products (Van den Hondel et al., 1991). On the other hand, the origin of the signal sequence does not reveal to cause a difference to the gene products. For example, in *A. nidulans*, the use of the signal sequence of either *glaA* or chymosin resulted in similar amounts of secreted chymosin (Cullen et al., 1987). The production of the *Fusarium solani pisi* cutinase in *A. awamori* did not need the use of cutinase pro-sequence since the use of the endogenous *exlA* leader peptide led to similar levels of production (van Gemeren et al., 1996). Also, in several researches, the effect of naturally occurring pro-sequences on the amount of gene products has been studied. A positive effect of a pro-sequence on gene products has been observed in the case of restrictocin protein (Brandhorst and Kenealy, 1995) and a negative effect in the production of cutinase and chymosin proteins (van Gemeren et al., 1996).

Optimization of codon usage of desired gene

Genes in both prokaryotes and eukaryotes show a nonrandom usage of synonymous codons (Gouy and Gautier, 1982; Ikemura, 1985; Sharp et al., 1988). In many prokaryotes and eukaryotes, well-expressed genes are highly biased towards a subset of the present synonymous codons (Karlin and Mrazek, 2000). It was early revealed that a significant heterogeneity in the codon usage exists among genes within species and the grade of codon bias is positively correlated with gene expression (Karlin and Mrazek, 2000). It is suggested that optimal codons assist to attain faster translation rates and high accuracy. Different factors have been suggested to be related to codon usage bias, including gene expression level (showing selection for optimizing translation process by tRNA abundance), %G+C composition (showing horizontal gene transfer or mutational bias), GC skew (showing strand-specific mutational bias), amino acid conservation, protein hydropathy, transcriptional selection, RNA stability, optimal growth temperature and hypersaline adaptation (Ikemura, 1981; Sharp and Li, 1987; Wright, 1990; Carbone et al., 2003; Wu et al., 2005; Puigbo et al., 2007; Ramazzotti et al., 2007).

It is possible to determine the optimal codons for any organism by analyzing and comparing the codon usage of genes encoding known highly-expressed versus low-expressed genes. Knowledge of the optimal codons used by an expression host allows the recognition of codons existed in desired recombinant genes that may limit the expression level of their encoded protein. Although, the use of synonymous codons vary widely between different genes and organisms, it can be optimized the codon usage of the desired gene to improve expression in each used transformants. Genes can then be manipulated with rare codons replaced by their optimal synonyms and then reconstructed by overlap extension of synthetic oligonucleotides. For instance, primary introduction of the native family 11 xylanase gene (*xynB*) from *Dictyoglomus thermophilum* Rt46B.1 into *T. reesei* by biolistic bombardment using tungsten microprojectiles showed no production of the enzyme (Hazell et al., 2000; Te'o et al., 2002) because of excessive differences in the codon usage between the heterologous gene and the expression host could prevent expression at the transcriptional level. But, after reconstructing the *D. thermophilum xynB* gene to accommodate the codon usage pattern of *Trichoderma,* northern blot analysis revealed the existence of mRNA transcripts of the expected size in the transformants tested (Te'o et al., 2000). They overcame this basic problem with constructing a synthetic xynB gene carrying changes in 20 codons was generated by advanced primer extension PCR using the codon preference of highly-expressed *T. reesei* genes (Te'o et al., 2000).

The construction and use of protease-deficient fungal strains

In fungi, protein folding, glycosylation, the formation of disulphide bridges, phosphorylation and subunit assembly are achieving in the endoplasmic reticulum (ER). ER-related events contribute to the folding of proteins (Penttila et al., 2004). The unfolded protein response mechanism detects the presence of unfolded proteins in the ER and induces the synthesis of folding enzymes (Conesa et al., 2001). Therefore, after mRNA translating, the carriers accomplish the translocation of the produced proteins into the ER; allow accurate folding and thereby protecting them from protease degradation. It has been shown that proteases are responsible for the degradation of many heterologous proteins (Van den Hombergh et al., 1997). Fungi can produce a diversity of extracellular proteases. Extracellular proteases of *Aspergillus*, such as aspergillopepsin (Berka et al., 1990), are responsible for the degradation of many heterologous proteins (Broekhuijsen et al., 1993). Intracellular or cell-wall localized proteases can also be responsible for low yields of secreted heterologous proteins as in the case of hIL-6 in *A. awamori* (Gouka et al., 1996).

So far, most researchers focused their efforts on selecting and using protease deficient strains as hosts for

recombinant DNA to overcome protease degradation problems. However, such strains are usually so debilitated and unstable for large-scale bioreactor and biocontrol applications. Therefore, it is often essential to match the protease loss of function strain to the desired protein and identify genes involved in regulating extracellular protease secretion, since knocking out all native proteases would likely be lethal (Katz et al., 2000). Fungal strains deficient in extracellular proteases have been constructed by random mutagenesis (Mattern et al., 1992; van den Hombergh et al., 1997) or molecular genetic approaches (Berka, 1990; van den Hombergh et al., 1997). The use of these protease deficient strains has resulted in the improvement of production level of heterologous proteins (Berka, 1991; Roberts et al., 1992; Broekhuijsen et al., 1993). The use of strains deficient in vacuolar proteases has led to increased level of heterologous proteins by *Saccharomyces cerevisiae* (Wingfield and Dickinson, 1993). The production of chymosin as well as porcine phospholipase 2 was improved in *A. awamori* by removal of the *pepA* gene encoding the aspartic proteinase aspergillopepsin A (Berka et al., 1990; Roberts et al., 1992). Also, a newly described species, *Aspergillus vadensis*, shows promise as a heterologous host due to its low native protease level (de Vries et al., 2004).

The construction and use of chaperones/foldases-overproduced fungal strains

Molecular chaperones and foldases help in the folding of newly synthesized proteins and they prevent transport of proteins to other compartments before they are folded properly. Incorrectly folded proteins are recognized by an unknown mechanism and are degraded by proteases in the cytoplasm. In normal cells, the concentrations of these foldases and chaperones are likely to be sufficient for proper folding, but in transgenic strains which some transformed gene are overexpressed, there is a greater flux of desired proteins being translocated into the ER, the folding, assembly, and secretion machinery may become saturated, leading to improperly folded structures or protein aggregates which are not secreted. One approach to overcome the problem of protein aggregation is to overexpress chaperones or foldases in the strains ER (Coux et al., 1996).

The use of native or artificial intron-containing genes in fungal strains

The requirement of introns for efficient mRNA cytoplasmic accumulation has been indicated for many eukaryotic genes (Nesic et al., 1993). It has been suggested that introns regulate gene expression at transcription (Morello et al., 2002) and post-transcription (Sivak et al., 1999) levels. It has been suggested that the existence of introns might protect pre- mRNA from undergoing degradation in the nucleus, facilitate polyadenylation or transport mRNA to the cytoplasm (Liu and Mertz, 1995). Moreover, it has also been shown that certain genes cannot be expressed without their introns (Jonsson et al., 1992). Two possible mechanisms for intron dependent mRNA accumulation are commonly accepted. One is that introns protect the pre-mRNA from degradation (Kurachi et al., 1995), either by accepting a stable secondary structure or by providing binding sites for factors protecting the pre-mRNA, such as oligo-U binding proteins (Gniadkowski et al., 1996) or heterogeneous nuclear ribonucleoproteins (Krecic and Swanson, 1999). Other mechanism is that spliceosome assembly onto the introns in pre-mRNA facilitates an association with enzymes involved in other aspects of RNA maturation (such as polyadenylation) and transport of the mRNA to the cytoplasm.

The phenomenon of intron-dependent mRNA accumulation was revealed to occur in mammalian and plant cells (Koziel et al., 1996). It also proposed that the small introns in the genes filamentous fungi may not only act as intervening elements, but may also play vital roles in gene expression by increasing the stability of the mRNA or by assisting the export of mRNA (Xu and Gong, 2003). In the ascomycete *P. anserine*, it was reported that the existence of at least one intron was needed for the expression of the gene encoding the ribosomal protein S12 (Dequard-Chablat and Rotig, 1997). It demonstrated that introduction of introns in modified *hph*-gene (replacing the AT-rich coding region with a more GC-rich sequence) resulted in even higher mRNA level in *Schizophyllum commune* (Schuren and Wessels, 1998). It was also shown that adding of one intron outside the translational unit of the *SC3* cDNA was enough to enhance *SC3* mRNA accumulation to a level similar to that observed with the genomic *SC3* gene which naturally contains five introns (Lugones et al., 1999a). In a trial, after introducing the coding genes of *ABH1* (*Agaricus bisporus*), *SC3*, *SC6* (*S. commune*) and *GFP* (*Aequorea victoria*), without introns, no or very low expression level were seen contrasting with the expression of intron-containing genes (Scholtmeijer et al., 2001). Xu and Gong (2003) showed that introns are required for AFP gene (a gene encoding antifungal protein in *A. giganteus*) expression in *T. atroviride* transformants, as demonstrated by the level of mRNA and confirmed by analysis of AFP synthesis. In contrast, in the ascomycetous yeast *S. cerevisiae* only 2 to 5% of the genes contain introns and no obligation for introns has been observed for vertebrate and plant genes expression (Hiraiwa et al., 1997; Lin et al., 1997). However, less is even known about intron-dependent mRNA accumulation in fungi and further researches should be accomplished.

The glycosylation of produced heterologous proteins

There are some evidences that improper glycosylation of

glycoproteins has been suggested as a problem for heterologous protein processing. Engineering protein glycosylation sites for improved usage *in vivo* has made large improvements in yield of several glycoproteins (van den Brink et al., 2006). N-glycosylation has been shown to improve the amount of secreted chymosin in *A. awamori*, although, the specific activity of the chymosin was reduced (Ward, 1989). It has been reported that the overexpression of mannosylphospho dolichol synthase encoding gene from *S. cerevisiae* in *T. reesei*, which is needed for O-glycan precursor synthesis, improved the production of CBHI (Kruszewska et al., 1999). Overexpression of a yeast glycosylating enzyme has improved protein yields in an *A. nidulans* mutant impaired in glycosylation (Perlinska-Lenart et al., 2005). 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. These observations could indicate that the problems encountered in glycoprotein production could be reducing by manipulating glycosylation. Systematic alteration of all three glycosylation sites in *T. reesei* transformant expressing XynB gene showed that the amounts of XynB increased since all three sites were eliminated but the activity of the enzyme was not affected (Teo et al., 2000). Earlier studies with endogenous *Trichoderma* cellulases have indicated that N-glycosylation of cellulases of *T. reesei* was not required for enzyme activity and secretion (Kubicek et al., 1987), but had an effect on their thermostability and resistance to proteolysis (Merivuori et al., 1985; Wang et al., 1996).

CONCLUSION

In this review, strategies were discussed to illustrate a few research focused on genetic manipulating of fungal strains. Fungi have long been employed in the fermentation industry as principal source of useful proteins such as antibiotics and enzymes and as biological control agents. Advances within the last decades in molecular genetics of fungi have provided commercially promising recombinant fungal strains, thus, led to a new era in fermentation biotechnology. Biotechnological production of small and highly volume drugs, chemicals and bioproducts is well justified economically. In field of biological control, the main reason of the scarcity of the broad-spectrum commercial use of biocontrol fungi is their lower efficiency and reliability compared with chemical fungicides. Many genetically improved strains have been produced, but few of them have yet been tested under field conditions.

With the isolation of a large number of genes encoding favorable traits and the availability of a large collection of wild type isolates, the breeding of strains with a better functional performance and an increase of the genetic diversity among them may be expected in the near future. Nevertheless, more researches are required to elucidate complexities observed about genetic stability and the expression of heterologous genes in fungal strains.

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