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Heterologous Protein Expression in Yeasts and Filamentous Fungi

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11.1. INTRODUCTION

Development of recombinant DNA technologies in the late 1970s laid the foundation for the advancement of various heterologous protein expression systems, including those based on microbial platforms. In the early 1980s, *Escherichia coli* and *Saccharomyces cerevisiae* expression systems were developed and used for heterologous protein expression (47). Many comprehensive reviews of these two expression systems are available (12, 29, 41, 48, 60, 72, 74, 110, 134). Development of other microbial expression systems, such as the methylotrophic yeast *Pichia pastoris* (72) and filamentous fungi (74), followed quickly, and the broad utility of these systems is becoming increasingly apparent.

Heterologous protein expression technologies are widely used in both academic research and industrial applications. In basic research, heterologous gene expression serves as an important tool to prepare proteins and enzymes that are either at limited levels or difficult to purify in their native context. Heterologously expressed proteins and enzymes are used across the entire spectrum of biology. Many enzymes and proteins are of commercial importance, and therefore improved processes for large-scale production of industrial enzymes and proteins are of great interest. Since different enzymes and proteins possess different molecular and biochemical characteristics, different expression systems with specific features are required to meet the growing need for heterologous protein expression at the level of both basic and industrial research.

With the steady advancement in gene expression technology, such as new vectors for gene delivery, improvement in throughput of screening for strain selection, and genetically engineered hosts, production of recombinant proteins has become more efficient with broader applications. In addition, introduction of fusion tags such as hexa-histidine (6× His) and inducible promoters have allowed rapid detection and purification of recombinant proteins and increased expression titers. Since enzymes and proteins have diverse physical and biochemical properties, expression yields vary widely from microgram to low-milligram (37, 57) to multi-gram levels (62). Choosing a suitable expression system for a protein of interest is one of the most critical steps for the development of a high-expression process. Some advantages and drawbacks for commonly used heterologous protein expression systems are listed in Table 1 (138). *E. coli* expression systems provide quick and easy molecular manipulation and, in general, require low-cost growth media. However, the protein expression in *E. coli* cannot be used if foreign proteins require transcriptional and/or posttranslational processing and modifications. By contrast, fungal protein expression systems including yeast and filamentous fungi can provide high expression levels and posttranscriptional and posttranslational processes and modifications of eukaryotic proteins. Similar to *E. coli* protein expression systems, fungal expression systems need low-cost culture media, the technologies needed for scale-up of fungal fermentation processes are well developed, and the organisms are Generally Regarded As Safe (GRAS) (24, 127). In contrast, insect and mammalian expression systems require special culture conditions and relatively high-cost culture media and often have lower expression titers.

When choosing a heterologous protein expression system, the following general criteria should be considered: (i) requirement of translational modification and processing of the protein; (ii) authenticity of the expressed proteins; (iii) protein expression level and scale-up requirement; (iv) localization, e.g., intracellular, membrane-bound, or secreted proteins; (v) cofactor requirement; and (vi) cost, safety, and other regulatory-related issues. Each protein expression system offers a unique set of properties, and for successful production of a heterologous protein, it is often required to tailor a process specifically based on the properties of the protein to be produced and the expression system used.

Many filamentous fungi and yeast species have been used for heterologous protein expression (32, 34, 53, 54, 89, 94, 101, 105, 110, 112, 132, 133). There are numerous reviews addressing foreign protein expressions in each of the fungal protein expression systems (14, 20, 21, 44, 63, 101, 107, 110). Most reviews are focused on one specific system. Furthermore, some address specific aspects of the expression system, such as protein secretion in *S. cerevisiae* (121) and glycosylation of proteins in *P. pastoris* (11). Significant progress has been made in recent years in the production of therapeutic proteins and antibodies using humanized yeast (40). This chapter covers the most commonly used fungal expression systems (*S. cerevisiae*, *P. pastoris*, and *Aspergillus* species), with a focus on vector systems, promoter and leader sequences, posttranslational modifications, and fermentation scalability.

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TABLE 1 Pros and cons for some commonly used heterologous protein expression systems

Expression hosts	Pros	Cons
E. coli	Easy genetic manipulation, rapid growth, simple media requirements and low cost to grow, no human virus carried	Inclusion bodies, no secretion into media, lack of posttranslational process
S. cerevisiae	Low-cost culture and easy to grow, genetic regulation well-known, secretion protein, no risk for human virus infection	Overglycosylation at N-linked sites, cell difficult to break, expression level limited
P. pastoris	High-level expression, low cost and easy to grow, genetic regulation well-known, secretion protein, no risk for human virus infection	Royalties can be expensive, potential for overglycosylation, cell difficult to break
Filamentous fungi	High level of expression, good secretion system, some level of posttranslational modification, culture medium is inexpensive	No good commercial vectors are available, longer culture time, spores are concerns for health problems, not well documented for production of therapeutic proteins
Insect cells	Easy to grow up to 400 liters, easy to manipulate genetically for baculovirus, can grow in the same-style tanks as mammalian cells	Relatively low expression, serum-free medium is expensive, royalties for the commercially available systems can be costly, few contractors have current Good Manufacturing Practice experience
Mammalian cell lines	Authentic protein expression and proper posttranslational processing and assembly for therapeutic proteins and other mammalian proteins of interest, extensively used, reasonable expression level, endotoxin is not a concern	Medium is expensive, special tank, require CO2, potential risk of human virus in culture, longer expression time

11.2. *S. CEREVISIAE* FOR HETEROLOGOUS PROTEIN EXPRESSION

S. cerevisiae, a unicellular yeast, is one of the most studied eukaryotic microorganisms. Its genetics, physiology, biochemistry, metabolism, and fermentation are well researched and a wealth of information is readily available. Thousands of genes from *S. cerevisiae* have been characterized (43) and its genome sequence has been determined (33). *S. cerevisiae* has been extensively used as a host for heterologous gene expression of eukaryotic proteins for research and industrial applications (44, 110).

S. cerevisiae is a GRAS organism and has been used for centuries in the brewing and baking industries. Similar to *E. coli*, *S. cerevisiae* requires simple culture media for growth and is easily scaled up for large-volume fermentations. On the other hand, unlike *E. coli*, *S. cerevisiae* is capable of accomplishing many posttranslational modifications, such as proteolytic processing, disulfide bond formation, glycosylation, and other posttranscriptional and translational processing unique to eukaryotic organisms. Many vector systems containing a variety of promoters and auxotrophic or dominant selectable markers have been developed allowing constitutive or regulated gene expression. Several methods for transformation of foreign DNA into *S. cerevisiae* with high efficiency have also been developed (31, 85). As a result, *S. cerevisiae* is one of the most widely used microbial hosts for heterologous gene expression. However, there are limitations to using *S. cerevisiae* for heterologous gene expression; for example, the specific rate of production can vary from protein to protein, ranging from as low as 0.03 g/kg/h to as high as 4.5 g/kg/h (44). The use of episomal vector systems for heterologous protein expression in *S. cerevisiae* often results in plasmid instability during fermentation, which may lead to a lower growth rate and reduced overall protein yield. Furthermore, since many proteins of therapeutic interest are secreted glycoproteins, the tendency of *S. cerevisiae* to hyperglycosylate proteins not only reduces the efficiency of protein secretion but also may lead to undesired changes in the immunogenic properties or biological activities of the expressed proteins (23, 39, 40, 44, 84).

11.2.1. Vectors

S. cerevisiae expression vectors usually are shuttle plasmids that contain sequences for propagation and selection in both *S. cerevisiae* and *E. coli*. Two types of vectors have been described based on their mode of replication: episomal and integrating vectors (Table 2) (16, 18, 27, 45, 73, 86, 108, 109, 111, 138). Episomal vectors can be characterized according to their copy numbers, mode of replication, and stability. There are three major types of episomal vectors: YRp, YCp, and YEp. YRp-type vectors contain an autonomous replication sequence from the *S. cerevisiae* genome and have an average copy number of 1 to 10 per cell, even though higher copy numbers (up to 100 copies per cell) have been reported (44). These vectors are unstable, and plasmid loss rates can be as high as 10% per generation (44). This instability greatly limits their use in large-scale fermentation processes. YCp-type vectors are derived from the incorporation of *S. cerevisiae* centromeres into YRp plasmids and have improved plasmid stability. However, YCp-type vectors have lower copy numbers, typically at 1 to 2 copies per cell. The most commonly used episomal vectors are of the YEp type derived from the naturally occurring plasmid called 2μ circle in *S. cerevisiae*. These vectors are present at an average of 40 copies per cell and exhibit higher stability than the YRp and YCp vectors (5, 16). Consequently, YEp vectors are the best-developed vectors for heterologous gene expression in *S. cerevisiae*. These vectors are especially useful for controlled expression of toxic proteins. More than 80 compact expression vectors have been developed based

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TABLE 2 Vector systems used for heterologous protein expression in *S. cerevisiae*

Vector	Copy number per cell	Reference
Episomal		
YEp: 2μ-based	25–200	27
YCp: Centromere	1–2	18
YRp: Replicating	1–20	86
Regulated copy number	3–100	16
Integrating		
YIp	>1	45
rDNA-integrating	100–200	73
*Ty*δ	<20	111
Transplacement	1	109

on the pRS series of centromeric and 2μ plasmids (26). The GATEWAY vectors (Invitrogen Life Technologies, Carlsbad, CA) incorporated some of these features into their systems in order to provide fast cloning and transfer of genes to different vectors for heterologous expression in most strains of *S. cerevisiae*. Other 2μ-based expression vectors available from commercial sources include YEpFLAG-1 from Sigma (St. Louis, MO), YES vectors from Invitrogen, and pESC vectors from Stratagene (La Jolla, CA).

Integrative vectors contain selectable *S. cerevisiae* genes and lack sequences for autonomous replication. These vectors are highly stable but usually present at low copy numbers. They include the YIp-type vectors, and their integration can be directed by homologous recombination between sequences carried on the plasmid and its homologous counterpart in the *S. cerevisiae* genome. Some integration vector systems use repetitive elements such as delta sequences, *Ty* elements, or tRNA genes for heterologous gene integration (119). A system for multiple-site integration of a heterologous gene into the ribosomal DNA <QU1> cluster of 9.1-kb segment has also been developed (73). Stability of the genome-integrated expression cassette can be maintained under selection. However, a decrease of copy numbers of the integrated expression cassette was observed under nonselective culture conditions (44).

11.2.2. Promoters

An array of *S. cerevisiae* promoters has been used for heterologous gene expression. There are constitutive promoters derived from genes such as *CYC1* (cytochrome C <QU2> oxidase), *TEF2* (translation elongation factor), and *GPD* (glyceraldehyde-3-phosphate dehydrogenase), as well as regulated promoters such as *GAL1* (galactokinase/galactose epimerase 1), *ADH2* (alcohol dehydrogenase 2), *CUP1* (metallothionein), and *PHO5* (acid phosphatase) (6, 10, 15, 46, 51, 55, 56, 58, 65, 66, 80, 97, 98, 124). It is often advantageous for improving expression titer to couple the protein production phase with cell growth phases if there is no toxicity issue for the protein of interest. Some inducible promoter systems that work well at the shake flask scale, such as temperature-regulated promoters, do not always function well in large-scale fermentation due to operational restraints (44). Hybrid promoters such as *GAP/GAL* and *GAL10/CYC1* have also been reported (6). Several reviews on promoters and their applications in *S. cerevisiae* are available (108, 128, 138).

11.2.3. Transcriptional and Translational Regulation

In order to fully utilize the transcriptional, translational, and posttranslational regulatory machinery and to get efficient heterologous gene expression in *S. cerevisiae*, it is very important to match the expressed protein with the proper expression vector system. There have been many studies and extensive reviews on these aspects (22, 44, 82, 123). Generally, the use of strong promoters and robust transcriptional terminators of *S. cerevisiae* is essential for maximal expression since most prokaryotic or higher eukaryotic transcription terminators are not active in *S. cerevisiae*. More importantly, mRNA levels are controlled by the rate of transcription initiation and the transcript turnover rate (mRNA stability). In addition to upstream regulatory elements, downstream activation sequences are also required for maximum transcription initiation in *S. cerevisiae* (123). Expression levels of some foreign genes with high AT content (>60%) in *S. cerevisiae* can be low or absent due to incomplete transcript elongation (108). Transcript-destabilizing sequence elements have been reported in the 5′ nontranslated regions, coding regions, and 3′ nontranslated regions of different mRNAs (22). Therefore, if the titer is low due to the instability of mRNA, it may be necessary to use a stronger promoter, to delete the transcript-destabilizing sequence elements, and to introduce alternative codons. It has been shown that secondary structure of mRNA is one of the most important factors affecting the rate of initiation and translational elongation (123). For secreted proteins, posttranslational processes such as proteolytic cleavage, N-terminal modification, and glycosylation are critical to both proper protein folding and high protein yields. Consequently, it is worthwhile to evaluate the effect of the posttranslational steps when strong promoters and different strains fail to produce desired product yields.

S. cerevisiae has been used for expression of many eukaryotic proteins in both intracellular and secreted forms. For production of secreted proteins, secretion signals can come from either native protein signal sequences or from *S. cerevisiae* secretion protein signal sequences. Due to the limited studies available in the literature on the effects of different signal peptides on the yield of heterologous protein expression in *S. cerevisiae* (120), it is a good starting point to include the signal sequence of the foreign protein in the initial test of expression cassettes. Alternatively, an *S. cerevisiae* signal from such genes as invertase (SUC2, 19 <QU3> amino acids), acid phosphatase (PHO5, 17 amino acids), and the most widely used α-factor pheromone (MFα1, 20 amino acids) can be incorporated into the recombinant construct(s).

An important step among posttranslational modification processes is glycosylation. Heterologous glycoproteins expressed in *S. cerevisiae* are glycosylated at both N-linked and O-linked sites (64). Little is known about O-linked glycosylation, but more information is available on the process of N glycosylation. In *S. cerevisiae*, a core sugar moiety consisting of two *N*-acetylglucosamines (GlcNAc), nine mannoses, and three glucoses is added to the N-amide of asparagine at the Asn-X-Ser/Thr sequence in the endoplasmic reticulum (ER) in the early process of N glycosylation (67). Three glucose residues and one mannose residue are subsequently removed in the early Golgi apparatus. It is in the Golgi apparatus where further modification takes place that results in major differences between *S. cerevisiae* and higher eukaryotes in oligosaccharide structures. In higher eukaryotes, additional mannose residues are removed and several other sugars such as galactose, sialic

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acid, and fucose are added (39, 40). *S. cerevisiae* maintains the core mannose scaffold and is often extended with a large number of additional mannose residues. This often results in a hyperglycosylated outer chain containing more than 50 mannose residues with many branch chains. Many examples of hyperglycosylation of heterologous proteins in *S. cerevisiae* have been reported (23, 68, 84). Since specific glycosylation patterns are important for the immune response in mammalian systems, wild-type *S. cerevisiae* is not a good system for the production of therapeutic proteins such as monoclonal antibodies that have complex sugar structures.

11.2.4. Industrial Protein Production in *S. cerevisiae*

Many heterologous proteins have been produced in *S. cerevisiae* and large-scale fermentation processes have been developed (43, 108). Nevertheless, most heterologous <QU4> protein production in *S. cerevisiae* remains in shaker flask cell cultures. The first therapeutic recombinant protein expressed in *S. cerevisiae* was human alpha interferon (47). Since then, many other therapeutic proteins have been expressed in *S. cerevisiae*. These include the hepatitis B surface antigen (126), human insulin (122), the human papillomavirus (HPV) vaccine Gardasil™ (93, 135), and many others. In the case of Gardasil, the four vaccine components were expressed in *S. cerevisiae*, which were transformed by pGAL110-based yeast expression vectors, each containing the gene of interest coding for HPV L1 types 6, 11, 16, or 18 (75, 104). An excellent review by Vasavada (128) details a comprehensive list of foreign proteins expressed in *S. cerevisiae* at small and large industrial scale. Various strategies have been reported to improve the expression levels of different heterologous proteins in *S. cerevisiae*, such as screening of various host strains with different expression vectors, altering host genetics by molecular engineering, and optimization of fermentation conditions (83, 115, 128). Since there are no defined conditions that can be used for all protein production, it is a general rule of thumb to examine different parameters to develop an optimized process for heterologous protein expression in *S. cerevisiae*.

11.3. *P. PASTORIS* FOR HETEROLOGOUS PROTEIN EXPRESSION

Pichia expression systems have been widely used for expression of heterologous proteins from a diverse array of organisms, including bacteria, fungi, protists, plants, invertebrates, nonhuman vertebrates, and humans (72). Similar to *S. cerevisiae*, protein expression systems in *Pichia* have the advantages of well-established, cost-effective fermentation technology and comprehensive understanding of the host molecular genetics. *P. pastoris* is a methylotrophic yeast and has strong preference for respiratory growth (72). Cell cultures can reach extremely high density, with dry weight mass reaching as high as 100 g/liter (44). Most of the *Pichia*-based protein expression systems use a tightly controlled and highly inducible promoter derived from the alcohol oxidase 1 gene (*AOX1*). Under methanol-induced conditions, a single protein such as alcohol oxidase can reach as high as 30% of the total cell proteins (44, 72). Similar methods used for the molecular genetic manipulation for *S. cerevisiae* can be applied to *P. pastoris*. Extensive studies have been published on the use of *Pichia* systems in heterologous protein expression. A recent review article by Cregg et al. (14, 20) listed 241 individual studies of heterologous proteins expressed in *P. pastoris*, more than 80 of which were human proteins. Expression levels for heterologous proteins differ greatly, ranging from microgram-per-liter to gram-per-liter levels. For example, a hydroxynitrile lyase from a tropical rubber plant (*Hevea brasiliensis*) was expressed at levels as high as 22 g/liter (42). There are numerous reports on heterologous protein expression in *P. pastoris* (9, 91, 94, 107, 113, 114, 130, 138).

11.3.1. Expression Vectors and Host Strains

A fully developed *P. pastoris* expression system is commercially available (Invitrogen). The *Pichia* vectors can be grouped into two categories based on their promoters used: (i) inducible promoters derived from the *AOX1* gene or the formaldehyde dehydrogenase gene (*FLD1*) (117), and (ii) a strong constitutive promoter derived from the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*). The methanol-inducible *AOX1* <QU5> promoter provides tighter control of gene expression. The *FLD1* promoter can be induced by either methanol as the sole carbon source or certain methylated amines such as methylamine as the sole nitrogen source. Alternatively, the constitutive *GAP* promoter does not require cultures to be shifted from one carbon source to another for the induction of heterologous gene expression. However, if the foreign proteins to be expressed are toxic to the host cells, constitutive expression of the enzyme or proteins may cause inhibition of cell growth or result in loss of the expression vector from the cell.

There are no stable episomal expression vectors available in *P. pastoris*, and the expression genes need to be integrated into the genome to obtain stable expression strains. The simplest way of integration is single crossover homologous recombination by digesting the vector at a unique site with a restriction enzyme within either the marker gene (e.g., *HIS4*) or the promoter sequences (e.g., *AOX1*) (117). In <QU6> the case of *HIS4* as the selectable marker, transformants of auxotrophic mutant strain (his4) can be selected for His^+ cell growth. Alternatively, expression strains can be generated with *AOX1* flanking sequences at both the 5′ and 3′ terminals by cutting the vector in the *AOX1* sequence region. The flanking *AOX1* sequences at both ends can stimulate gene replacement events at the *AOX1* site in the *Pichia* genome, and this can result in a gene replacement integration. Heterologous proteins expressed in *P. pastoris* can be retained inside cells (intracellular expression) or secreted into medium depending on signal sequences in the expression vector systems. Commercial vectors for secreted <QU7> protein expression often carry a leader sequence from α-MF (mating factor) of *S. cerevisiae* as a secretion signal (117). Other leader sequences, such as the alkaline phosphatase (PHO) signal sequence from *P. pastoris* and some native protein leader sequences, have also been used for secretion of foreign proteins in *Pichia* (79). To facilitate protein detection and purification, most vectors have 6× His (histidine) or *c-myc* tag sequences fused at the C-terminal or N-terminal end.

A variety of selection markers are available for *Pichia* protein expression. Some can be used for selecting multicopy gene integration, such as the drug resistance markers zeocin and blasticidin, while others provide auxotrophic selection such as $his4^-$ mutant strains. Commercially available *Pichia* host strains for protein expression include X-33, GS115, SMD1168, and KM71. Additional host strains for

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P. pastoris heterologous protein expression are reviewed by Cregg et al. (20).

11.3.2. Protein Folding and Glycosylation in *P. pastoris*

P. pastoris, similar to *S. cerevisiae* and other fungi species, can perform protein posttranslational modifications, such as cleavage of signal sequences for secreted proteins, N- and O-linked glycosylation, and formation of disulfide bonds (71, 72). It has been well documented that overexpression of heterologous proteins in *P. pastoris* can have a deleterious effect on its secretion pathway. Protein folding in the ER and Golgi is often the rate-limiting step for heterologous protein expression. Overloading the secretion pathway can lead to accumulation of partial or misfolded heterologous proteins in cells. Existence of high levels of unfolded or misfolded proteins in cells may induce a stress reaction termed the "unfolded protein response" (UPR), which triggers the expression of folding chaperones and enzymes in addition to other physiological responses. Both heat shock protein 70-type chaperone (BiP) and a protein disulfide isomerase (Pdi1) were reported to be induced when cells face UPR in *Pichia* (44, 72). BiP and Pdi1 showed synergistic effects on improving secretory capacity (136).

Glycosylation is one of the most important posttranslational modifications for heterologous protein expression, since it not only affects proper folding and secretion of the protein but also has a direct impact on the biological activities of proteins. Both N- and O-linked glycosylation have been reported using *Pichia* systems for heterologous protein expression (20). The consensus sequence for N-linked glycosylation in *Pichia* is the same as that in *S. cerevisiae* (Asn-X-Thr/Ser), in which Ser or Thr provides a hydroxyl group for glycosylation. Similar to *S. cerevisiae* and other fungal organisms, *Pichia* transfers a lipid-linked oligosaccharide unit, $Glc_3Man_9GlcNAc_2$, to amino acid (Asn) in the ER. This oligosaccharide core is further modified in the Golgi. Unlike hyperglycosylation (>50 Man) occurring in *S. cerevisiae*, the most commonly found N-glycosylation structure in *P. pastoris* is $Man_{14}GlcNAc_2$. There has been no α-1,3-mannosyltransferase activity detected in *P. pastoris*; only α-1,6 and α-1,2 linkages were found in glycosylated proteins in *Pichia* (11). The O-linked oligosaccharides in *Pichia* are also reported, but a consensus sequence for O-linked glycosylation is not available. The O-linked oligosaccharides in *P. pastoris* are generally short (<5 residues) and contain only α-1,2-linked mannose units. Phosphorylation of oligosaccharide units has been found in *P. pastoris* expression systems, but the exact sites of phosphate linkage on saccharides are unknown (72). In order to produce antibodies for therapeutic use, extensive glycoengineering efforts have been carried out to make humanlike glycoforms in *Pichia* (39, 40). Fully humanized sialylated antibody was produced successfully in a humanized *Pichia* strain (39). It is noteworthy that unlike antibodies produced in mammalian cells, antibodies produced in humanized *Pichia* do not contain fucose in the core carbohydrate structures and the afucosylated antibodies showed increased binding affinity to Fcγ receptor IIIA, which in turn results in higher antibody-dependent cellular cytotoxicity (70).

11.3.3. Heterologous Protein Production in *Pichia*

Expression levels of heterologous proteins in *P. pastoris* vary greatly depending on copy numbers of the foreign gene integrated and the stability of both mRNA and protein. Since expression levels are correlated with gene copy number, it is important to achieve high copy numbers of gene integration. To obtain multiple copies of the target gene integrated in *Pichia*, one can screen large numbers of transformants in a 96-well plate format, applying high concentrations of the selection agents (e.g., zeocin and blasticidin), or construct multiple copies of the gene in tandem repeats in vitro.

In addition to the high-copy-number transformants, optimization of the expression process is vital to achieve high-level expression of heterologous proteins in *Pichia*. Although some proteins can be expressed well in flask cultures, it is common that expression levels are greatly enhanced in a controlled environment. Cultures in fermentor vessels offer several advantages over flask cultures, such as control of pH and dissolved oxygen levels, and continuous feeding of carbon and other components during cell growth and protein expression (49, 72). Since *P. pastoris* cells have a preference for respiratory growth, cultures can be grown to extremely high cell densities (up to 500 absorbance units at 600 nm) (20, 21). This is particularly important for secreted proteins since the concentration of the expressed proteins is often proportional to the concentration of cells in culture. Common defined media for *P. pastoris* protein expression are composed of glucose and glycerol as the carbon source and casamino acids and/or ammonium salts as the nitrogen source in addition to biotin and trace elements. The *Pichia* fermentation for heterologous protein expression can be separated into two phases. The first phase is that of growth using glycerol as a carbon source, and at this stage the *AOX1* promoter is fully repressed. After depletion of glycerol, a transitional phase may be added to feed more glycerol at a growth-limiting rate. The second phase of feeding methanol starts induction of the *AOX1* promoter and heterologous gene expression. Detailed protocols for fermentation of *P. pastoris* can be found in various articles (30, 50, 72, 90, 96).

Similar to other heterologous protein expression systems, proteolytic hydrolysis often causes instability of foreign proteins in *Pichia*. In addition to use of proteinase-deficient strains, several fermentation strategies can be applied to control proteolysis of foreign proteins. Some common approaches that have been used successfully to reduce hydrolysis of heterologous proteins in *Pichia* include: (i) adding amino acid-rich supplements such as peptone or casamino acids; (ii) culturing at more acidic pH values (*Pichia* cultures grow well between pH 3 and 7); (iii) using a protease (*PEP4*, vacuolar protease gene)-deficient strain; and (iv) controlling culture temperature (44, 72). Another limitation of heterologous protein expression in *Pichia* is inefficient secretion of some mammalian proteins. In these cases, alternative protein secretion sequences can be tested first, followed by the consideration of more extensive molecular engineering approaches to improve protein chaperone and folding machinery.

11.4. HETEROLOGOUS PROTEIN EXPRESSION IN FILAMENTOUS FUNGI

Filamentous fungi, most notably *Aspergillus* species, can produce high levels of proteins and are capable of secreting proteins into the media. Many industrial enzymes, such as amylase, invertase, pectinase, and cellulases, that are used widely in the food industry for production of high-fructose corn syrup, fruit juice, and wine are produced in *Aspergillus*

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species. The most well-known species for producing commercial enzymes include *A. oryzae* and *A. niger*; both have been given GRAS status by the U.S. FDA (116) for production of enzymes and proteins (35). The GRAS status makes them more attractive for use in the production of enzymes and proteins for the food industry and for pharmaceutical products such as therapeutic proteins and antibodies (29, 131). However, in comparison with *S. cerevisiae* and *P. pastoris*, filamentous fungi are less studied for heterologous protein production with respect to their transcription and translation control, mRNA stability, glycosylation, and regulation of protein secretion. With growing demand for the production of therapeutic proteins including antibodies, there is renewed interest in developing cost-effective fungal expression systems including yeast and *Aspergillus* species for heterologous protein production. In recent years, many advances have been made in the use of filamentous fungi for protein production (74, 87, 129).

11.4.1. Host Strains and Selection Markers for Transformation

Unlike *Pichia* and *Saccharomyces*, there are no commercially available protein expression systems for *Aspergillus*. However, there are several Web-accessible organizations such as the ATCC (American Type Culture Collection), the FGSC (Fungal Genetics Stock Center), the NRRL (Agricultural Research Service Culture Collection), and the BCCP (Belgium Culture Collection) from which the necessary tools for heterologous protein expression in filamentous fungi can be obtained.

For heterologous protein expression in *Aspergillus* species, the first step is to select a suitable host strain. The expression host must be either auxotrophic or antibiotic susceptible so that it can be used for efficient transformation and selection of transformants. The most genetically accessible strain is *A. nidulans*, but *A. niger*, *A. awamori*, and *A. oryzae* are more extensively used because of their capacity for high-level expression of commercial enzymes. *A. niger* can efficiently secrete many enzymes including food-related enzymes. *A. oryzae* has become more amenable to molecular analysis, and current research is focused on the identification of strong promoters to improve expression of industrially valuable enzymes. Due to the limited number of studies on *Aspergillus* strains and patent restrictions on the use of some strains and markers, more studies are warranted to fully utilize the potential of *Aspergillus* host strains for heterologous gene expression.

Transformation of host strains can result in chromosomal integration of plasmids, either through a random mechanism or through site-directed integration. Markers commonly used for transformation of auxotrophic strains are listed in Table 3 (7, 13, 59, 81, 100–102, 125, 137, 138). The *pyrG* gene (uracil/uridine auxotrophs) from *A. niger* is a common marker found in many plasmid vectors. The *A. nidulans argB* gene is also used extensively for efficient transformation of suitable auxotrophic host strains. For example, Buxton et al. (13) described the complementation of an auxotrophic *A. niger* strain with the *A. nidulans argB* gene. More recently, there was a report on the complete disruption of the *argB* gene on chromosome I in *A. niger* (69). The *A. niger argB* deletion strain can be complemented by the *argB* genes from both *A. niger* and *A. nidulans* and can serve as a useful host strain for heterologous protein expression in *A. niger*.

Although plasmids can be readily integrated into the chromosome (stable and multicopy integration) during transformation of *Aspergillus* species, it is desirable to have a replicative plasmid for heterologous protein expression in filamentous fungi. Sequences have been isolated that allow one to perform replicative transformation in *A. nidulans* (2). Initial work with linear plasmids containing human telomeric elements was encouraging, but later these were shown to be unstable (3). To date, a synthetic *Aspergillus* replicative plasmid of wide utility is not yet available (74).

11.4.2. Expression Vectors and Promoters

Promoters commonly used for heterologous protein expression in *Aspergillus* species are listed in Table 4 (17, 25, 35, 38, 61, 99, 103). Constitutive promoters include the *trpC* and the *gpdA* promoters from *A. nidulans*. Other frequently used promoters are the starch-inducible promoters, α-amylase and the glucoamylase promoters from *A. oryzae* and *A. niger*. The glucoamylase promoter responds to maltose as a carbon source and is repressed by glucose. More recently the *TEF1*-α promoter of *A. oryzae* has been studied for use as a strong constitutive promoter (61).

TABLE 3 Selectable markers used for heterologous gene expression in *Aspergillus* species <QU9>

Gene	Enzyme	Cellular function	Species	Reference
Auxotrophic				
argB	Ornithine carbamoyltransferase	Arginine biosynthesis	*A. nidulans*	13
pyr4	Orotidine 5′-phosphate decarboxylase	Uracil biosynthesis	*Neurospora crassa*	7
pyrG	Orotidine 5′-phosphate decarboxylase	Uracil biosynthesis	*A. niger*	81
trpC	Phosphoribosylanthranilate isomerase	Tryptophan biosynthesis	*A. nidulans*	137
amdS	Acetamidase	Acetamide or acrylamide assimilation, N_2 source	*A. nidulans*	59
niaD	Nitrate reductase	Nitrate assimilation, N_2 source	*A. niger*	125
Dominant				
hph [HmB^r]	Hygromycin B phosphotransferase		*E. coli*	100
phleo^r	*E. coli* transposon Tn*5* phleo^r DNA			102

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TABLE 4 Promoters used in heterologous gene expression in *Aspergillus* species

Gene	Enzyme	Cellular function	Species	Reference(s)
Strong inducible				
alcA	Alcohol dehydrogenase 1 (ADH1) (ethanol inducible)		*A. nidulans*	25
α-Amylase	Starch inducible		*A. oryzae*	17
glaA	Glucoamylase (starch and maltose inducible)		*A. nidulans*	36
Constitutive				
gpdA	Glyceraldehyde-3-phosphate dehydrogenase		*A. nidulans*	99, 103
trpC	Phosphoribosylanthranilate isomerase	Tryptophan biosynthesis	*A. nidulans*	38
TEF1-α	Translation elongation factor 1-α		*A. oryzae*	61

Another suitable promoter for the expression of heterologous proteins in filamentous fungi is the ethanol-regulated promoter *alcA* for alcohol dehydrogenase from *A. nidulans*. Genes for ethanol utilization in *A. nidulans* are efficiently transcribed. The *alcA* promoter is tightly regulated through *alcR*, a *trans*-activating gene, and is subject to repression by the major carbon catabolite repressor CreA (25). In a recent report, expression of antisense *creA* in *A. nidulans* increased levels of expression for glucose-repressible enzymes in the presence of glucose (8). The de-repression effect by the *creA* antisense DNA did not alter cell morphology and growth parameters, as observed in a *creA* null mutant. The *alcR-alcA* regulatory system from *A. nidulans* has been transformed into *A. niger*, which does not possess an inducible *alc* system (88). The *alcR-alcA*-transformed strain of *A. niger* showed similar levels of heterologous expression of the reporter enzyme β-glucuronidase, as in *A. nidulans*.

Ishida et al. (52) reported that the tyrosinase gene promoter (*melO*) was four times stronger for the heterologous expression of β-glucuronidase from *E. coli* in a submerged culture as compared with other gene promoters, such as *amyB*, *glaA*, and *agdA*, previously used for heterologous gene expression in *A. oryzae*. The *melO* promoter was used to produce the *glaB*-type glucoamylase in a submerged culture of *A. oryzae* and achieved maximum yield of 3.3 g/liter and 99% purity. This work establishes the *melO* promoter for high-level expression and high-purity production of heterologous proteins in *A. oryzae*.

11.4.3. Posttranslational Regulations

Large numbers of mammalian enzymes and proteins have been expressed through genetic engineering; however, secretion of heterologous proteins is often much less efficient than the secretion of native fungal proteins. During the last 10 years, the production levels of heterologous proteins in *Aspergillus* have been improved by fusing the protein of interest to the 3′ end of a highly expressed gene encoding a protein that is efficiently secreted from the host. The gene fusion or carrier protein strategy has been used with the glucoamylase gene from *A. niger* or *A. awamori*. Increases of 5- to 1,000-fold in protein production levels have been observed (87). Gouka et al. (36) extensively reviewed the production levels of different proteins fused to the glucoamylase carrier gene. They also performed detailed experiments on the production levels of various proteins with carrier fusions to the glucoamylase protein in isogenic, single-copy strains of *A. awamori*. The glucoamylase gene can be divided into three distinct domains: the catalytic N-terminal domain, the C-terminal starch-binding domain, and a flexible linker region that separates the two domains. The C-terminal starch-binding domain can be replaced by a heterologous protein. The glucoamylase carrier proteins are believed to improve translocation in the ER, to enhance proper protein folding, and possibly to afford protection against proteolysis. In the secretory pathway, the carrier protein can be cleaved by a KEX2-like protease at a specific proteolytic cleavage site separating the glucoamylase carrier and the protein of interest. The amino acid sequence (NVISKR) of the cleavage site is derived from the propeptide of glucoamylase. The carrier approach appears to be the first choice in attempts to produce secreted proteins in *Aspergillus* species. There are many reports on the use of carriers for heterologous protein secretion. A recent study reported a successful secretion of laccase (*lac*1) (an industrially useful enzyme for the oxidation of *p*-diphenols) from *Pycnoporus cinnabarinus* that used either the native laccase signal sequence or the *A. niger* glucoamylase pre-pro-sequence as a carrier for the heterologous enzyme expression in *A. niger* (106). The fusion of the glucoamylase pre-pro-sequence increased expression levels 80-fold in comparison to the levels observed with the laccase signal peptide sequence. Another advantage of using the glucoamylase pre-pro-sequence as a carrier for laccase in *A. niger* is the production of the 70-kD protein, which is similar to the molecular weight of the native protein (106). However, it was reported that laccase expressed in *P. pastoris* had an estimated 110-kD molecular weight due to hyperglycosylation in the yeast (76, 77, 106). Human interleukin-6 (IL-6) secretion was also improved using the native glucoamylase as a carrier when expressed in *A. niger* (74). It has been reported (4) that the UPR, i.e., the deliberate upregulation of "foldases" and chaperones, can also improve the secretion of heterologous proteins.

Efficient heterologous protein production in fungi cannot always be achieved by alleviating the limitations of transcription. Posttranslational regulations such as glycosylation, protein folding, and secretion often pose challenges for successful expression of heterologous proteins in *Aspergillus* species. Another major limiting factor in protein production in filamentous fungi is proteolytic degradation by high levels of intracellular and extracellular proteases. *A. niger* has four predominant extracellular proteases, two aspartyl proteases (PEPA and PEPB) and two serine carboxypeptidases (PEPF and PEPG). In *A. nidulans*, neutral and alkaline proteases often are the dominant ones affecting the

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yields of heterologous proteins. Efforts to eliminate specific protease activities through mutagenesis or gene disruption have improved protein production levels. Van den Hombergh et al. (127) reviewed fungal proteases and described the development of a set of tester strains of *A. niger* that may be used to identify the appropriate genetic background for a particular protein.

Posttranslational modifications such as N glycosylation are particularly important for many pharmaceutically relevant proteins because of their impact on biological activities (19). High mannose-type glycosylation is observed in filamentous fungi, but it is much less extensive than that seen in proteins produced in *Saccharomyces* and *Pichia* species. Maras et al. (78) have nicely reviewed the fungal glycosylation pathway and approaches and compared the carbohydrate structures with proteins produced in mammalian cells.

Successful expression of *Drosophila melanogaster* xanthine dehydrogenase in *A. nidulans* is a recent example of proper posttranslational modification of a heterologous enzyme in filamentous fungi (1). In comparison to the baculovirus expression system, which produced only about 10% of active xanthine dehydrogenase enzyme due to incomplete incorporation of the cofactor molybdenum and two iron-sulfur clusters, *A. nidulans* produced 40% of active enzyme with relatively more complete cofactor incorporation.

11.4.4. Scale-up of Fermentation and Protein Production in *Aspergillus*

In addition to the molecular genetic approaches, a combination of host strain selection, strain development, and production process development including media optimization have resulted in commercially attractive protein yields in *Aspergillus* protein expression systems (101). For example, IL-6 gene carrier fusion in *A. nidulans* in combination with host strain development and growth optimization resulted in a 200-fold increase in production of active IL-6 (101). A combination of gene dosage and growth condition optimization for the improvement of protein expression in *A. oryzae* resulted in a high production titer of phospholipase A_1 (PLA1) at the industrial scale (118). Transformants with 15 to 20 copies of the *PLA1* gene were integrated in the chromosome of *A. oryzae* and produced 60- to 7,500-fold higher activity than the native expression level. In addition, improvement of the mycelial morphology from a pellet form to a filamentous form improved production of PLA1 in scaled-up fermentations and resulted in production of PLA1 at 1.8 g/liter consistently. This is consistent with the observation that improvements in oxygen transfer and broth rheology obtained with the pellet form of growth often resulted in increased protein production.

It has been recognized that fungal morphology plays an important role in determining process productivity in mycelial fermentations. This has led to investigations into alternative ways to conduct fermentations to optimize production yields. Papagianni et al. investigated the effect of cell immobilization on protein production and secretion (92). They reported on cells of *A. niger* that were immobilized by attachment to metal surfaces or by spore entrapment, and subsequently grown on Celite beads. They noted that cell attachment on metal surfaces decreased secretion of proteases and increased glucoamylase production. It was observed that growth on Celite beads in shake flasks reduced the secreted proteases by half and almost doubled the specific activity of glucoamylase. In bioreactor cultures, conditions can be better controlled than in flask culture and effects of culture conditions on protein yields are much greater.

11.5. FUTURE PROSPECTS

The demand for production of a variety of proteins and enzymes in heterologous hosts for research and industrial applications is growing continuously. It is of great interest to develop and optimize diverse host systems for heterologous protein expression to meet the growing demand. Fungal expression systems offer many advantages over other expression systems and have been widely used for production of heterologous proteins from many organisms. Discovery of new promoters and development of new vectors will provide more choices for different protein expression needs. Greater understanding of transcriptional control, mRNA stability, glycosylation, protein folding, and other posttranslational processes will allow us to overcome the difficulties associated with heterologous protein expression in fungi, such as hyperglycosylation in yeasts and poor secretion associated with some mammalian proteins. It has been documented that the use of protease-deficient strains can increase the yield of recombinant protein production (28, 95) and that the use of specific mutants may eliminate hyperglycosylation problems with some proteins expressed in *Saccharomyces* (115). The exponential increase in fungal genomics and proteomics in recent years will no doubt fuel the advancement of filamentous fungi as heterologous protein expression hosts (74). Collectively, fungal protein expression systems have been and will continue to serve as a choice for heterologous protein production in small or large quantities.

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AUTHOR QUERIES

QU1 Phrase "cluster of 9.1-kb segment" OK?

QU2 Should this be G*APDH*?

QU3 Change "genes" to "proteins"? Or, change to "from genes such as those encoding invertase..."?

QU4 "shake flask"?

QU5 Should this be G*APDH* or G*PD* as above?

QU6 Sentence correct as worded? Including italicization of HIS4 here and in next sentence?

QU7 MFα1 as above?

QU8 Should this be a page range?

QU9 Please check expanded column format of Tables 3 and 4 and fill in empty cells if possible. Italicize "phleo" if a gene. Note that Table 3 title specifies Aspergillus species, but the table also includes *N. crassa*.