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MINI-REVIEW

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Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts

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Abstract Bulk production of xylanases from thermophilic microorganisms is a prerequisite for their use in industrial processes. As effective secretors of gene products, fungal expression systems provide a promising, industrially relevant alternative to bacteria for heterologous enzyme production. We are currently developing the yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* for the extracellular production of thermophilic enzymes for the pulp and paper industry. The *K. lactis* system has been tested with two thermophilic xylanases and secretes gram amounts of largely pure xylanase A from *Dictyoglomus thermophilum* in chemostat culture. The *T. reesei* expression system involves the use of the cellobiohydrolase I (CBHI) promoter and gene fusions for the secretion of heterologous thermostable xylanases of both bacterial and fungal origin. We have reconstructed the AT-rich *xynB* gene of *Dictyoglomus thermophilum* according to *Trichoderma* codon preferences and demonstrated a dramatic increase in expression. A heterologous fungal gene, *Humicola grisea xyn2*, could be expressed without codon modification. Initial amounts of the XYN2 protein were of a gram per liter range in shake-flask cultivations, and the gene product was correctly processed by the heterologous host. Comparison of the expression of three thermophilic heterologous microbial xylanases in *T. reesei* demonstrates the need for addressing each case individually.

Key words *Trichoderma reesei* · *Kluyveromyces lactis* · Thermophilic xylanases · Heterologous expression

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Introduction

Enzymes of industrial interest are routinely expressed in various microbial hosts to increase the yields to satisfy the needs of both the manufacturer and the end user. The economics required for a particular application varies according to the volume and the final product from the process. For industrial processes of large volume, such as wood pulp bleaching, enzymes must be produced at a several grams per liter range to provide a competitive alternative/additive to eliminate or decrease the amount of chlorine dioxide used in pulp delignification (Table 1). For enzymatic pulp bleaching to be cost competitive, enzyme should be produced at less than US$2.25 per gram and the final cost of enzyme treatment should not exceed US$4.50 per tonne of pulp. Therefore, cost-effective production of enzymes for the processing industries makes the appropriate selection of the host-vector expression system critical.

Most commercial xylanases are mesophilic enzymes produced by the filamentous fungi *Trichoderma reesei* and *Aspergillus niger*. This process reflects well the fact that filamentous fungi are naturally excellent protein secretors and can produce enzymes in industrially feasible amounts. However, endogenous fungal xylanases may not be efficient enough in all conditions used in paper mills where enzymes active at high temperatures (up to 85°C and pH 9 or more) would be desirable. Consequently, enzymes from thermophilic microorganisms have gained attention, and a number of genes encoding hemicellulases have been isolated from

Table 1. Culture volumes required to provide enzyme for an average-sized pulp mill

Organism	Approximate enzyme yield (g) per liter of culture supernatant	Culture volume (l) requirement per tonne of pulp[a]	Culture volume (l) requirements per week for average-sized pulp mill[b]
Kluyveromyces lactis	0.3	3.3	26,400
Trichoderma reesei	5.0	0.2	160

[a]Based on an average requirement of 1 g enzyme per tonne of pulp
[b]Based on an average mill processing 8,000 tonnes pulp/week

many thermophilic bacteria (Kulharni 1999) and some thermophilic fungi (Iikura et al. 1997; Paloheimo et al. 1998; Lin et al. 1999; Takashima et al. 1999; Poças-Fonseca et al. 2000; Faria et al. 2000). Low productivity, considerable technical problems, and unfavorable production economics associated with the cultivation of thermophilic microorganisms on a large scale (reviewed in Robb and Place 1995; Adams 1996) have led to a search for other, industrially exploited microorganisms as production hosts for thermophilic proteins.

Genes of bacterial origin are usually expressed in heterologous bacterial systems, typically *Bacillus licheniformis* or *B. amyloliquefaciens* for efficient secretion of gene products for industrial applications (Debabov 1982). Even though yields of some secreted enzymes from genetically improved *Bacillus* systems can reach several grams per liter (Yoneda 1982), many heterologous gene products suffer from the aggressive proteases produced by the host organism (Lundström 1984; Kallio et al. 1986). Recently, exoprotease-deficient mutants of *B. brevis* have been used as expression hosts for heterologous gene products (Kajino et al. 1999). Expression of hydrolases for industrial applications in *Escherichia coli* is not a feasible option because of poor secretion, relatively low yields, and the pathogenic nature of the host organism. However, expression in *E. coli* will continue to serve as a tool for the synthesis of recombinant proteins for initial structural and functional studies. Development of other bacterial production systems with industrial potential as expression hosts, such as lactic acid bacteria (Kuipers et al. 1997) or *Streptomyces* (Binnie et al. 1997), is underway.

Mutant strains of filamentous fungi can excrete tens of grams of endogenous extracellular protein into their growth medium (reviewed in Nevalainen et al. 1994; Berka et al. 1991). However, there are only few examples of successful expression of bacterial genes in fungal hosts. Many bacterial enzymes produced in fungi seem to remain trapped in the fungal mycelium (van den Hondel et al. 1991; Jeenes et al. 1991). With some notable exceptions, the best yields reported for bacterial enzymes secreted by fungi have been of the order of 10–20 mg/l (Gwynne et al. 1987). Although fungal systems have a great potential for protein secretion, more research is needed into the bottlenecks associated with heterologous gene expression. We have chosen to explore the potential of the unicellular yeast *Kluyveromyces lactis* and the filamentous fungus *Trichoderma reesei* as hosts for heterologous protein production to allow significant scale-up and to simplify downstream processing of thermophilic enzymes with the objective of being able to use enzymes secreted into the growth medium without further treatment. In this article, we review our recent experiments on the expression of thermophilic xylanases in the two fungal hosts and compare the efficacy with other expression systems developed for thermophilic enzymes.

Kluyveromyces lactis as an expression host

Kluyveromyces lactis is a unicellular organism related to *Saccharomyces cerevisiae*, whose entire genome has been sequenced. Therefore, it is expected that development of *K. lactis* as an expression host will benefit from the current large-scale effort to determine gene functions in *S. cerevisiae* because there are significant similarities between genes sequenced from the two yeasts (Ozier-Kalogeropoulos et al. 1998). Standard *S. cerevisiae* markers such as *TRP1*, *URA3*, and *LEU2* genes have been used in *K. lactis*, where they complement mutant genes (Wésolowski-Louvel et al. 1996). Various heterologous genes have been expressed in *K. lactis* with protein yields from 20 mg/l (amylase) to a few grams per liter (bovine prochymosin) from both plasmid-borne and integrated genes (reviewed in Wésolowski-Louvel et al. 1996; Gellissen and Hollenberg 1997).

Construction of the pKD1-based (Bianchi et al. 1987) expression vector pCWK1 (Fig. 1) used for our studies has been described previously, as has the insertion of the *Dictyoglomus thermophilum* and *Thermotoga maritima* FjSS3B.1 *xynA* genes (Walsh and Bergquist 1997; Walsh et al. 1998a, b). *K. lactis* transformant strains examined were able to promote secretion of the *Thermotoga* xylanase XynA with the same electrophoretic mobility as the enzyme produced in *E. coli*. Recombinant XynA was by far the most abundant protein present in transformed *K. lactis* culture supernatants (Fig. 2) and comprised more than 95% of the total protein secreted from strain CBS1065 (Centraalbureau voor Schimmelculturen, Utrecht, The Netherlands).

Several parameters influence xylanase secretion levels, including plasmid architecture and composition of the growth medium. Significantly higher levels of extracellular enzyme were attained through *LAC4*-directed XynA expression on full pKD1 sequence vectors as compared to expression with other pKD1 *ori* plasmid derivatives that we have constructed (Walsh et al. 1998a). The carbon source influenced secretion levels by modulating promoter strength, which in turn affects plasmid mitotic stability

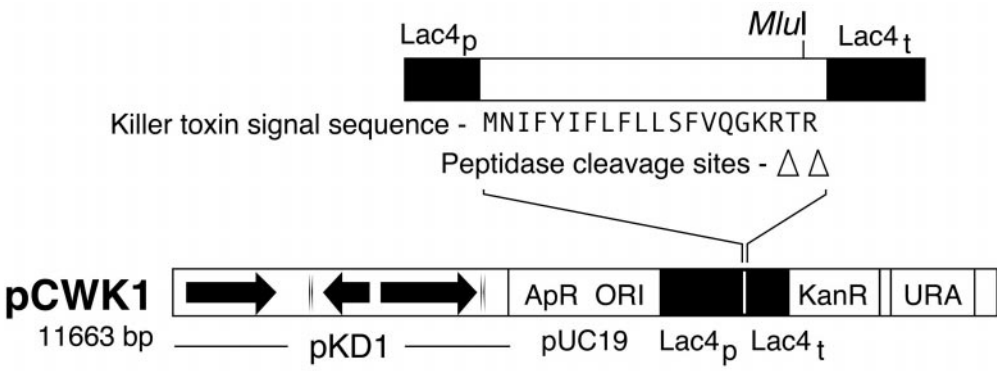

Fig. 1. Schematic of the *Kluyveromyces lactis* expression vector pCWK1. The 11.66-kb vector consists of the pKD1 plasmid of *Kluyveromyces drosophilarum*, the pUC19 plasmid, and the *LAC4* promoter and terminator region from *K. lactis*. The vector uses the killer toxin signal sequence with a single *Mlu*I site to allow cloning and expression of the heterologous gene as a fusion to the signal sequence. The kanamycin resistance gene (KanR) from transposon Tn903 confers geneticin resistance. The *URA3* gene of *Saccharomyces cerevisiae* is for integration of the construct into the yeast genome or selection in antibiotic-free medium when applicable

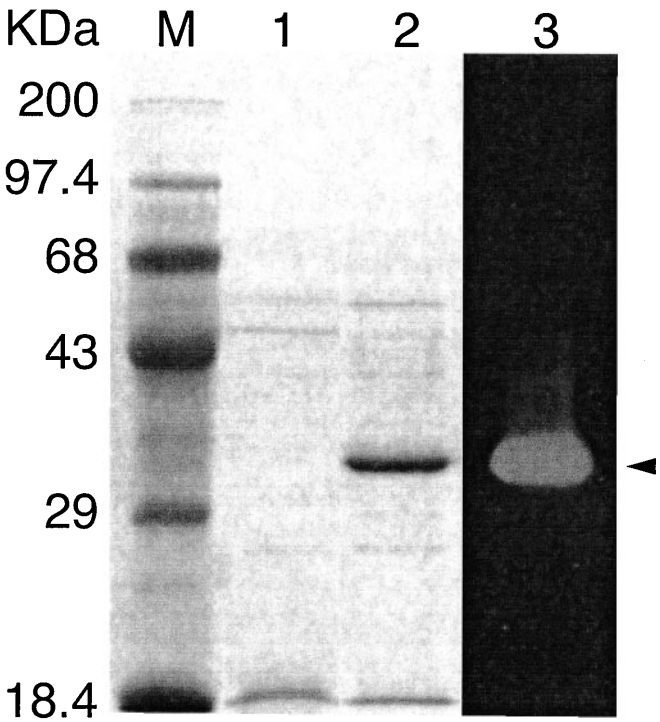

Fig. 2. Polyacrylamide gel electrophoresis of supernatants from strain CBS1065 with the plasmid pCWK1 (*lane 1*) and pCWK-*xynA* of *Thermotoga maritima* FjSS3B.1 (*lane 2*). *Lane 3* is a zymogram showing xylanase activity (the 33-kDa band is indicated by an *arrowhead*) after protein renaturation and Congo red staining of an SDS-PAGE gel containing oat spelts xylan. *Lane M*, molecular weight markers. For more details, see Walsh et al. (1998a)

(Walsh and Bergquist 1997). In noninducing glucose medium, *xynA* expression directed by *LAC4* is incompletely repressed in CBS1065, although plasmid stability remains high. Full induction of the *LAC4* promoter on galactose (without glucose) resulted in a drastic reduction in plasmid stability in strain CBS1065, and consequently lower levels of xylanase secretion were observed. The killer toxin secretion signal (Tanguy-Rougeau et al. 1988) is processed by *K. lactis* signal peptidase to give cleavage after Gln-Gly to release of correctly processed XynA.

The XynA enzyme from *Thermotoga* produced in *K. lactis* has optimal activity at 90°C and a half-life comparable to the results reported for this enzyme produced in *E. coli* (Saul et al. 1995). Similarly, the *Dictyoglomus* XynA protein produced in *K. lactis* was shown to have identical biochemical characteristics to its counterpart produced in *E. coli* (Walsh and Bergquist 1997). The two recombinant xylanases produced in *K. lactis* are biologically active (Walsh and Bergquist 1997; Walsh et al. 1998a) and are not hyperglycosylated, as we found previously for expression of the thermophilic *Caldicellulosiruptor saccharolyticus* xylanase, XynA, in *S. cerevisiae* (Donald et al. 1994).

Using the strain CBS1065 as the expression host combines reasonable secretion levels on nonselective medium without optimization of induction (120 mg/l in shake-flask culture) with high plasmid stability on YEP plus glucose. Thus, this recombinant is suitable for scaled-up production of *Thermotoga* xylanase A in chemostat culture where 20- to 30-fold improvements have been recorded in preliminary experiments with *K. lactis* transformant strains harboring the recombinant *xynA* gene (Harris and Bergquist, unpublished data). The next step for further improvement of the yields of recombinant xylanases in *K. lactis* involves optimization of fermenter cultivations for the manufacture of substantial amounts of enzyme for other basic biochemical studies and for larger-scale bleaching trials. To reach this goal, it will be necessary to develop an appropriate induction strategy to minimize the effects of plasmid instability with pCWK1 seen in the absence of geneticin selection.

Trichoderma reesei as an expression host

Trichoderma reesei is an asexually reproducing filamentous fungus in which the vegetative growth is mycelial and conidiation is induced by light. A series of vectors have been constructed by us and others (Mäntylä et al. 1998; Penttilä 1998;

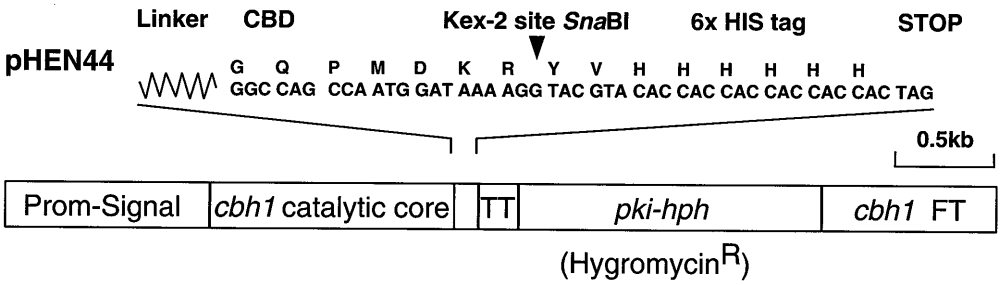

Fig. 3. Schematic of a *Trichoderma reesei* expression vector featuring a C-terminal 6× HIS tag for purification of the heterologous protein and a KEX2 peptidase site for correct cleavage to regenerate the mature protein from the CBHI catalytic core linker. The residues glycine-477 and glutamine-478 of the CBHI cellulose-binding domain have been retained to allow cleavage by an uncharacterized *T. reesei* proteinase (Penttilä 1998). The vector can be linearized to allow targeted integration at the *cbh1* locus. *TT*, *cbh1* truncated transcriptional terminator; *cbh1 FT*, full *cbh1* transcriptional terminator; *pki-hph*, hygromycin resistance gene

Te'o et al. 2000a) using the strong main cellobiohydrolase *cbh1* promoter with provision for in-frame fusions of heterologous genes to either the *cbhl* signal sequence or downstream of the *cbh1* catalytic core-linker region (for an example, see Fig. 3).

The traditional method for the transformation of *Trichoderma* is the introduction of the transforming DNA into protoplasts in the presence of polyethylene glycol (Mach and Zeilinger 1998). However, protoplast transformation is both is time consuming and results in a high number of unstable transformants. This process may also be unsuitable for many industrially utilized high protein-secreting *T. reesei* strains that have undergone a rigorous strain improvement program by random mutagenesis and screening (Nevalainen et al. 1994) that may have affected their ability to regenerate the cell wall (Nevalainen et al. 1995). Thus, alternative transformations such as direct bombardment of intact conidia are advantageous, providing simplicity, savings in time, and a stable outcome.

Biolistic transformation with single plasmids has been applied to *Trichoderma harzianum*, currently developed as a biocontrol agent (Lorito et al. 1993), and *T. longibrachiatum*, which has been used as a heterologous expression host (White and Hinde 1996). We have developed this technique further to introduce two individual plasmids into *T. reesei* conidia in one hit with a cotransformation efficiency of about 90% (Hazell et al. 2000). The procedure is convenient as *T. reesei* conidia are haploid, there is no requirement for osmotic stabilizers or elaborate transformant purification, and the transformants are mitotically stable (Hazell et al. 2000). A modification of the procedure in which the "gene gun" is adapted to carry seven barrels instead of one has allowed an entire standard petri dish to be bombarded to accommodate up to 50 well-defined colonies (Te'o et al. 2000b). This procedure allows the acquisition of large numbers of transformants by simply increasing the number of plates bombarded.

Thermophilic bacterial xylanases expressed in *Trichoderma*

A number of heterologous fungal hydrolase genes have been successfully expressed in *T. reesei* (Paloheimo et al. 1993), but there are only few published reports on the expression of genes encoding thermophilic or thermostable enzymes in this organism. These genes include a xylanase gene from the actinomycete *Actinomadura flexuosa* (Paloheimo et al. 1998), a xylanase gene, *bcx-2,* from *Bacillus circulans* (White and Hinde 1996), modified to increase thermostability of the enzyme (Campbell et al. 1995), and the xylanase gene, *xynB,* from *Dictyoglomus thermophilum* (Te'o et al. 2000a). The heterologous *A. flexuosa* xylanase was produced from the *cbh1* promoter as a fusion protein to the *T. reesei* mannanase core-linker containing amino acid sequences for the processing by a KEX2-like protease (Paloheimo et al. 1998). The improvement was more than 200 fold when compared to xylanase levels in the bacterial gene donor. Comparison of the relative xylanase activites showed that the highest activity was obtained in transformants where the protein was effectively cleaved from the carrier (Paloheimo et al. 1998). The recombinantly produced xylanase retained its thermal properties (stable at 70°C for 100 h at pH 7) and allowed 15%–20% reduction in the consumption of chlorine chemicals in a mill-scale experiment. The DNA sequence of the *Actinomadura* xylanase discussed earlier has not been disclosed; however, there is a very strong bias toward C at the wobble position in the *Actinomadura* sp. FC7 xylanase II gene sequence, which strongly resembles that seen with the most powerfully expressed main cellobiohydrolase *cbh1* of *T. reesei* (Table 2). Similarity of the *Actinomadura* sp. FC7 xylanase II with the *T. reesei* xylanases at the amino acid level is 47.5% for xylanase I and 41.5% for xylanase II, respectively.

The genetically modified *B. circulans* xylanase (BcX-2) has a temperature optimum of 70°C at pH 6.5. The BcX-2 and *T. longibrachiatum* (*T. reesei*) xylanase II are 65% similar at the amino acid level (White and Hinde 1996). When the *bcx-2* gene was expressed under the *cbh1* promoter and secretion signal, only about 1% of the total xylanase activity was secreted outside into the culture medium, whereas the majority of the enzyme accumulated inside the mycelium and became proteolytically degraded. This observation was suggested to result from the nonprocessing of the secretory signal peptide from the BcX-2 protein. It was noted that the combined amount of intra- and extracellular recombinant xylanase activity in the transformants was about 50-fold

Table 2. Comparison of the codon usage among the following genes: *Bacillus circulans bcx-2* (Campbell et al. 1995), *Actinomadura* sp. FC7 xylanase II (*xylII*, accession number U08894), *T. reesei* xylanase I (*xyn1*, accession number S51973), xylanase II (*xyn 2*, accession number S51975), and cellobiohydrolase I (*cbh1,* Penttilä et al., unpublished data)

AA	Codon	*Bc bcx-2*	*Am xylII*	*Tr xyn1*	*Tr xyn2*	*Tr cbh1*	AA	Codon	*Bc bcx-2*	*Am xylII*	*Tr xyn1*	*Tr xyn2*	*Tr cbh1*
Gly	GGG	4	2	2	1	2	End	TAG	0	0	0	1	0
Gly	GGA	8	2	1	7	12	End	TAA	1	0	1	0	1
Gly	GGT	10	1	2	5	10	Tyr	TAT	8	0	0	2	4
Gly	GGC	6	37	22	12	40	Tyr	TAC	8	12	17	9	21
Glu	GAG	0	10	6	7	16	Phe	TTT	4	0	4	5	2
Glu	GAA	4	1	1	1	3	Phe	TTC	5	12	5	3	13
Asp	GAT	4	0	2	2	15	Ser	AGT	5	0	0	5	2
Asp	GAC	0	25	2	5	9	Ser	AGC	6	12	6	10	18
Val	GTG	3	8	5	4	4	Ser	TCG	3	5	5	3	7
Val	GTA	3	1	0	0	0	Ser	TCA	1	0	0	2	3
Val	GTT	4	0	1	7	7	Ser	TCT	4	0	4	4	12
Val	GTC	4	20	12	11	13	Ser	TCC	4	12	13	5	16
Ala	GCG	2	11	4	2	4	Arg	AGG	0	1	0	1	0
Ala	GCA	2	0	0	1	2	Arg	AGA	2	0	0	0	1
Ala	GCT	1	1	3	6	10	Arg	CGG	1	3	1	1	1
Ala	GCC	2	36	6	5	17	Arg	CGA	0	0	0	0	1
Lys	AAG	1	13	5	1	12	Arg	CGT	2	0	1	4	1
Lys	AAA	3	0	0	0	2	Arg	CGC	1	19	7	1	7
Asn	AAT	11	0	1	3	2	Gln	CAG	3	22	9	7	21
Asn	AAC	8	35	19	17	31	Gln	CAA	5	0	1	4	2
Met	ATG	1	8	2	5	7	His	CAT	2	0	0	1	0
Ile	ATA	1	0	0	0	0	His	CAC	1	6	3	4	5
Ile	ATT	4	0	3	1	4	Leu	TTG	0	0	0	0	4
Ile	ATC	4	16	6	7	8	Leu	TTA	3	0	0	0	0
Thr	ACG	1	9	8	1	10	Leu	CTG	1	13	3	4	13
Thr	ACA	4	0	0	3	7	Leu	CTA	1	0	0	0	1
Thr	ACT	2	0	0	9	14	Leu	CTT	0	2	0	4	5
Thr	ACC	9	19	9	9	27	Leu	CTC	0	11	4	3	5
Trp	TGG	6	11	6	6	9	Pro	CCG	2	6	3	0	5
Cys	TGT	0	0	0	0	4	Pro	CCA	3	0	2	2	2
Cys	TGC	0	7	1	1	20	Pro	CCT	0	0	0	3	10
End	TGA	0	0	0	0	0	Pro	CCC	2	10	5	3	11

This table was generated using the Wisconsin Genetics Computer Group (GCG) Sequence Analysis Program "Codon Frequency" (Devereux et al. 1984)

AA, amino acid; *Bc*, *Bacillus circulans*; *Am*, *Actinomadura* FC7; *Tr*, *Trichoderma reesei*

lower than the endogenous xylanase in the untransformed host (White and Hinde 1996). This result implied further problems in the transcription of the bacterial gene and translation of the gene product in the heterologous fungal host. Transcription may be hampered by the fact that there are three codons in the *B. circulans bcx-2* gene, GTA (V), ATA (I), and TTA (L) that are never used in the *Trichoderma xyn1*, *xyn 2*, or *cbh1* (see Table 2).

The extremely thermophilic bacterium *D. thermophilum* produces a xylanase, XynB, that has optimal activity at 85°C and pH 6.5. The enzyme has been tested in large-scale bleaching of eucalyptus pulp with excellent results (Morris et al. 1998). We introduced the native *xynB* gene into *T. reesei* to boost the enzyme yield, but preliminary experiments showed no expression. One possible reason for this result was considered to involve the choice of synonymous codons, which proved to be very different in *T. reesei* and *D. thermophilum*. Efficiently expressed *Trichoderma* genes exhibit a strong bias against A or T at the anticodon wobble position (Goller et al. 1998) whereas *D. thermophilum xynB* prefers A or T at the third codon position (Morris et al. 1998). In addition, the overall AT content of *xynB* is 61% compared to less than 40% in a typical *T. reesei* cellulase gene; this may cause problems in the fungal host by the formation of truncated mRNA transcripts because of to incorrect processing of AU-rich elements and potential underpresentation of isoacceptor tRNAs for effective peptide synthesis (Gouka et al. 1996). The similarity of the *D. thermophilum* xylanase B to *T. reesei* xylanase I and II is about 45% at the amino acid level in both cases.

The effect of codon usage on the expression of heterologous proteins in filamentous fungi has not been addressed in detail. Most often the foreign gene to be expressed in a fungal host has had the native codons of the donor organism. For reasons already discussed, we reconstructed the *D. thermophilum xynB* gene to accommodate the codon usage pattern of *Trichoderma* CBHI. Altogether, 20 codons were altered by primer extension PCR; some examples are shown in Table 3. Full details of the experimental methods are published elsewhere (Te'o et al. 2000a). Recombinant *E. coli* colonies exhibiting the largest halos in the xylan-overlay assay (Teather and Wood 1982) were chosen for

Table 3. Examples of codons changed to achieve expression of the *Dictyoglomus thermophilum* Rt46B.1 *xynB* gene in *Trichoderma reesei*

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

plasmid DNA isolation and sequencing to confirm that the synthetic gene was intact. DNA isolated from one selected transformant was transferred into a proteinase-defective *T. reesei* strain under the *cbh1* promoter as a fusion to *cbh1* signal sequence and as a fusion with the catalytic core-linker of the mature CBHI protein. Northern analysis revealed the presence of mRNA transcripts of the expected size for both plasmid types in the transformants tested (Te'o et al. 2000a).

The three examples discussed here indicate that prediction of the outcome of the expression of a bacterial xylanase gene in *Trichoderma* is not straightforward. With the *Actinomadura* xylanase, satisfactory levels of the enzyme seemed to have been obtained by the carrier protein strategy. *Actinomadura* codon usage resembles that of *Trichoderma cbh1* (see Table 2). Production of *B. circulans* xylanase in *T. longibrachiatum* was hampered, to start with, by the inefficient cleavage of the *cbh1* secretion signal in the expression host. There were also three codons that were never used by *T. reesei xyn1, xyn2*, or *cbh1* genes. Prerequisite for the expression of the AT-rich *D. thermophilum xynB* gene in *T. reesei* was the construction of a synthetic gene according to the codon usage of *Trichoderma* genes, including the highly expressed *cbh1*. In this case, fusion to a mature endogenous carrier protein was not necessary for secretion and did not improve enzyme yields detected in the culture medium.

In general, secreted recombinant xylanase has been visualized as multiple protein bands in Western blots (White and Hinde 1996; Paloheimo et al. 1998), and activity gel assays (Fig. 4). The bands have been interpreted as differently processed or modified forms of the heterologous enzyme. In our case, the *D. thermophilum xynB* contains four putative N-glycosylation sites and may thus be glycosylated in the fungal host, therefore resulting in the multiplicity of protein bands separated by SDS-PAGE. It is noteworthy that, in all three examples discussed here, a different *Trichoderma* expression host was used and that in at least two cases the host was a high-secreting mutant strain. Therefore, there may be other differences between the host strains made by random mutagenesis that are unknown or have not been explored in detail involving, for example, glycosylation and proteolytic activity (Nevalainen et al. 1998; Nevalainen 2001). Yields of the heterologous bacterial xylanases produced in *Trichoderma* have not been disclosed in grams per liter terms. In general, starting levels of about 0.5 g/l allow considerable improvement of the yields by fermentation using industrially exploited mutant strains to achieve economically feasible amounts of the enzyme.

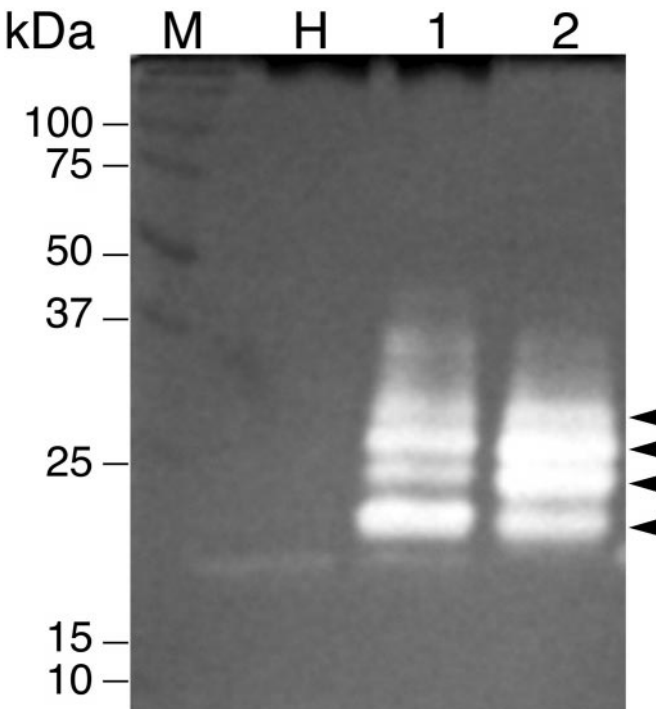

Fig. 4. Zymogram activity assay showing the different-sized forms of the heterologous *Dictyoglomus thermophilum* XYNB enzyme produced in *T. reesei*, assayed at 70°C. *Lane M*, low molecular weight protein markers; *lane H*, untransformed *T. reesei* host strain; *lanes 1* and *2*, *T. reesei*-XYNB transformants with multiple bands exhibiting xylanase B activity shown as clearing around the protein bands (*arrowheads*). Proteins in the *T. reesei* culture supernatants were separated on a 12% SDS-PAGE gel containing 0.5% oat spelts xylan

Expression of xylanase genes from thermophilic fungi in *Trichoderma reesei*

Thermophilic fungi can grow at temperatures exceeding 45°C and produce hydrolases that are in general more heat stable than the corresponding enzymes produced by mesophilic fungi (Maheswari et al. 2000). Paloheimo et al. (1998) screened about 150 thermophilic fungal isolates to discover novel xylanases with industrially relevant characteristics. As a result, several strains were found that produced xylanase activity that was stable at 70°C. Xylanase-encoding genes were isolated from the best strains and expressed in *T. reesei* under the *cbh1* promoter. Expression levels and bleaching properties of the recombinant xylanases were reported to be similar to the native xylanases, allowing large-scale trials.

A cDNA gene encoding a family 11 xylanase (*xyn2*) has been recently isolated from the thermophilic fungus *Humicola grisea* var. *thermoidea* (Faria et al. 2000). The *Humicola* XYN2 gene product is highly active at 70°C,

Fig. 5. An alignment of the N-terminal region of the recombinant *Humicola* enzyme (HXYN2) compared to the *T. reesei* xylanases I and II (TRC30XYN1 and -2). A KEX2-like secondary peptidase processing site is indicated by an *arrowhead*. *Shaded boxes* represent amino acids common to all three sequences

pH 6.5 (Faria et al., unpublished data). We have cloned the *xyn2* gene into *T. reesei* as a fusion to the *cbh1* signal sequence and the CBHI core-linker (Faria et al. 2002). 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 exhibited xylanase activity on a zymogram 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 XYNI and II, showing that it was processed in a similar manner to give mature enzyme secreted into the supernatant (Faria et al. 2002) (Fig. 5). The recombinant XYN2 yields were of the order of 0.5–1 g/liter in nonoptimized shake-flask cultivations and have been improved further by fermentation.

Conclusions

The requirements for large-scale protein production are low cost, simple cultivation, and minimal amounts of downstream processing. These goals 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 biotechnically relevant heterologous proteins in filamentous fungi include studies into gene regulation, protein glycosylation, occurrence of intra- and extracellular proteases, manipulation of chaperones and foldases assisting other proteins in the secretory pathway, and initiatives in fungal genomics and proteomics (reviewed in Nevalainen 2001). However, there is no single pathway to success. In each case, such as with bacterial xylanases as discussed here, the end result seems to depend on the matching of the gene to be expressed in a particular heterologous host at the molecular level. A wealth of basic information can be extracted from public databases to assist in the construction of an expression plasmid "interpretable" to the production host. Each gene can be modified, for example, in terms of codon usage and by introducing signals for extracellular transport. In addition, it is possible to add posttranslational processing sites for the gene product, change the biochemical properties of the protein, or modify sequences recognized by host proteases. Finally, it is evident that more studies are needed into the expression biology of the chosen host organism, especially regarding conditions for effective product fermentation. It is reasonable to assume that the yields of recombinant xylanases produced in fungal hosts will be considerably improved during the next few years following the strategies discussed here.

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