How to achieve high-level expression of microbial enzymes
Strategies and perspectives

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Microbial enzymes have been used in a large number of fields, such as chemical, agricultural and biopharmaceutical industries. The enzyme production rate and yield are the main factors to consider when choosing the appropriate expression system for the production of recombinant proteins. Recombinant enzymes have been expressed in bacteria (e.g., Escherichia coli, Bacillus and lactic acid bacteria), filamentous fungi (e.g., Aspergillus) and yeasts (e.g., Pichia pastoris). The favorable and very advantageous characteristics of these species have resulted in an increasing number of biotechnological applications. Bacterial hosts (e.g., E. coli) can be used to quickly and easily overexpress recombinant enzymes; however, bacterial systems cannot express very large proteins and proteins that require post-translational modifications. The main bacterial expression hosts, with the exception of lactic acid bacteria and filamentous fungi, can produce several toxins which are not compatible with the expression of recombinant enzymes in food and drugs. However, due to the multiplicity of the physiological impacts arising from high-level expression of genes encoding the enzymes and expression hosts, the goal of overproduction can hardly be achieved, and therefore, the yield of recombinant enzymes is limited. In this review, the recent strategies used for the high-level expression of microbial enzymes in the hosts mentioned above are summarized and the prospects are also discussed. We hope this review will contribute to the development of the enzyme-related research field.

Introduction

Native and recombinant enzymes benefit major sectors of the biopharmaceutical, agricultural and chemical industries. Products of these industries, in turn, augment the fields of medicine, diagnostics, food, nutrition, detergents, textiles, leather, paper, pulp and plastics.1 Enzymes are synthesized by all living organisms as part of their natural metabolism, serving as biocatalysts to increase the rate of the metabolic reactions. The enzyme production rate, yield, quality and functionality are the most important factors to be considered when industrial recombinant enzymes are produced in hosts.1,2

The choice of an expression system for the high-level production of recombinant enzymes depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, post-translational modifications and biological activity of the protein of interest, as well as regulatory issues in the production of the therapeutic proteins.3 Moreover, the cost breakdown in terms of process and design and other economic considerations is required for the selection of a particular expression system.4 Recombinant enzymes have been expressed in Escherichia coli, Bacillus (e.g., Bacillus subtilis) and lactic acid bacteria (e.g., Lactobacillus lactis), filamentous fungi (e.g., Aspergillus) and yeasts (e.g., Pichia pastoris).5-9 Bacterial expression systems remain very attractive due to their ability to grow rapidly and at high density on inexpensive substrates, their well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains.10 The advent of recombinant DNA technology has revolutionized strategies for protein production. Due to the well-characterized genome and a variety of mature tools available for genetic manipulation, E. coli is still the most common workhorse for recombinant protein production.1,10,11 However, despite the extensive knowledge on the genetics and molecular biology of E. coli, not every gene can be efficiently expressed in this organism.

Due to the multiplicity of physiological impacts arising from high-level gene expression and host strains, the overproduction goal can hardly be achieved, and therefore, the yield of recombinant enzymes is limited. The mechanistic link between the stress response and deteriorated culture performance of most host stains is still not completely understood. Most of the genetic strategies for engineering the cell physiology developed thus far are still limited to single-gene manipulation and their feasibility appears to be target-protein dependent. Another bioprocess obstacle is the lack of suitable techniques for monitoring the cell physiology.

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High-level expression of enzymes by transcriptional regulation in E. coli. A promoter suitable for high-level protein synthesis must be strong, and its tight regulation is essential for the synthesis of proteins that may be detrimental to the host E. coli. The most widely used promoters for large-scale protein production are thermal or chemical inducers. Cold-responsive promoters have been shown to facilitate efficient gene expression at reduced temperatures. The activity of the phage λ P L promoter is highest at 20°C and declines as the temperature is raised. The rationale behind the use of cold-responsive promoters for gene expression is based on the proposition that the rate of protein folding will be only slightly affected at 15–20°C. This will provide sufficient time for protein refolding, yielding active proteins and avoiding the formation of inactive protein aggregates.

Figure 1. Systems-level engineering strategies to achieve high-level expression of recombinant proteins.

The regulation of recombinant protein expression is a complex system consisting of interaction elements. The expression plasmid is important for the expression of a particular recombinant protein. However, the construction of an expression plasmid requires several elements, the configuration of which is crucial for the highest levels of the enzyme synthesis. The essential elements of an E. coli expression vector include the promoter, ribosome-binding site (RBS), transcription terminator and copy number. Among them, the promoter is positioned approximately 10–100 bp upstream of the RBS and is under the control of a regulatory gene. The promoters of E. coli consist of a hexanucleotide sequence located approximately 35 bp upstream of the transcription initiation base (−35 region) separated by a short spacer from another hexanucleotide sequence (−10 region). The transcription terminator is located downstream of the coding sequence and serves both as a signal to terminate the transcription and as a protective element composed of stem-loop structures, protecting the mRNA from exonucleolytic degradation and extending the mRNA half-life. Additionally, the copy number of the plasmid is determined by the origin of replication. The use of runaway replicons resulted in a massive amplification of plasmid-encoded proteins. There are several strategies for achieving high-level expression of recombinant proteins in E. coli (Fig. 1).
of the Pseudomonas putida F1 cym and cym operons to control target expression at the transcriptional level by using β-isopropyl-β-D-ribofuranosylbenzoate (cymate) as an inducer, use of a “plating” method, the increase of the repression-to-operator ratio, induction by infection with mutant phage, attenuation of promoter strength on high-copy-number vectors, use of transcription terminators in combination with antiterminators, use of an inducible promoter within a copy-number-controllable plasmid, "cross-regulation" systems, co-transformation of plasmids utilizing the 39S RNA polymerase and use of antisense RNA complementary to the mRNA of the cloned gene. The above approaches have provided important tools for the armamentarium of gene expression.

High-level expression of enzymes by translational regulation in E. coli. The determinants of protein synthesis initiation are difficult to decipher. In E. coli, AUG is the preferred codon by two- or 3-fold, while GUG is only slightly better than UUG. The secondary structure at the translation initiation region of mRNA plays an important role in the efficiency of gene expression. Upon addition of the cap analog, the translation from the 5′-terminal region of p53 mRNA is enhanced, and the level of IacZ expression increases with the increase in the loop size of the local mRNA.

The SD and other sequences in the mRNA are important for an efficient translation. The translation was enhanced upon interaction with bases 485–466 of the 16S RNA. One of the translation enhancers was the U-rich sequences in the 5′-untranslated region (UTR) of the mRNAs. Significant enrichment was observed for U-rich motifs within 100 nucleotides downstream of 5′ splice sites, if both classes of exons with the highest enrichment were located between positions +6 and +30.

The mRNA stability also plays an essential role in maintaining critical cellular levels of a given protein in E. coli. It was indicated that the stability of mRNA in a cell-free extract could be controlled using engineered T7 terminator sequences, and the relative expression level of target proteins could be manipulated by employing the T7 terminator of adjusted stem lengths. The design of expression vectors frequently includes the insertion of all three stop codons to prevent possible ribosome skipping. The termination efficiencies significantly vary depending on both the stop codon and the fourth nucleotide, ranging from 80% (UAA/U) to 7% (UGAC); therefore, the UAA stop codon is preferred in E. coli. The increase of the activity of the prokaryotic release factor RF3, a stimulatory protein, can markedly improve the efficiency in decoding strong stop signals in vivo.

Enhancement of the expression of enzymes by different protein formations in E. coli. Inclusion bodies are significant cytoplasmic aggregates. Several experimental approaches have been used to reduce the formation of inclusion bodies and improve protein folding. Among others, these include culture at lower temperatures, expression of DnaK/DnaJ (Hsp70), selection of different E. coli strains, change of pH, a diminished hydrophobic effect at elevated pressures, molecular chaperones, regulation of cytosolic Ca2+ limiting growth conditions, and co-expression. The oxidative environment of the periplasm has the important function of facilitating the proper folding of proteins. The cleavage of signal peptides in vivo during translocation to the periplasm is more likely to yield the authentic N-terminus of the target protein. Strategies for the improvement of the translocation of proteins to the periplasm include the supply of components involved in protein transport and processing: overproduction of the signal peptidase I, co-expression of the pBAD and secE genes, deleration of the twin-arginine translocation motif, type III secretion chaperone (Ochly culpata chimatoris SIC1), mutations in secE, and addition of endoplasmic reticulum (KDEL) or Golgi (YQRL) retention sequences.

The expression of enzymes into culture media is preferred due to several advantages such as simplified downstream processing, high expression level, simpler purification, improved protein folding and level of proteolysis. However, E. coli secretes few proteins and the manipulation of the various transport pathways to facilitate secretion of foreign proteins is an important task. In order to improve the protein secretion the limited leakage of the outer membrane was induced. The reported strategies include the optimization and mutation of signal peptides, the over-expression of the twin-arginine translocation (Tat) ABC, the synergistic use of EDTA and lysisyme, the addition of detergents, periplasmic chaperones, fusion proteins, SDS, glycine, Carboxylic acids, or Na+; the delayed supplementation of glycine, translation engineering, and the employment of the 19-residue propeptide of staphylococcal nuclease.

Improving the yield of enzymes by fusion proteins or molecular chaperones in E. coli. Several fusion proteins have been used for improving the high-level production of enzymes. The remarkable increase in protein yield is thought to be due to the protection of the target protein from proteolysis, improved folding and the presence of regulatory regions in the T7 polymerase gene, thus enhancing the expression level of transmembrane proteins and efficient mRNA translation. Among others, fusions with the gfa cell stimulating factor (GSF) were used for improving the production of different target recombinant proteins. The aquaporin Z (AqpZ) expression was improved by fusion with TrxA in E. coli. The expression of recombinant proteins as fusions with the small ubiquitin-related modifier (SUMO) protein significantly increased the yield of difficult-to-express proteins in E. coli. Rice protein expression was improved by fusion with thioredoxin at the N-terminus in E. coli. Additionally, it was noted that the expression of poorly expressed heterologous genes was improved by translational fusion with fragments of the ropE gene.

Molecular chaperones can assist the folding of proteins in the cell under normal and stress conditions. It is especially difficult to heterologously produce some enzymes in E. coli and the use of molecular chaperones could reduce their aggregation tendency and provide higher yields of correctly folded, biologically active proteins. The combined use of molecular chaperones and target proteins from the same species is another strategy that has been successfully employed, as exemplified in the case of the production of the soluble gp37 by co-expression with two bacteriophage T4-encoded chaperones in a two-vector system in E. coli. Moreover, the heterologous production of the antimalarial drug target GTP cyclohydrolase 1 (PGCH) was improved.
by co-expressing the *Plasmodium falciparum* molecular chaperone. The most efficient expression system studied was suitable for the expression of membrane protein complexes. Several recombinant enzymes were expressed in the *B. subtilis* expression system. The alkaline α-amylase from *B. alcalophilus* was overproduced in *B. subtilis* W16800 using the vector pMA5, and its titer was 7.9 times higher than that in the wild-type host. Individual secretion machinery components with their special functions are involved in the total flow of proteins from the cytoplasm to the medium. However, the multiple regulators can affect the expression of secretion machineries as well as their post-transcriptional functions for protein secretion, resulting in complicated networks. The secretion of heterologous proteins can be enhanced by engineering components involved in the late stages of secretion. The overproduction of the molecular chaperone PsaL lipoprotein enhanced the secretion of α-amylase from *B. stearothermophilus* (4-fold) in *B. subtilis*. The promoter system is also an important factor for the expression strength of *B. subtilis*. In order to improve the P1 promoter system, site-directed mutagenesis of several nucleotides downstream its transcription origin site was adopted. Thereafter, the mutated P1 promoter was obtained, and the production of β-galactosidase reached values 1.8 times as high as that of the wild-type promoter in *B. subtilis*.

High-level expression of microbial enzymes in *B. megaterium*, *B. megaterium* is a well-studied prokaryote, which is used for heterologous protein production. This expression host has several favorable features, including low protease activity, structural and segregational stability of plasmids and the ability to grow on a wide variety of substrates. Different heterologous proteins (e.g., GFP, sugar-modifying enzymes and hydrolases) have been successfully produced in *B. megaterium*. For example, keratinase has been expressed under the PdxA and Pnp/p promoters in recombinant *B. megaterium*, while the penicillin G acylase gene (pac) was highly expressed in the *B. megaterium* pac minus mutant. However, enzyme production processes in *B. megaterium* use regulation-differentiation mechanisms for the degradation of enzymes and the production of co-enzyme genes is repressed during the exponential growth by transition-state regulators. In order to counteract the repression, the regulon Deg/SU transcriptional control promoted the hyper-secretion of a heterologous *B. amylopliticus* α-amylase in *B. megaterium*. High-level expression of microbial enzymes in *B. brevis*. *B. brevis* is also an interesting host for heterologous protein production. Heterologously expressed enzymes are secreted directly into the culture medium, and they are accumulated at high levels in a relatively pure state. The secreted enzymes are usually correctly folded, soluble and biologically active. Since *B. brevis* has a very low level of extracellular protease activity, the secreted proteins are stable and not significantly degraded. For example, a hyper-thermophilic cellulase derived from *Pyrococcus horikoshii* and the recombinant phospholipidinositol-specific phospholipase C from *B. thuringiensis* have been successfully produced with the *B. brevis* host-vector system. Additionally, the secretion of heterologous proteins, which is important for the high-level production can be improved by engineering methods. In fact, the

High-level expression of enzymes by codon optimization in *E. coli*. Heterologous protein production in *E. coli* may be diminished by biased codon usage. The expression of enzyme genes in *E. coli* shows a non-random usage of synonymous codons. The competition for rare tRNA may also adversely affect the expression of host genes or elicit a stringent response. The arginine codons AGA and AGG are particularly rare in *E. coli*, and they have been shown to elicit a stringent response as well as lower protein expression. Codon optimization could improve the translational fidelity and thus enhance the expression of enzymes. The codon bias can be mitigated by targeted mutagenesis to change rare to more commonly used codons or by co-expressing genes that encode the tRNA. The codon-optimized and mRNA secondary structure-free sequences could be obtained by synthesis of complete genes. The heterologous expression of numerous genes was improved by synthetic DNA. Moreover, the expression of genes with rare codons could be rescued by co-expression of rare cognate tRNA genes.

Fermentation optimization of enzyme production in *E. coli*. The production of enzymes in *E. coli* can be greatly improved by the use of high-density culture systems. The methods achieving high cell concentration include batch, fed-batch and continuous cultures. The composition of the growth medium must be optimized and monitored because it significantly affects the metabolic effects on both the cells and enzyme production. Nutrient composition and fermentation variables such as temperature, pH and other parameters affect the production levels of enzymes. However, high-cell-density culture suffers from several factors such as limited availability of dissolved oxygen at a high cell density and high carbon dioxide levels. The resulting decreased growth rates enhance acetate formation and reduce the mixing efficiency. The accumulation of acetate is a problem in the production of recombinant protein at high cell density culture. The acetolactate synthase introduced into *E. coli* has a rate and CO2 production rate are also important factors affecting the expression level of enzymes.

High-Level Expression of Microbial Enzymes in Bacilli

As well as *E. coli*, gram-positive Bacilli strains are popular organisms for recombinant enzyme production. Compared with the well-known *E. coli*, Bacilli strains have a high secretion capacity and can export proteins directly into the extracellular medium. Furthermore, few enzymes expressed in the Bacilli strains are isomerized.

High-level expression of microbial enzymes in *B. subtilis*. *B. subtilis* is an interesting alternative system for heterologous gene expression. The ability of secreting proteins directly into the medium is one of its greatest advantages. A number of expression systems for heterologous protein production have been constructed. After comparison of different *B. subtilis* expression

systems, it was found that the subtilin-regulated gene expression (SURE) system was the most efficient expression system developed.
secretion system of *B. brevis* was enhanced by the use of fungal protein disulfide isomerase (PDI). The fusion with PDI increased the extracellular production of geranylgeranyl pyrophosphate synthase in *B. brevis.*

**High-Level Expression of Microbial Enzymes in Lactic Acid Bacteria**

For the development of biotechnological, genomic and proteomic tools, the lactic acid bacteria are becoming promising potential hosts for the high-level expression of recombinant enzymes. Apart from the cheap and easily scalable protein production associated with the microbial nature of lactic acid bacteria, since they do not have endotoxins in their membrane, these species are optimal and safer expression hosts for the production of recombinant proteins of food and medical interest. The key tools (e.g., modified strains, optimal expression vectors, suitable promoters and enhanced induction and secretion systems) for the recombinant protein production in these strains have been developed.

**L. lactis** is a promising lactic acid bacterium in terms of recombinant protein production. Some recombinant proteins have been successfully expressed in *L. lactis.* The *L. lactis* MG 1363 strain was engineered as a recombinant live β-galactosidase delivery system using food-grade protein-expression techniques and selected probiotics as vehicles. The food-grade β-galactosidase was successfully expressed in that system. Additionally, different inducible expression systems have been constructed for the expression of recombinant proteins in *L. lactis.* Ni- and Cu-inducible gene expression is the most widely used inducible system in *L. lactis.* This system affords tightly controlled expression and relatively high protein yield. Moreover, *Bifidobacterium longum* NRRL B-41049 1-arabinose isomerase (I-AI) was cloned and overexpressed in *L. lactis* by using a phosphate-depletion-inducible expression system. The *L. brevis* S-layer protein (SlpA) was cloned into *L. lactis* under the transcriptional control of the xylose-inducible expression system (XIES) and secreted into the extracellular medium. The enhancement of the mRNA stability achieved with the inclusion of 3′-untranslated leader sequences enhanced the production of the recombinant amylase in *L. lactis* by several folds. Furthermore, secretion is another area that can be exploited for improving the production of recombinant enzymes in *L. lactis.* Secretion by fusion with the Ugp45 signal peptide generally obtained satisfactory efficiency levels. A nitrile kinase from *B. subtilis* was successfully secreted into the medium by using that system.

**High-Level Expression of Microbial Enzymes in Yeasts**

Due to advantages such as genetic manipulation, rapid growth and ability of producing eukaryotic post-translational modifications (e.g., glycosylation), yeasts are popular industrial hosts for the expression of recombinant enzymes. The yeast expression hosts used for recombinant enzymes expression mainly include *P. pastoris,* *Saccharomyces cerevisiae,* *Hansenula polymorpha,* *Kluyveromyces lactis,* *Schizosaccharomyces pombe,* *Yarrowia lipolytica,* *Arxula adeninivorans* and *Candida boidinii.* These yeasts use carbon sources, rapidly reach high cell densities and are thermo-tolerant and halo-tolerant. In order to improve the production of recombinant enzymes, several yeast hosts were engineered to include further advantages (e.g., lack of proteases, optimized transformation system and efficient expression) (Table 1).

**Table 1. The advantages of different yeast hosts of recombinant proteins**

| Hosts                        | Advantages                                                        | References |
|-----------------------------|-------------------------------------------------------------------|------------|
| *Pichia pastoris*            | Signal peptide cleavage; protein folding; posttranslational modifications inside the cell; the ability of secretion into medium with normal function | 84         |
| *Saccharomyces cerevisiae*   | In addition to the advantages of yeast systems; safe (GRAS) strain | 125        |
| *Hansenula polymorpha*       | Strong, tunable promoters derived from genes of the methanol utilization pathway; MOX (methanol oxidase) promoter significantly repressed under glucose limitation or starvation; allowing for a methanol-free process; high cell densities; efficiently secrete proteins | 125, 126   |
| *Lactobacillus lactis*       | A strong, inducible promoter (LAC) the ability to utilize cheap substrates; ability to secrete high molecular weight protein | 125, 127   |
| *Schizosaccharomyces pombe*  | Higher eukaryotes; morphologically well-defined glycoproteins; the quality control mechanism of glycoproteins folding closer to mammalian cells | 125, 128   |
| *Yarrowia lipolytica*        | The ability to secrete high molecular weight proteins; the high secretion of large molecular enzymes by the co-translational translocation pathway; does not ferment sugars; high cell density fermentation; safe (GRAS) strain | 125, 129   |
| *Arxula adeninivorans*       | A temperature-dependent dimorphic yeast; secrete higher concentrations of proteins; can grow on a range of compounds as the sole source of carbon and energy | 125, 130   |
| *Candida boidinii*           | An efficient host for secretory production of enzyme              | 131        |
auxotrophic mutants GS115 and protease-deficient strains (e.g., SMD 1365, SMD 1366), are derived from the wild-type strain NRRL-Y 11430. P. pastoris has three phenotypes (i.e., Mut -, Mut + and Mut ) related to methanol utilization. There are many advantages of this expression system and the most important one is its perfect protein processing mechanism, including signal peptide cleavage, protein folding, post-translational modifications inside the cell and the ability of secreting its products into the medium with normal function.125,126,133,139,140 Many recombinant activities have been expressed in P. pastoris. For example, an alkaline α-amylase gene from alkaliphilic Alkalimonas sp. was successfully expressed in P. pastoris GS115.134 A poly(vinyl alcohol) dehydrogenase (PVADH) from Sphingopyxis sp. was also highly expressed in P. pastoris GS515 by using a 3.3 kb bioreactor and reached an activity of 902 U/mL.135

The optimal system for the production of recombinant enzymes in P. pastoris includes several crucial parts: a suitable vector, the codon-optimized gene, the signal sequence, the ability of proteolytic cleavage and fermentation optimization. Among them, gateway-compatible vectors (pBGPI-DEST and pPICZ-α-DEST) have been developed to enable a rapid and convenient preparation of expression plasmids for the production of secretory proteins in P. pastoris.135 Additionally, the choice of the inducer and the induction mechanism for heterologous gene expression highly affects product yields of enzymes. In P. pastoris fermentations, since methanol is an inappropriate inducer for the production of proteins used in the food industry, glycerol, aldehyde-3-phosphate dehydrogenase (GAP) promoter has been widely preferred as an alternative to the AOX1 promoter.136 In order to improve the expression levels of the GAP promoter, the combination of AOX1 and GAP promoters to co-express recombinant proteins has been developed, and the secreted protein concentration was approximately 2-fold higher compared with the concentration obtained using the GAP promoter alone, with appreciable differences in the cell concentration.137

For high-level expression of recombinant enzymes in high-cell-density fermentation of P. pastoris, the medium includes a basal salt medium, trace salt solution (PTM1) and ammonium hydroxide. In particular, ammonium hydroxide was used to maintain the pH, while different combinations of glycerol and methanol were supplied as carbon source. Although the use of glycerol as co-substrate is one of the most common strategies, when glycerol is used for the recombinant enzyme production, the excess glycerol in the medium represses the AOX1 promoter.138 An alternative to the glycerol and methanol co-feeding strategy, different feeding strategies were adopted, such as sorbitol as a co-substrate.139,140,144 Due to the oxygen used in the oxidation of methanol to formaldehyde as a side-reaction, high oxygen transfer rates are required in the methanol-utilization pathway.135 High agitation and oxygen-enriched air can be used to minimize the transfer resistance, and thus, the dissolved oxygen is kept around 20–30%.145,146 The post-secretory degradation of the recombinant enzymes by host-specific proteases is also a problem that affects the production yield, especially in high-density fermentation processes. As a solution, several approaches have been attempted, including the control of the cultivation conditions (e.g., pH), addition of protease inhibitors (e.g., casein and amino acids) and different medium compositions.134,125,136 Although the optimum temperature for the growth is 30°C, the recombinant protein production was improved by decreasing the induction temperature. When the induction temperature was decreased from 30°C to 20°C, the highest yield of recombinant alkaline α-amylase reached 130 U/mL.143

High-level expression of microbial enzymes in S. cerevisiae
In addition to the advantages having been acknowledged in P. pastoris, S. cerevisiae is recognized as generally regarded as safe (GRAS) strain. Therefore, the recombinant enzymes produced in this host are mainly used in the food and drug industries. The most important recombinant enzyme product on the market that is made in S. cerevisiae is urate oxidase.145 However, there are other recombinant enzymes that have been successfully expressed in S. cerevisiae (e.g., an esterase from Thermus thermophilus HB827).146 Additionally, a novel peroxiredoxin (Prx) isolate from Acinetobacter sp. SM04 has been expressed in the extracellular supernatant with a titer of 0.24 mg/mL.146 However, several factors can affect the expression yield of recombinant enzymes in S. cerevisiae. In this context, there are several strategies aiming to enhance the expression yield of proteins. An endoglucanase from Paenibacillus barcinonensis has been expressed in S. cerevisiae by using different domains of the cell wall protein Pk4 as translational fusion partners.147 The manipulation of the unfolded protein response (UPR) pathway regulator Hac1p can stimulate the secretory pathway of S. cerevisiae to improve the secretion of recombinant enzymes. Overexpression of S. cerevisiae HAC1 yielded a 2.0-fold enhancement in the secretion of the endogenous invertase.148 After screening of a S. cerevisiae mutant library, the deletion of the gene MON2 enhanced the secretion of the recombinant luciferase, which encoded a scaffold protein for vesicle formation located in the late Golgi.149 The supplementation of the growth medium with amino acids significantly improved the culture growth and cellulose production in S. cerevisiae.150

High-level expression of microbial enzymes in other yeast hosts.
The enzymes produced in H. polymorpha are xylose isomerase, glucose oxidase, glycolate oxidase, catalase, phytase and isopenicillin-N synthase.151,152,153,154 A mixture of glycerol and methanol was added to enhance the enzyme production during the last hours of the fermentation in H. polymorpha. Kortmeeier et al. found that secondary substrate-limited batch fermentation could divert the metabolic flux toward protein production and found the yield of GFP to increase 1.9-fold.155 V. lipolytica is also an increasingly popular host for high-level expression of heterologous recombinant enzymes with the advantage of secretion of high-molecular-weight enzymes through the co-translational translocation pathway and high-cell density fermentation. A chitosanase from the marine bacterium Pseudomonas sp. OUC1 was overexpressed in Y. lipolytica when the expression vector carrying two copies of the chitosanase gene was transformed.156 A thermostable α-amylase from Thermobifida fusca NTU22 was cloned into Y. lipolytica P01 g host strain using the vector pYLS1C1, and high levels of extracellular amylase production (750 U/L) were obtained.157
K. lactis has been successfully used for the production of recombinant enzymes such as lipase, chitosanase, extracellular galactosidase and glucoamylase.\textsuperscript{145,146,147} \textbf{A} glucose repression-defective mutant of \textit{K. lactis} was used to improve the production yield of glucoamylase.\textsuperscript{148}

The dimorphic species \textit{A. adeninivorans} uses adenine, xanthine, uric acid, putrescine, glucose and \textit{n}-alkylamines. Among the few recombinant enzymes that have been expressed in \textit{A. adeninivorans}, a tannase has been successfully over-produced using plasmids carrying two expression modules.\textsuperscript{149} \textit{S. pombe} is an attractive host for the expression of recombinant enzymes. An endopolygalacturonase from \textit{S. cerevisiae} PGU1 was successfully expressed in \textit{S. pombe}.\textsuperscript{150} The deletion of its proteases improved the secretion of recombinant enzymes in the \textit{S. pombe}. In fact, a multi-protease-deficient \textit{S. pombe} strain A8 obtained by deleting protease genes resulted in a 36-fold increase in the secretion of IGH, a proteolytically sensitive model protein.\textsuperscript{151}

The methylothrophic yeast \textit{C. boidinii} was investigated as an efficient host for the secretory production of enzymes. The acetylperoxidase oxidase (ASOD)-coding sequence was placed under the control of the \textit{AODI} promoter and overexpressed in the \textit{C. boidinii} strain \textit{ade1}.\textsuperscript{152}

**High-Level Expression of Microbial Enzymes in Filamentous Fungi**

Due to the superior capacities of hyper-producing and secret- ing proteins, filamentous fungi are extraordinary hosts for the overexpression of recombinant enzymes. The industrial strains include \textit{Aspergillus}, \textit{Trichoderma}, \textit{Penicillium} and \textit{Rhizopus} species. In particular, \textit{Aspergillus} and \textit{Trichoderma} species can produce and secrete very high levels of recombinant enzymes.

**High-level expression of microbial enzymes in \textit{Aspergillus} species.** \textit{Aspergillus} species are particularly important industrial filamentous fungi employed in the large-scale production of both homologous and heterologous enzymes. \textit{A. oryzae} and \textit{A. niger} are on the GRAS list of the Food and Drug Administration (FDA) in the United States. Although \textit{A. fumigatus} and \textit{A. nidulans} are the natural filamentous fungal production hosts, they produce toxins and are therefore not relevant as hosts for the production of recombinant enzymes.\textsuperscript{153,154} \textit{A. niger} represents a promising host for the expression of recombinant enzymes. Some homologous and heterologous enzymes were overexpressed in \textit{A. niger} by using a standard- ized expression cassette. For example, a manganese peroxidase gene (\textit{mnp}) from \textit{Phanerochaete chrysosporium} was transformed by exploiting the resistance to hygromycin B, and successfully expressed in \textit{A. niger}.\textsuperscript{155} A \textit{Hyphomycetes scorpii} endoglucanase \textit{Ct7B} was expressed in a recombinant \textit{A. niger}, which was grown on spent hydrolysates (stillage) from sugarcane bagasse and spruce wood.\textsuperscript{156} The features that influence protein production and secretion could be employed to improve enzyme production levels in industrial bioprocesses via protein engineering. Protein sequence features were explored in relation to the produc- tion of overexpressed extracellular proteins by \textit{A. oryzae}. It was found that tyrosine and asparagine composition positively influence the high-level production of recombinant enzymes.\textsuperscript{157} Specific induction of a regulatory site of the \textit{actR} (actin related) promoter may be used to enhance the heterologous protein production in \textit{A. niger}. For example, the induction of the \textit{catR} promoter with 0.2% \textit{H2O2} and 1.5% \textit{CaCO3} in the culture medium increased the expression of the alkaline xylanase by 2.63- and 2.20-fold in \textit{A. niger}, respectively.\textsuperscript{158}

\textit{A. oryzae} is an especially interesting host for the production of recombinant proteins because of its demonstrated capacities for hyper-produce and secrete enzymes. For example, a glucose-stimulated \textit{β}-glucosidase from the termite \textit{Nesititermes bokensensis} was successfully expressed in \textit{A. oryzae}.\textsuperscript{159} Two termite endogenous \textit{β}-1,4-endoglucanases (\textit{RbEG} and \textit{NtEG}) from the salivary gland of \textit{Reticulitermes speratus} and the midgut of \textit{Nesititermes takahasai} were respectively expressed in \textit{A. oryzae} as the host.\textsuperscript{159} Proteolytic degradation by secreted protease into the culture medium is a significant problem that remains to be solved in the heterologous protein production by filamentous fungi. In order to enhance the production of bovine chymosin in \textit{A. oryzae}, two (\textit{ppk4} and \textit{ppk3}) and five (\textit{ppk4}, \textit{ppk6}, \textit{ppk8}, \textit{dppb} and \textit{dppv}) protease genes were disrupted.\textsuperscript{160} Since the post-transcription processing limits enzyme yield, the vacuolar protein-sorting gene \textit{VPS10} was mutated to investigate the extracellular production levels of heterologous proteins in \textit{A. oryzae}.\textsuperscript{161}

**High-level expression of microbial enzymes in \textit{Trichoderma} species.** The \textit{Trichoderma} spp includes \textit{T. reesei}, \textit{T. atroviride} and \textit{T. virgilius}. \textit{T. reesei} has an extraordinary ability to secrete proteins and represents a principal target cellulase host in the quest to replace gasoline with cellulose-derived ethanol. However, a more efficient heterologous expression system for enzymes from different organisms is needed to further improve cellulase mixture in \textit{T. reesei}. For example, the strong \textit{ehb3} (cellulohydrolase I) promoter of \textit{T. reesei} was used in the heterologous expression of cellulases.\textsuperscript{162} Lv et al. constructed two \textit{T. reesei} expression vec- tors, \textit{pWEEF3} and \textit{pWEF3}, which are useful for the large-scale expression of the gene encoding the enzyme.\textsuperscript{163} Three endoxylanase genes (\textit{Ct xyn1A}, \textit{Ct xyn1B} and \textit{Ct xyn1C}) from the thermophilic fungus \textit{Chaeomium thermophilum} CBS 730.95 were expressed in \textit{T. reesei} under the control of the strong \textit{T. reesei cell7A (chh1)} promoter.\textsuperscript{164} The lipase gene (\textit{Lip}) of \textit{A. niger} was de novo synthesized and expressed in the \textit{T. reesei} under the pro- moter of the \textit{chhl}.\textsuperscript{165} Moreover, RNAi-mediated gene silencing could effectively suppress \textit{ehb3} gene expression, and the reduction of \textit{CBH1} resulted in a clear improvement in the production of recombinant enzymes.

**High-level expression of microbial enzymes in other fila- mentous fungi species.** The white rot fungi \textit{Penicillium} and \textit{Rhizopus} spp have the potential for the production of recombi- nant enzymes. For example, two novel lignin peroxidase genes isolated by RT-PCR and RACE-PCR were transformed into the white rot fungus \textit{P. annulispora} \textit{YK-62} uracil auxotrophic mutant UV-64, and successfully expressed and secreted in an active form.\textsuperscript{166} A strain with high production of pectin lyase (PL) was transformed with the plasmid pAN32ppg2, containing the gene encoding polygalacturonase (PG) of \textit{P. griseoroseum}, under the control of the \textit{gpd} promoter from \textit{A. nidulans}. The activities of
PL and PG in the recombinant strain *P. griseoroseum* were 266- and 274-fold higher, respectively, than those in the wild-type strain. Additionally, carbon sources can affect the secretion of enzymes in *Penicillium* species. Navarrete al. found that the acetylated xylan and sugar beet pulp exerted strong regulatory control over the enzymes secreted in *Penicillium camemberti* 

Meanwhile, *Rhizopus arrhizus* has been used for the production of enzymes such as glucoamylase and lipase. Mertens et al. have created three plasmid vectors using the glucoamylase A (amyA), pyruvate decarboxylase (*pdhA*) or phosphoglycerate kinase (*pgk1*) promoter to drive the expression of heterologous proteins.

**Conclusions**

Recombinant microbial enzymes can be expressed in cell cultures of bacteria (e.g., *E. coli*, Bacillus or lactic acid bacteria), yeasts or filamentous fungi. Large proteins (> 100 kD) are usually expressed in eukaryotic systems, while smaller ones (< 30 kD) are expressed in prokaryotic systems. The *E. coli* expression system continues to dominate the bacterial expression systems. Yeasts can be grown rapidly to high densities, and the level of product expression can be simplified by regulation of the medium. *Bacillus* systems are high secretors and host strains and are mainly preferred for the homologous expression of recombinant enzymes (e.g., proteases and amylases). Since filamentous fungi can secrete high levels of biosynthetic proteins in the wild-type strain, they became attractive hosts for the recombinant DNA technology. Although the lactic acid bacteria are safe and highly effective expression systems that can revolutionize the field of recombinant protein production in food and drug, the application of lactic acid bacteria as host factories for the overexpression of recombinant proteins needs to be further explored.

Due to the wide variety of applications of recombinant enzymes, their need will increase keratinase increase in the future. The challenges to meet the growing demand are multifaceted in terms of quality, quantity and cost-effectiveness. The production of large amounts of recombinant proteins is of interest and needs to employ high-throughput expression technologies. A comprehensive coverage of the proteins in a proteome is demanded for systems-level understanding. With a large variety of vectors, promoters and selection markers to choose from, combined with the accumulated knowledge on industrial-scale fermentation techniques and current advances in post-genomic technology, it is possible to design improved cost-effective expression systems in order to meet the increasing demand for enzymes.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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