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Production of Recombinant Bleaching Enzymes from Thermophilic Microorganisms in Fungal Hosts

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Abstract

Cost-effective production of enzymes for industrial processes makes the appropriate selection of the host-vector expression system critical. We have developed two systems for the bulk production of bleaching enzymes from thermophiles. *Kluyveromyces lactis* has been developed as a secretion host employing expression vectors based on the 2μ-like plasmid pKD1 of *Kluyveromyces drosophilarium*. Our second system involves the filamentous fungus *Trichoderma reesei*. Fusion and nonfusion vectors have been constructed using the strong cellobiohydrolase 1 (*cbh1*) promoter. The KEX2 protease cleavage site and a 6 × HIS-tag have been incorporated to facilitate both cleavage and purification of the mature foreign proteins.

Index Entries: *Trichoderma reesei*; *Kluyveromyces lactis*; thermophilic xylanases; heterologous expression; gene redesign.

Introduction

Over the last 5 yr in particular, the advantages of expressing recombinant proteins in eukaryotic cells have become apparent in terms of correct

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processing of complex gene products to functional proteins *(1)*. However, expression of foreign proteins at high levels, to the full potential of a particular system, has been difficult to achieve. Cost-effective production of extremophilic enzymes for commercial or industrial processes makes the appropriate selection of the host-vector expression system critical. Although there have been recent reports of high-level protein production in the milk of transgenic animals *(1)*, we have chosen to explore the potential of fungi as hosts for heterologous protein production to allow significant scaleup and to simplify downstream processing with the objective of being able to use enzymes secreted into the growth medium without further treatment.

Filamentous fungi such as *Aspergillus* and *Trichoderma* traditionally have been the organisms of choice for large-scale processes associated with the production of bulk industrial enzymes. However, their natural abilities such as growth on relatively simple and cheap (waste) media, secretion of gram amounts of gene products into the cultivation medium to facilitate product harvesting, and their eukaryotic machinery for posttranslational modifications of proteins make them attractive potential hosts for the synthesis of any high-value protein or biocatalyst. Protein production (transcription, translation, and secretion) in uni- and multicellular fungi follows the same principles and uses the same basic mechanisms as in other eukaryotes. *Kluyveromyces lactis* and *Trichoderma reesei* are relatively simple organisms that possess the eukaryotic machinery for protein processing. However, there are also differences. *K. lactis* is a unicellular organism related to *Saccharomyces cerevisiae* whose entire genome has been sequenced, and there is currently a large-scale effort to determine the function of every gene in the yeast genome. *K. lactis* has the advantage that it does not hyperglycosylate heterologous proteins as seen with *S. cerevisiae (2)*. *T. reesei* is an industrially exploited multicellular organism and one of the most powerful secretors of extracellular proteins, because of its ability to excrete in the order of 40 g/L of protein into the culture medium. In this article, we review our recent results on the expression of enzymes from thermophiles in these two hosts.

Materials and Methods

K. lactis System

The construction of the vector pCWK1 and the insertion of the *Dictyoglomus thermophilum* and *Thermotoga maritime* FjSS3B.1 *xynA* genes have been described previously *(3–5)*. The vector is composed of the 2μ plasmid of *K. drosophilarum* for replication in *K. lactis (6)*, the origin and ampicillin-resistance gene of pUC19 for replication in *Escherichia coli*, the pLAC4 promoter in front of the killer toxin signal sequence with a single *Mlu*I site for cloning the heterologous gene as a fusion, an LAC4 terminator, the kanamycin-resistance gene from Tn903 (conferring resistance to Geneticin in yeasts), and the URA3 gene of *S. cerevisiae* for integration and

selection in antibiotic-free medium (Fig. 1A). Other deletion derivatives have been developed, but they were unstable and rapidly segregated as a result of deletion of two inverted repeat sequences believed to be required for stable partition *(7)*.

Trichoderma reesei System

A series of vectors has been constructed using the strong main cellobiohydrolase *cbh1* promoter with provision for in-frame fusions of the heterologous genes to either the *cbh1* signal sequence or downstream of the *cbh1* core-linker region. These vectors, which are shown in Fig. 1B as stylized representations, may include either a N- or a C-terminal 6×HIS-tag for subsequent purification of the foreign protein, and a KEX2 site (PMDKR or RDKR) has been inserted for correct cleavage to regenerate the mature protein from the CBHI core-linker. Glycine 477 and glutamine 478 of the CBHI cellulose-binding domain have been retained to allow cleavage by an uncharacterized *T. reesei* proteinase *(8)*. Suitable restriction sites have been introduced into the vectors to allow linearization for targeted integration at the *cbh1* locus after the removal of the pUC19 portion of the vector (gapped vector). All vectors have the pUC19 replicon for replication in *E. coli* and utilize the hygromycin B gene as a selective marker in *Trichoderma*.

Synthesis of Dictyoglomus xynB *Gene with Altered Codon Usage*

XynB from the bacterium *D. thermophilum* has been shown to be quite effective in the bleaching of kraft pulp *(9)*. Preliminary experiments showed that the native gene was not expressed in *Trichoderma* and that it was necessary to change its codon usage to resemble that of the fungus. A primer extension polymerase chain reaction (PCR) strategy was employed to change 20 codons of *xynB*. The 603-bp-long catalytic domain was reconstructed in a two-step procedure using eight 85 to 107-bp oligonucleotides containing overlapping regions of 14–24 bp (Fig. 2A). The synthetic gene was ligated into the vector pHEN54 and then transformed into *E. coli (10)*. About 300 *E. coli* recombinants were tested using the xylan-overlay assay *(11)*, of which 12 exhibited a clear halo around the colony indicating expression of an active xylanase (Fig. 2B). From a total of 19 transformants in *Trichoderma*, 5 exhibited thermostable xylanase activity in the plate test carried out following overnight incubation at 70°C (Fig. 3). There was no obvious correlation between the halo size and the type of plasmid used (fusion to *cbh1* signal sequence or the catalytic core). Full details of the experimental methods are published elsewhere *(10)*.

Analytical Techniques

Details of the biolistic transformation developed for conidia have been published *(12)*. Standard protein analysis procedures on polyacrylamide gels have been described *(4)*, as have enzyme assay procedures *(9,13)*.

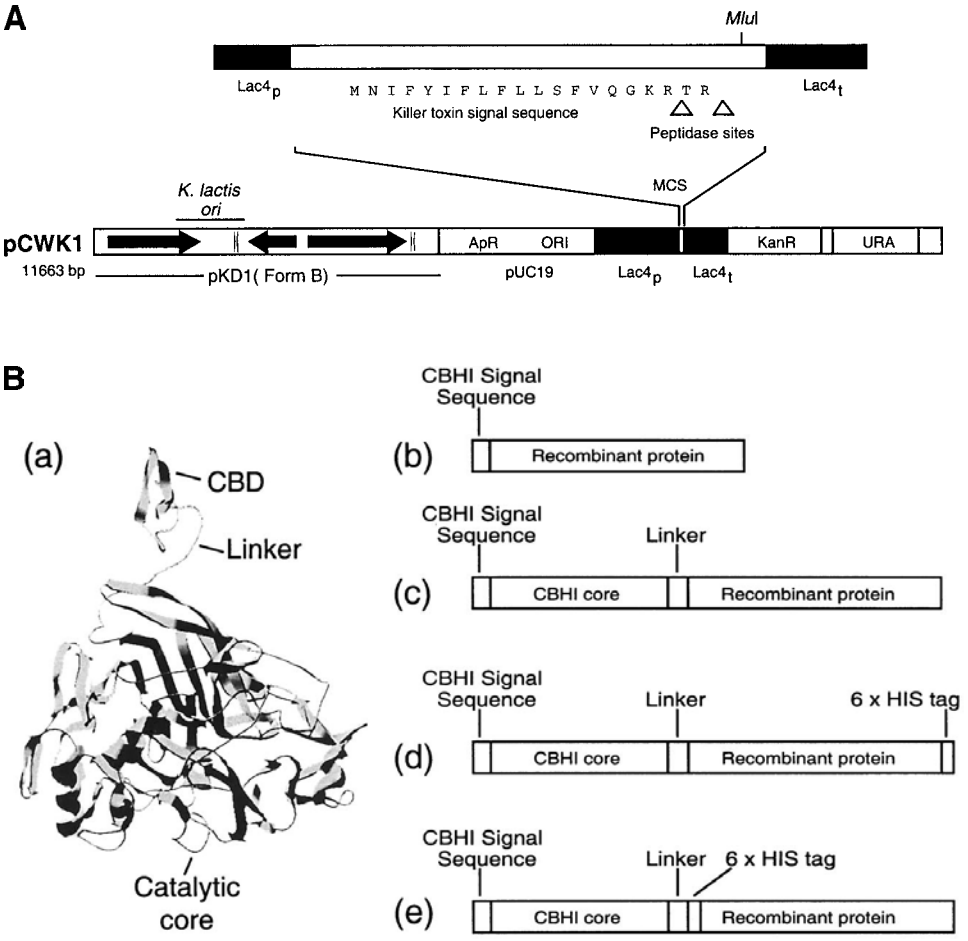
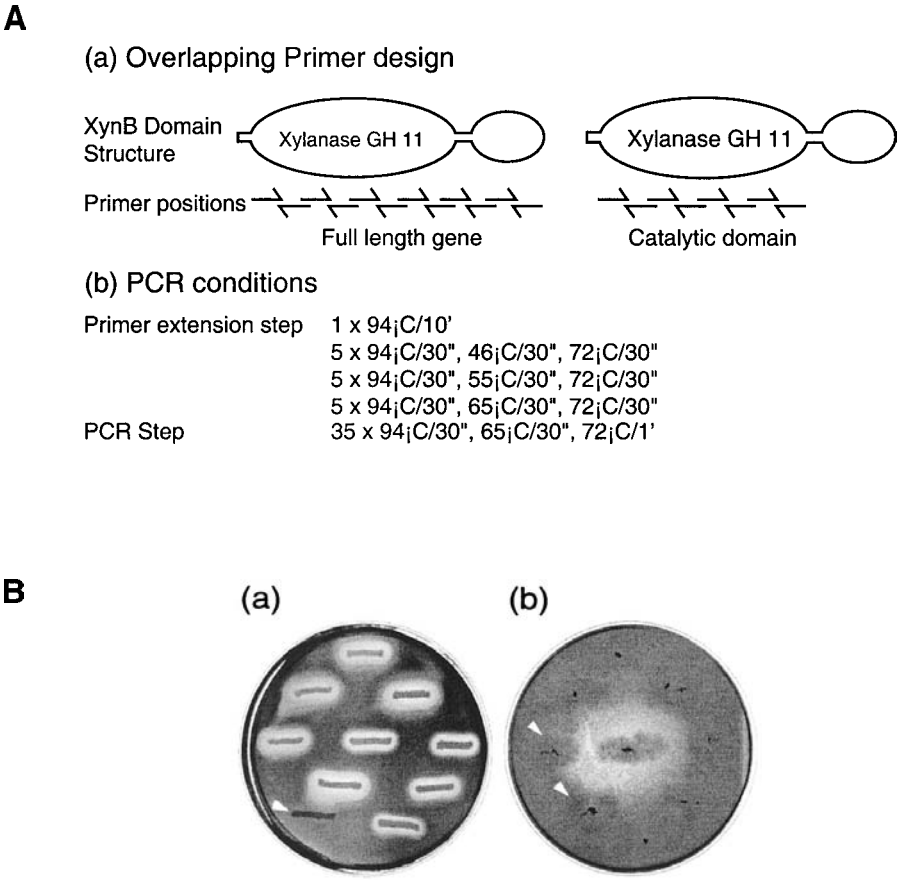


Fig. 1. **(A)** Schematic of *K. lactis* expression vector pCWK1. The 11.66-kb vector consists of the 2-μ plasmid of *K. drosophilarum*, the pUC19 plasmid, and the LAC4 promoter and terminator region from *K. Lactis*. The killer toxin signal sequence ends in an *Mlu*I cloning site. The gene for kanamycin resistance allows selection on media containing Geneticin and the *Saccharomyces* URA3 gene provides for integration of the plasmid into the chromosome or selection in a *ura*-minus strain. **(B)** Ribbon diagram of CBHI from *T. reesei* (a) and schematic of *Trachoderma* expression vectors (b–e). Vector b has only the CBHI signal sequence fused to the heterologous protein, whereas vector c has the foreign protein fused to the CBHI core-linker sequences. Vectors d and e have either 3′- (d) or 5′-terminal 6 × HIS tags incorporated to aid in downstream purification.

Results and Discussion

K. lactis strains harboring a pKD1-based expression vector efficiently secrete the recombinant *Thermotoga* xylanase at high levels (Fig. 3 and refs. *4* and *5*). All strains examined were able to promote secretion of the xylanase with the same electrophoretic mobility as the control enzyme



Fig, 2. **(A)** PCR strategy for alteration of codon usage of *D. thermophilum* Rt46B.1 *xynB* gene to allow expression in *T. reesei*. (a) Overlapping primers for primer extension to generate the full-length gene and the catalytic domain. (b) PCR conditions for primer extension and PCR of the synthetic gene. **(B)** Transformants for *xynB*-expressing xylanase in *E. coli* (a) and *T. reesei* (b). Plates were overlaid with oat spelts xylan in agarose and stained with Congo Red after incubating overnight at 70°C.

produced in *E. coli* (approx 33 kDa). Recombinant xylanase is by far the most abundant protein present in transformed *K. lactis* culture supernatants and the *Thermotoga* XynA protein comprises >95% of total proteins secreted from *K. lactis* strain CBS1065 as determined by densitometry of scanned gels (Fig. 3).

Several parameters influence xylanase secretion levels, including plasmid architecture and composition of the growth medium. The carbon source influenced secretion levels by modulating promoter strength, which in turn affects plasmid mitotic stability *(4)*. In noninducing glucose medium, *xynA* expression directed by LAC4 is partially repressed in CBS1065, although plasmid stability remains high. Full induction of the LAC4 pro-

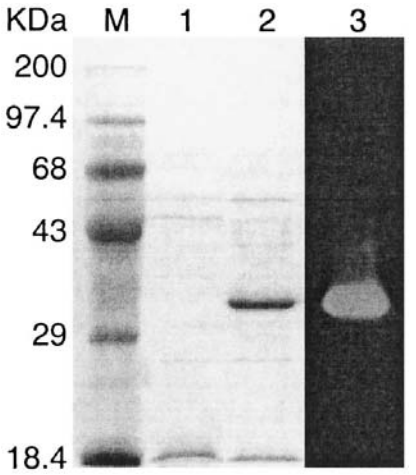


Fig. 3. Polyacrylamide gel electrophoresis of superatants from strain CBS1065 with plasmid pCWK1 (lane 1) and pCWK-*xynA* of *T. maritime* FjSS3B.1 (lane2). Lane 3, activity gel showing xylanase activity afterrenaturation and Congo red staining of pCWK-*xynA* supernatant fractions; lane M, molecular weight markers. For more details, see ref. *4*.

moter on galactose results in a drastic reduction in plasmid stability in strain CBS1065, and, consequently, lower levels of xylanase secretion are observed *(4)*.

The killer toxin secretion signal is processed by *K. lactis* signal peptidase to produce cleavage after Gln-Gly in the killer toxin signal sequence and the release of correctly processed XynA *(14)*. Recombinant xylanases produced in *K. lactis* are biologically active as shown by activity gels *(3,4)* and are not hyperglycosylated, as found previously for expression of a thermophilic xylanase in *S. cerevisiae (2)*. Since little is known of glycosylation patterns in *K. lactis* strains *(15)*, further investigation would help future studies of expression by this organism.

The XynA enzyme from *Thermotoga*, produced in *K. lactis*, has optimal activity at 90°C and a half-life comparable with the results reported for this enzyme produced in *E. coli (16)*. Similarly, the *Dictyoglomus* XynA protein produced in *K. lactis* also was shown to have identical biochemical characteristics to its counterpart produced in *E. coli (3)*. The next step involves the scaleup of the production system in fermentors for the production of substantial amounts of enzyme for larger-scale bleaching trials that require kilogram quantities of enzyme. It will be necessary to develop an appropriate induction strategy to minimize the effects of plasmid instability with pCWK1 in the absence of Geneticin selection, as well as the rapid metabolism of the inducer, galactose, in wild-type strains. The galactose concentration falls rapidly, limiting the level of heterologous enzyme produced (unpublished data). This may be overcome by introducing the mutant galactokinase gene *gal1-209* to provide a gratuitous induction system *(17)*.

Transformation of Trichoderma

T. reesei is an asexually reproducing filamentous fungus in which the vegetative growth is mycelial and conidiation is induced by light. The spores (conidia) develop in a condiaphore, from which they are released and germinate under suitable conditions, reestablishing mycelial growth. The traditional method for the transformation of *Trichoderma* is the introduction of the transforming DNA into protoplasts in the presence of polyethylene glycol (PEG). However, protoplast transformation is time-consuming and produces a high number of unstable transformants. Furthermore, many industrially utilized high-protein-secreting *T. reesei* strains have resulted from a strain improvement program involving random mutagenesis and screening *(18)*. However, exposure to mutagenic agents may change the fungal cell wall *(19)*, causing complications in standardizing the protoplasting protocol (unpublished data) and regenerating the cell wall. Thus, alternative transformation methods avoiding the use of fungal protoplasts, such as direct bombardment of intact conidia, are advantageous, providing simplicity, savings in time, and stable recombinants. Nuclear transformation by microprojectile bombardment typically results in the integration of several copies of the transforming plasmid into the fungal genome as well as in increased mitotic stability of the transformants *(20,21)*. Biolistic transformation with single plasmids has been applied to *T. harzianum (20)* and *T. longibrachiatum (22)* by others.

We have developed a technique in which DNA is introduced into *T. reesei* conidia using bombardment with tungsten microprojectiles carrying precipitated plasmid, gapped plasmid DNA, or two different plasmids *(12)*. The cotransformation frequencies obtained are sufficient to avoid the need for cloning the gene of interest in the same plasmid as the selection marker. The procedure is convenient because conidia are usually haploid; there is no requirement for osmotic stabilizers or elaborate transformant purification, and the transformants are mitotically stable. The copy numbers of the integrated hygromycin resistance gene varied from 1 to 6 and occurred at several different places on the genome *(12)*. A modification of the procedure in which the "gene gun" is adapted to carry seven instead of one barrel allows an entire standard Petri dish to be bombarded. Bombardment conditions have been optimized and up to 50 well-defined *Trichoderma* colonies can be accommodated on a standard Petri dish *(23)*.

Heterologous Bacterial Thermophile Xylanase Genes Expressed in Trichoderma

For commercial exploitation, it is necessary to express the gene *xynB* of *D. thermophilum* in a high-yielding organism familiar to the fermentation industry. We introduced this gene into *T. reesei*, but preliminary experiments showed no expression. Optimization of the levels of heterologous protein expression in fungal systems has targeted gene copy number, gene and protein fusions, and the use of strong promoters *(24)*. Less attention has

Table 1
Examples of Codons Changed to Achieve expression of *D. thermophilum* Rt46B.1 *xynB* Gene in *T. reesei*

Amino acid	Codon	Usage in *T. reesei* (%)	Usage in *xynB* (%)	New Codon
Ile	ATA	4	42	ATC
Thr	ACA	10	47	ACC
	ACT	20	33	ACC
Cys	TGT	24	80	TGC
Tyr	TAT	25	62	TAC
Leu	TTA	1	43	CTG
	CTT	14	30	CTC
Phe	TTT	38	33	TTC
Gin	CAA	19	60	CAG
Pro	CCA	12	50	CCC

been paid to mRNA stability, processing, and translational efficiency, which, at least to some extent, depend on the coding sequence of an individual gene. The choice of synonymous codons may vary widely among different genes and organisms. For example, efficiently expressed *Trichoderma* genes exhibit a strong bias against A or T at the anticodon wobble position *(25)*, whereas *D. thermophilum xynB* prefers A or T at the third codon position *(9)*. In addition, the overall A-T content of *xynB* is 61% compared with <40% in a typical *T. reesei* cellulase gene. These observations may be a source of potential problems for the expression of genes with high A-T content, such as the *D. thermophilum xynB*, in the fungal host by the formation of truncated mRNA transcripts because of incorrect processing of A-U-rich elements and potential underrepresentation of isoacceptor tRNAs for effective peptide synthesis *(26)*.

The strategy of modification of the sequence of the incoming gene without altering the amino acid sequence of the gene product has been successfully applied for the expression of mammalian gene products in *E. coli (27)*. Published reports on the synthesis and expression of heterologous genes exhibiting the codon preference of highly expressed genes in filamentous fungi have appeared only recently *(10)*. We have reconstructed the *D. thermophilum xynB* gene to accommodate the codon usage pattern of *Trichoderma*. Some of the more significant codons altered are given in Table 1.

Northern analysis revealed the presence of mRNA transcripts of the expected size for both plasmid types in the transformants tested *(10)*. Expression from the signal fusion plasmid was more effective than that from the fusion to mature cellobiohydrolase I (CBHI) core. The N-terminal fusion partner may play a role in stabilizing the recombinant mRNA, serving as a carrier to facilitate the translocation of foreign proteins in the secre-

tory pathway and protecting the heterologous portion from degradation. In the case of *xynB*, the mRNA-stabilizing effect of the fusion was not obvious, and preliminary results from liquid cultivation suggest that the signal fusion strain also secreted more thermostable xylanase activity in the culture medium (not shown).

Our results with the *D. thermophilum xynB* in *T. reesei* indicate that extreme differences in the codon usage between the incoming foreign gene and the expression host could arrest expression at the transcriptional level. Modification of the codon usage of the *xynB* has proved instrumental in restoring effective transcription for the production of the thermophile enzyme in the mesophilic fungal host.

Expression of a Xylanase Gene from a Thermophilic Fungus in T. reesei

A cDNA gene encoding a family 11 xylanase (*xyn2*) has been isolated from the thermophilic fungus *Humicola grisea* var *thermoidea*. The XYN2 protein produced in *E. coli* is confined in inclusion bodies and is difficult to purify and refold *(28)*. Therefore, the *xyn2* gene was retransformed into *T. reesei* as a fusion to the *cbh1* signal sequence and the CBHI corelinker with and without a polyhistidine tag (Fig. 1B). The plasmids were transformed into several *T. reesei* strains using the biolistic particle bombardment system. Selected recombinants were grown in liquid medium suitable for the induction of the *cbh1* promoter *(29)*. Culture medium from a transformant harboring the plasmid with *xyn2* fused to the *cbh1* secretion signal showed a prominent band at the correct molecular mass that also showed xylanase activity on an in-gel assay. This band was isolated and the N-terminal sequence determined. The sequence obtained, QVTPNAE, was shown to coincide with a KEX2-like cleavage site in alignments against *T. reesei* xylanases XYN1 and XYN2, showing that it was processed in a similar manner to give mature enzyme secreted into the supernatant. The recombinant XYN2 yields were of the order of 0.5–1 g/L in nonoptimized shake-flask cultivations and are currently being improved by fermentor culture. The methods used and the results are more fully described elsewhere *(35)*.

Conclusion

The *Kluyveromyces* system uses a multicopy plasmid vector and is convenient because of its relative ease of transformation, genetic manipulation and verification, and rapid growth. *K. lactis* does not hyperglycosylate proteins in the manner of *S. cerevisiae (2,3)*. Although protein concentration is usually about 100 mg/L in shake-flask culture, we achieved much higher levels in fermentor culture in which the pH can be kept constant, although conditions have not been fully optimized. There are few promoter systems available, and the PLAC promoter is the most frequently used. This sequence has several *E. coli*-like promoter sequences present, and, consequently, there

tends to be leakage in steps involving replication in the bacterium. Therefore, it has been difficult to clone and maintain other genes encoding products toxic to the cell in the shuttle vector (unpublished data). Another disadvantage is the instability of the plasmid vector in the absence of Geneticin selection. Although this is host strain dependent, it is possible to prevent segregation of plasmidless cells in the absence of selection by careful attention to physiologic conditions during growth. We have yet to explore enzyme production from genes recombined into the chromosome.

The *T. reesei* system relies on the integration of the transforming DNA into the fungal genome, resulting in a better stability of trans-formants than when using autonomously replicating plasmids. Our biolistic transformation procedure allows relatively simple and reproducible transformation of industrial strains with a complex history of mutational events aimed at increasing enzyme secretion. The vectors constructed provide a variety of N- and C-terminal modifications for ease of purification and processing.

Even though heterologous fungal proteins can be produced in filamentous fungi in grams/liter amounts, the yield of gene products of higher eukaryotic origins have remained considerably lower *(8,31)*. The main cause of low yields lies in the processing/secretion of the proteins *(32,33)*. The basic requirements for a host suitable for large-scale protein production with good economics are the cost and simplicity of cultivation and minimal downstream processing. This can be achieved with a production system in which the enzyme is effectively secreted into the cultivation medium. In this regard, fungi provide an attractive option to industrial bacterial systems.

Current strategies to improve the processing and yields of biotechnologically relevant heterologous proteins in filamentous fungi include studies of gene regulation, protein glycosylation, occurrence of intra- and extracellular proteases, manipulation of chaperones and foldases assisting other proteins in the secretory pathway, and fungal genomics and proteomics *(34,35)*. Basic information for the construction of an expression plasmid suitable to a particular production host is available from public databases. Each gene can be modified, e.g., in terms of codon usage and by introducing or deleting signals for a particular cellular location. It is possible to add posttranslational processing sites for the gene product, change the biochemical properties of the molecule to be expressed to facilitate translocation, or delete sequences recognized by host proteases. Finally, closer examination of the "expression physiology" of the chosen host organism is necessary, especially regarding conditions for effective product formation. By following the strategies discussed here, it is expected that the yields of recombinant xylanases and other bleaching enzymes produced in fungal hosts will be considerably improved.

Acknowledgments

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