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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Expression of *Trichoderma reesei* cellulases CBHI and EGI in *Ashbya gossypii*

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Abstract To explore the potential of *Ashbya gossypii* as a host for the expression of recombinant proteins and to assess whether protein secretion would be more similar to the closely related *Saccharomyces cerevisiae* or to other filamentous fungi, endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from the fungus *Trichoderma reesei* were successfully expressed in *A. gossypii* from plasmids containing the two micron sequences from *S. cerevisiae*, under the *S. cerevisiae PGK1* promoter. The native signal sequences of EGI and CBHI were able to direct the secretion of EGI and CBHI into the culture medium in *A. gossypii*. Although CBHI activity was not detected using 4-methylumbelliferyl-β-D-lactoside as substrate, the protein was detected by Western blot using monoclonal antibodies. EGI activity was detectable, the specific activity being comparable to that produced by a similar EGI producing *S. cerevisiae* construct. More EGI was secreted than CBHI, or more active protein was produced. Partial characterization of CBHI and EGI expressed in *A. gossypii* revealed overglycosylation when compared with the native *T. reesei* proteins, but the glycosylation was less extensive than on cellulases expressed in *S. cerevisiae*.

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Introduction

The filamentous hemiascomycete *Ashbya gossypii* (Ashby and Nowell 1926) is a biotechnologically important producer of vitamin B2 (riboflavin; Demain 1972; Wickerham et al. 1946). Based on rDNA sequences, *A. gossypii* is more closely related to *Saccharomyces cerevisiae* than *Neurospora crassa* or *Aspergillus nidulans* (Wendland et al. 1999). *A. gossypii* has one of the smallest known eukaryotic genomes (Dietrich et al. 2004), a high homologous recombination efficiency allowing simple gene knockout strategies and precise positioning of gene constructs (Steiner et al. 1995; Wendland et al. 2000), extrachromosomal replication of plasmids bearing an autonomous replicator (Wright and Philippsen 1991), efficient introduction of heterologous DNA, and the fungus lacks the extensive duplication of chromosomal segments observed in *S. cerevisiae*, making it an extremely attractive candidate not only for riboflavin production, but also for the production of other industrial products such as recombinant proteins. However, little is known about the secretion of proteins by *A. gossypii*. Extracellular lipase has been detected, but activity was low in most conditions (Stahmann et al. 1997). An endo-β-1,4-glucanase (celA1) from *Streptomyces halstedii* has previously been expressed in *A. gossypii* with limited success (Althöefer et al. 2001). However, other filamentous fungi which do not secret large amounts of native enzymes/proteins have been found to still be good producers of recombinant proteins (Royer et al.

1995). Since *A. gossypii* is a filamentous fungus, it might be expected to have efficient protein secretion, but its close relationship to *S. cerevisiae* may suggest limited secretion ability. We therefore expressed two cellulase genes from the filamentous fungus *Trichoderma reesei* in *A. gossypii* in order to assess its ability to produce and secrete recombinant proteins.

T. reesei is one of the most extensively studied cellulolytic organisms (Kubicek et al. 1993). It produces three types of enzyme activities needed for degradation of crystalline cellulose into glucose. These are cellobiohydrolases (EC 3.2.1.91), which act as exoenzymes and release cellobiose as a main product from crystalline cellulose, endoglucanases (EC 3.2.1.4), which have high affinity towards soluble cellulose derivatives and attack these by endo-action, and β-glucosidases (EC 3.2.1.21), which hydrolyse cellooligosaccharides and the disaccharide cellobiose into glucose.

T. reesei CBHI and endoglucanase I (EGI) are useful as model proteins for recombinant protein secretion, since they have been extensively studied in a variety of expression systems, as well as in *T. reesei*, and appear to be challenging for other organisms to produce. Expression of CBHI in *Escherichia coli* (Laymon et al. 1996; Teeri 1987) and yeast (Godbole et al. 1999; Penttilä et al. 1988; Reinikainen et al. 1992) has resulted in the production of either insoluble or low activity enzyme. Whereas *E. coli* produced misfolded CBHI peptide and/or CBHI inclusion bodies from which active enzyme could not be obtained, yeasts (*S. cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*) produced hyperglycosylated cellulases with low activity on either soluble substrates or amorphous cellulose. Even when *T. reesei* cellulases have been expressed in the efficient protein producing fungus *Aspergillus oryzae*, the activity of both CBHI and CBHII toward Avicel were relatively low when compared with the native enzymes, possibly because of differences in the modes of glycosylation (Takashima et al. 1998). *Aspergillus niger* var. *awamori* (Jeoh et al. 2008) produced functional enzyme, but it was more glycosylated than the native enzyme. Reports of successful expression of *T. reesei* CBHI from non-*T. reesei* hosts have been limited to one example of insect cell (*Spodoptera frugipedra*) production, following baculovirus transfection (von Ossowski et al. 1997).

Heterologous expression of EGI from *T. reesei* has been reported in *S. cerevisiae* and *Y. lipolytica* (Park et al. 2000; Penttilä et al. 1987b; Van Arsdell et al. 1987). The recombinant EGI produced by *S. cerevisiae* was hyperglycosylated and significantly larger than the native enzyme produced by *T. reesei*. There were similarities in the extent of hyperglycosylation, but differences in the types of glycosylation between *Y. lipolytica* and *S. cerevisiae* (Park et al. 2000). Apart from the differences in the extent of asparagine-linked glycosylation, recombinant EGI from *Y. lipolytica* appeared to be processed in a similar manner to the processing of the native enzyme by *T. reesei*. EGI seems to be slightly easier than CBHI to produce in non-*Trichoderma* recombinant hosts.

In this paper, we evaluate the potential of *A. gossypii* as a host for recombinant protein production, using the *T. reesei* cellulases CBHI and EGI as model proteins. These proteins were chosen because they represent a challenge in recombinant protein production and have previously been expressed in several expression platforms. Because of the close genetic relationship between *A. gossypii* and *S. cerevisiae*, the comparison with expression in *S. cerevisiae* was of particular interest.

Materials and methods

Strains

A. gossypii ATCC10895 was obtained from Prof. P. Philippsen (Basel University) and maintained on agar (20 g l^{-1}) on *Ashbya* full medium (AFM; 10 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 1 g l^{-1} myo-inositol, 20 g l^{-1} glucose). *A. gossypii* EGI (VTT D-101398) and *A. gossypii* CBHI (VTT D-101399) were obtained by transformation of ATCC10895 as described below. *S. cerevisiae* H81 (α, *his3Δ1 leu2–3,112 ura3–52 trp1–289* Cyh^R) was used as host to obtain comparable transformants of *S. cerevisiae*.

Spores were prepared by collecting 7–10 day-old mycelium from agar solidified plates, digesting the mycelia with zymolyase (150 mg ml^{-1}) for 2 h and washing two times with a solution of 0.8% (*w*/*v*) NaCl, 20% (*v*/*v*) glycerol, and 0.025% (*v*/*v*) Tween 20. Aliquots were suspended in the same solution and were stored at −80°C.

Culture media

LB medium supplemented with 100 μg ampicillin ml^{-1} was used for *E. coli* cultivation. AFM supplemented with 200 μg ml^{-1} G418 was used for the selection and maintenance of *A. gossypii* transformants containing the G418 resistance gene (Dünkler and Wendland 2007; Wendland et al. 2000). For regeneration of protoplasts, AFM with 20 g agar l^{-1} was supplemented with 1 M sorbitol. To detect cellulase-producing strains, agar solidified AFM was supplemented with 0.1% (*w*/*v*) barley β-glucan or 0.1% (*w*/*v*) carboxymethylcellulose (CMC). Synthetic complete defined medium (SCD) contained complete amino acid supplement (Sherman et al. 1986), yeast nitrogen base (Difco), 20 g l^{-1} glucose, 1 g l^{-1} $CaCo_3$, and 1 g l^{-1} agar.

Plasmid construction

Plasmid pMI519 (Fig. 1a) contained *T. reesei egl1* cDNA and plasmid pMI514 (Fig. 1b) *T. reesei cbh1* cDNA. Plasmids pMI519 and pMI514 were constructed from plasmids pTTc11 (Penttilä et al. 1987b) and pTTc1 (Teeri et al. 1987), respectively, together with B609 (Ruohonen et al. 1995), using the primers listed in Table 1. Both genes were under the control of the *S. cerevisiae PGK1* promoter and terminator obtained from plasmid pAJ401 (Saloheimo et al. 1994). The *kanMX* expression module was derived from a modified pUG6 vector, with the *kan*^{r} gene from the *E. coli* transposon Tn*903* under control of the *TEF* promoter from *A. gossypii* (Güldener et al. 1996; Wach et al. 1994) and the *ADH1* terminator from *S. cerevisiae*, to confer to the transformants resistance to the aminoglycoside antibiotic G418 (Jimenez and Davies 1980; Webster and Dickson 1983).

The control vector pMI516 without a cellulase insert was constructed by digesting pMI514 with *Pac*I–*Asc*I, filling in the ends using T4 polymerase and circularizing the 6.8 kb fragment.

A. gossypii transformation and screening of transformants

Transformation of *A. gossypii* was carried using protoplasts as described by Penttilä et al. (1987a), and selection was carried out on medium containing G418. Spores from single colonies were collected as described above and stored at −80 °C for further analysis.

Transformants were screened in two ways. Initial screening was carried out by PCR using specific primers (Table 1). Template DNA was extracted by a rapid-miniprep method in which a piece of mycelium was transferred to a microfuge tube and lysed with lysis buffer (400 mM Tris–HCl pH 8.0; 60 mM EDTA, pH 8.0; 150 mM NaCl; 1% *w*/*v* SDS) at room temperature for 10 min. Proteins were precipitated by adding 150 µl of 3 M potassium acetate pH 4.8, vortexing and centrifuging at 13,000×*g* for 1 min. The supernatant was transferred to a clean tube, and the DNA was precipitated by adding an equal amount of isopropanol. The sample was mixed by inverting and centrifuged at 13,000×*g* for 2 min. The DNA was washed with ethanol 70% (*v*/*v*) and air-dried, before being dissolved in 50 µl TE buffer. PCR products were amplified with polymerase DNA2 MGII (Finnzymes) using 5 µl sample DNA with, F5 buffer (Finnzymes), 2 µl 2 mM dNTP mix, 0.2 µl oligo (5 nmol µl^{-1}) EGI, CBHI, or G418-specific primers.

After the initial screening, mycelia from several colonies were grown in test tubes containing 5 ml AFM supplemented with 200 µg ml^{-1} G418 and samples of the culture medium assayed for secreted EGI or CBHI using 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma) as the substrate. Based on these results, two strains, VTT D-101398 (expressing EGI) and VTT D-101399 (expressing CBHI), were selected for further study.

S. cerevisiae H81 was transformed with the same plasmids as *A. gossypii* using the LiAc transformation method (Gietz et al. 1995) and screened in the same way as *A. gossypii* transformants.

Culture conditions

Transformed strains of *A. gossypii* were grown at 30°C or 24°C in flasks (250 ml flasks containing 50 ml medium) at 200 rpm on AFM or SCD medium with 20 g l^{-1} glucose as carbon source and 200 µg ml^{-1} G418. Samples were collected to determine cell concentration (absorbance measured at 600 nm) and to obtain supernatant by filtration through glass fiber GF/C filters or by centrifugation (10 min at 4,000×*g* and 4°C).

Dry weight was determined by converting OD at 600 nm with a conversion factor obtained from a calibration curve, where one unit OD at 600 nm was found to correspond to

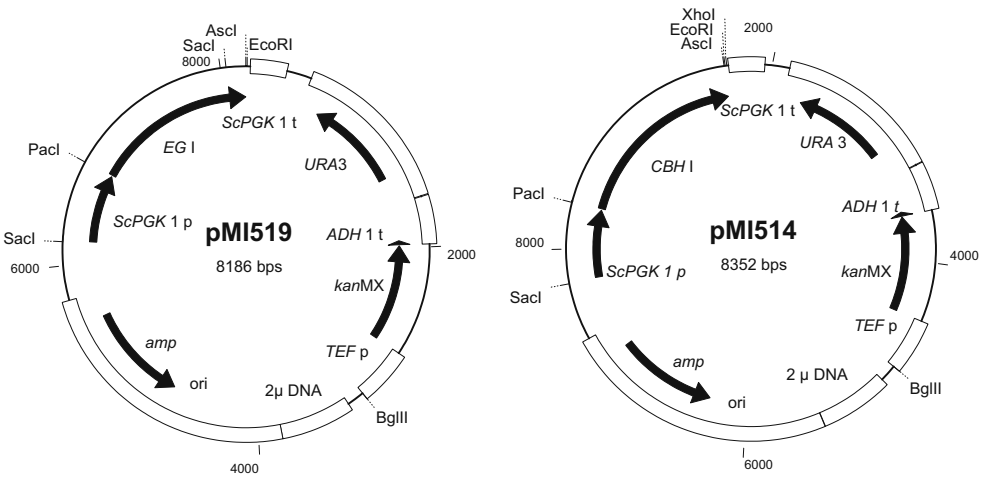

Fig. 1 Plasmids for expression of *T. reesei* CBHI or EGI in *A. gossypii*. cDNA was inserted under the control of the PGK promoter and terminator from *S. cerevisiae*

Table 1 Oligonucleotides used for construction of plasmids and strain screening

Oligonucleotide	Sequence
373 EGL1 WT EcoRI-PacI-ATG	GCGTT*GAATTC**TTAATTAA*ACAATGGCGCCCTCAGTTACACT
374 EGL1 WT TAG-AscI-EcoRI	GCGAT*GAATTC**GGCGCGCC*CTAAAGGCATTGCGAGTAGTAGTCG
379 ScPGK1prom-786 SacI + ApaI	GCGTT*GAGCTC**GGGCCC*TAATTTTTATTTTAGATTCCTGACTTCAAC
380 ScPGK1prom EcoRI-PacI	GCGTT*GAATTC**TTAATTAA*GTAAAAAGTAGATAATTACTTCCTTG
387 CBH1 WT EcoRI-PacI-ATG	GCGTT*GAATTC**TTAATTAAA*CAATGTATCGGAAGTTGGCCGTCATCTC
392 CBH1 WT TAA-AscI-EcoRI	GCGAT*GAATTC**GGCGCGCC*TTACAGGCACTGAGAGTAGTAAGG

The restriction sites used for the construction of plasmids are highlighted in *bold* and *underlined*

1.3 g l^{-1} DW for *A. gossypii* and 3.9 g l^{-1} DW for *S. cerevisiae* strains.

Cellulase activity on soluble substrates and insoluble substrates

Cellulase activities were determined using soluble MULac as substrate. The reaction consists in adding 50 µl MULAc (4 mM in DMSO) to 50 µl culture supernatant and incubating at room temperature. Reactions were stopped after 30 min incubation by adding 100 µl of 1 M Na_2CO_3, and liberation of 4-methylumbelliferone (MU) was detected by fluorescence measurement (excitation wavelength= 355 nm and emission wavelength=460 nm) with a Varian Varioscan spectrofluorometer in a black, flat bottom microtiterplate. MU (Sigma) was used as a standard. Volumetric enzyme activity was measured as micromoles of MU formed per minute per liter under the assay conditions. Specific activity is given as micromoles MU formed per minute per gram dry weight mycelium. Protein concentrations in the cell-free broth were measured with Bio-Rad protein reagent, using bovine serum albumin as standard.

Cellulase activity was also detected by the presence of a yellow halo after Congo red staining of CMC or β-glucan containing agar solidified medium, according to the method of Teather and Wood (1982) using 0.1% (*w*/*v*) CMC or 0.1% (*w*/*v*) β-glucan. To detect activity on agar solidified medium, 10 µl of sample was loaded into small wells punctured in the agar and the Petri dishes were incubated at 30°C for 20 h. Supernatants were first filtered through 0.22-µm pore size nylon filters, concentrated, and washed with 50 mM sodium acetate buffer pH 5.0 in 10 kDa Vivaspin concentrators (Sartorius). After 20 h, the Petri dishes were flooded with Congo red (0.1% *w*/*v*) for 1 h and washed with 1 M NaCl.

Binding to Avicel

For qualitative analysis of binding, 50 µl culture supernatant containing 0.6 mg of total protein, previously concentrated thirty-fold, were mixed with 12 mg Avicel in 50 mM sodium acetate pH 5.0. After incubation at 4°C overnight with end-over end rotation, the mixtures were centrifuged at 13,000×*g* for 5 min to sediment the substrate and bound proteins. The cellulose was washed with 50 mM sodium acetate pH 5.0 and then with 50 mM sodium acetate, pH 5.0, containing 1 M NaCl. The cellulose with bound proteins was mixed with 50 µl SDS sample buffer (250 mM Tris–HCl pH 6.8; 5% *v*/*v* glycerol; 10 mg ml^{-1} SDS; 2.5 % *v*/*v* β-mercaptoethanol; 0.05 mg ml^{-1} bromophenol blue) at 95°C for 3 min, and the supernatant was analyzed by SDS-PAGE in 12% (*w*/*v*) gels, followed by Western blot.

Enzymatic deglycosylation

To determine whether the recombinant cellulases were *N*-glycosylated, the proteins were digested with endoglycosidase H (Endo H, Roche) and PNGAseF (New England Biolabs) according to the manufacturer's instructions. In control samples, enzyme was replaced by water. Samples were incubated overnight, separated by 12% (*w*/*v*) SDS-PAGE, and visualized by Western blot.

Zymograms

Zymograms were made according to the general procedure of Flint et al. (1994), to visualize enzyme activity and estimate approximate molecular weight. The stacking gel was 4% (*w*/*v*) polyacrylamide, and the separating gel contained 12% (*w*/*v*) polyacrylamide with 0.1% (*w*/*v*) CMC (Sigma) added prior to polymerization. After electrophoresis, the gels were washed in 1% (*v*/*v*) Triton X100 (32×200 ml, 20 min per wash), soaked in sodium phosphate buffer (50 mM, pH 6.5) to allow renaturation of the enzymes (2 h at 4°C), and then incubated overnight at 37°C in sodium phosphate buffer (50 mM, pH 6.5). Following incubation, the gels were stained with 0.1% (*w*/*v*) Congo red for 1 h, and then destained with sodium chloride (1 M) for a further 1 h.

SDS-PAGE and Western blot analyses

Proteins were separated using denaturing SDS-PAGE with 12% (*w*/*v*) gels, as described by Laemmli (1970). Proteins

were blotted onto 0.45 micron nitrocellulose membrane (Hybond) and assayed with monoclonal antibodies specific for *T. reesei* CBHI and EGI (Aho et al. 1991). The nitrocellulose membrane was blocked for 1 h in TBS (10 mM Tris–HCl pH 8.0; 150 mM NaCl) containing 5% (*w*/*v*) non-fat milk powder and 0.01% (*v*/*v*) Tween 20 (Sigma). After washing with TBST (10 mM Tris–HCl pH 8.0; 150 mM NaCl; 0.05% *v*/*v* Tween 20), the monoclonal antibody was added. Goat anti-mouse Fc labeled with alkaline phosphatase was used as secondary antibody and BCIP/nitro-blue tetrazolium chloride substrate (Bio-Rad Laboratories, Hercules, CA) was used to visualize CBHI and EGI. The reaction was stopped by washing with distilled water and drying at room temperature. All washes and incubations were conducted at room temperature with gentle shaking.

Nucleotide sequence accession numbers

The nucleotide sequence for EGI is in GenBank database under accession number M15665 and CBHI under the number E00389.

Results

Transformation and heterologous expression of EGI and CBHI in *A. gossypii*

In order to express *T. reesei* cellulases CBHI and EGI in *A. gossypii*, protoplasts were transformed with plasmids pMI514, pMI519, and pMI516, with a transformation efficiency of 70–80 transformants per microgram plasmid DNA.

Transformants with the highest cellulase activity, based on the MULac assay as described in Material and methods, were grown in batch cultures in rich AFM and SCD medium at 30°C or 24°C at 200 rpm. Production of cellulases did not reduce the specific growth rate of the recombinant strains, i.e., expression of EGI (or CBHI) did not appear to impose a metabolic load on the host. *A. gossypii* CBHI had a specific growth rate of 0.12±0.01 h^{-1}, *A. gossypii* EGI of 0.11± 0.01 h^{-1} and the negative control of 0.09±0.00 h^{-1} in AFM containing 20 g l^{-1} glucose in flask cultures. Similarly, the production of *T. reesei* EGI did not reduce the specific growth rate of *S. cerevisiae* (Penttilä et al. 1987b).

Western blot analyses (Figs. 2 and 3) using monoclonal antibodies raised against the catalytic domain of the EGI or CBHI protein confirmed that *T. reesei* EGI (Fig. 3) and CBHI (Fig. 2) were present in the supernatant. CBHI was only detected by Western blot after removal of *N*-linked glycans, concentrating the protein to a single band (Fig. 2a, lane 2 and Fig. 2b, lane 4), demonstrating that the amount of secreted protein was very low.

Growth temperature may affect expression level, protein solubility, protein degradation, or cell viability, such that expression at a lower temperature increases the yield of the recombinant protein (Li et al. 2001; Shi et al. 2003). In *A. gossypii*, cellulase expression was not enhanced when the temperature was lowered from 30°C to 24°C (data not shown) either in rich AFM or in SCD medium.

Activity of recombinant cellulases produced in *A. gossypii*

Several methods were used to detect and evaluate whether the recombinant EGI and CBHI expressed in *A. gossypii* were in an active form. Secretion of active EGI was demonstrated by Congo red staining after incubation of concentrated sample of culture supernatant in CMC (Fig. 4a) or β-glucan (Fig. 4b) Petri dishes. The presence of a clearing zone indicated endoglucanase activity due to degradation of the substrate. Zymograms containing 0.1% (*w*/*v*) CMC in the resolving gel confirmed that EGI secreted by *A. gossypii* was active against CMC (Fig. 5, lane 5–6). Cellulases have a bimodal nature, with a cellulose binding domain (CBD) as well as an active site. We confirmed that the carbohydrate binding module was also present in the *A. gossypii* recombinant protein by incubation of recombinant EGI with Avicel. The protein was eluted with SDS-PAGE sample buffer and detected by Western blot analysis (Fig. 3, lane 3), confirming that both the cellulose binding domain and the active catalytic domain were present. CBHI activity was not detected in the plate assay or in zymograms containing 0.1% (*w*/*v*) Avicel, confirming that less CBHI was secreted than EGI or that the secreted protein was not active.

Heterologous expression of CBHI and EGI was also analyzed by measuring the activity on the soluble substrate MULac (Fig. 6). EGI activity in *A. gossypii* supernatant was maximal (400 μmol min^{-1} l^{-1}, 1.3 nmol min^{-1} μg^{-1} secreted protein) after 50 h growth in batch culture (Fig. 6a), at the end of the decelerating growth phase. In *S. cerevisiae*, maximal activity of 1,000 μmol min^{-1} l^{-1} (2.2 nmol min^{-1} μg^{-1} secreted protein) was detected after 60 h (Fig. 6b), also as the cells entered stationary phase. Specific EGI production (activity per gram biomass) for both organisms was highest (200–450 μmol min^{-1} g^{-1} dry weight) during exponential growth, as expected for proteins produced under the *PGK* promoter (Fig. 6c). CBHI activity was not detectable in *A. gossypii* cultures with the MULac assay (Fig. 6a), but *S. cerevisiae* produced a maximum of 348 μmol min^{-1} l^{-1} (1 nmol min^{-1} μg^{-1} secreted protein; Fig. 6b). Although protease activity was negligible in the culture supernatant of *A. gossypii* cultures (data not shown), EGI appeared to be degraded during the stationary phase (Fig. 6), probably when cell lysis occurred, and intracellular proteases were released into the culture medium, as has sometimes been observed for recombinant proteins in other

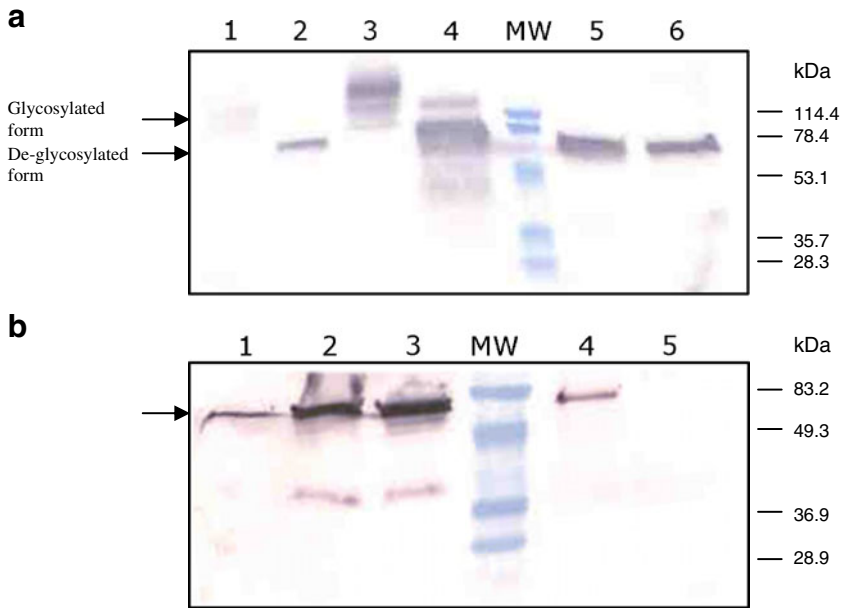

Fig. 2 Western blot analysis of recombinant CBHI treated with **a** endoglycosidase H or **b** PNGase F. **a** *Lane 1*, *A. gossypii* recombinant CBHI before Endo H treatment; *lane 2*, *A. gossypii* recombinant CBHI after Endo H treatment; *lane 3*, *S. cerevisiae* recombinant CBHI before Endo H treatment; *lane 4*, *S. cerevisiae* recombinant CBHI after Endo H; *MW* molecular weight standard (sizes given at right); *lane 5*, *T. reesei* CBHI before Endo H treatment; *lane 6*, *T. reesei* CBHI after Endo H treatment. **b** *Lane 1*, *T. reesei* CBHI without treatment; *lane 2*, *T. reesei* CBHI after PNGaseF treatment; *lane 3*, *T. reesei* CBHI incubated with water instead of PNGaseF; *lane 4*, *A. gossypii* CBHI after PNGaseF treatment; *lane 5*, *A. gossypii* CBHI without PNGaseF treatment. *Lanes* were loaded with 130 μg total protein. Protein bands are indicated with *arrows*

fungi (Archer et al. 1992; Broekhuijsen et al. 1993; Roberts et al. 1992; van den Hombergh et al. 1997).

Partial characterisation of recombinant cellulases secreted by *A. gossypii*

Zymogram analysis revealed that EGI secreted by *A. gossypii* had a higher molecular weight than the native enzyme from *T. reesei* (Fig. 5, lane 1), but was similar to the recombinant EGI in *S. cerevisiae* (Fig. 5, lane 2–4).

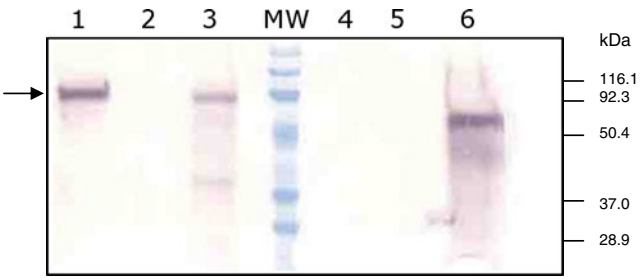

Fig. 3 Binding of EGI to Avicel. Six hundred micrograms of total protein was mixed with Avicel as described in Materials and methods. Legend: *lane 1*, recombinant protein that did not bind to Avicel; *lane 2*, washing Avicel with sodium acetate buffer 50 mM, pH 5.0 and 1 M NaCl; *lane 3*, EGI eluted from Avicel; *MW*, molecular weight standard (sizes given at right); *lane 4*, supernatant of *T. reesei* EGI after incubation with Avicel; *lane 5*, washing Avicel with sodium acetate buffer 50 mM, pH 5.0, and 1 M NaCl; *lane 6*, *T. reesei* EGI eluted from Avicel. Protein bands are indicated with *arrows*

Even though N-terminal sequence was not evaluated, the 20 amino acids composing the signal peptide sequence, being too small, would not account for the band shift observed in the zymogram (Fig. 5) or Western blot analysis (Figs. 2 and 3). The higher molecular weight indicated that the protein secreted by *A. gossypii* was probably glycosylated, as shown in Figs. 2 and 3, in which endoglycosidase treatment was used to remove glycans attached to the protein. Comparing lanes 2 and 5 of Fig. 5, *S. cerevisiae* appeared to produce more of the most highly glycosylated EGI than *A. gossypii* produced. After treating EGI from *A. gossypii* with PNGaseF (Plummer and Tarentino 1991), a minor band shift occurred (data not shown). PNGaseF was used to deglycosylate EGI produced in *A. gossypii* because it is known that EGI has a variety of glycosylation sites and that these have different susceptibilities to Endo H treatment. For example, Endo H is not able to completely deglycosylate recombinant EGI produced by *S. cerevisiae* (Penttilä et al. 1988; Van Arsdell et al. 1987). The reduction in molecular weight of *A. gossypii* produced EGI following enzymatic treatment was strong evidence that the recombinant enzyme was glycosylated (Trimble and Maley 1984; Maley et al. 1989), but not as extensively as EGI produced in *S. cerevisiae*.

Although only small amounts of CBHI were produced by *A. gossypii*, Western blot analysis after glycan removal, demonstrated that the CBHI produced by *A. gossypii* was also hyperglycosylated (Fig. 2a, lane 2 and Fig. 2b lane 4).

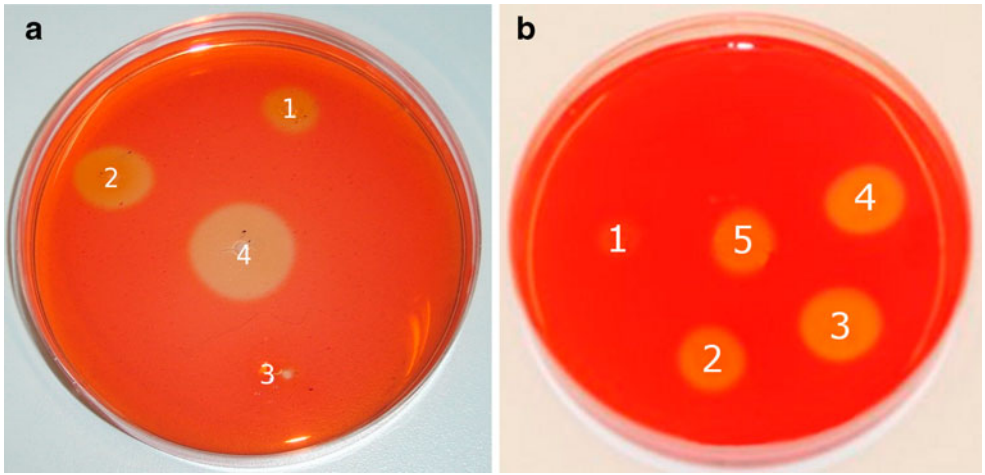

Fig. 4 Plate assay for detection of activity towards **a** carboxymethylcellulose (CMC) and **b** β-glucan. Thirty times concentrated protein samples, containing 60–120 μg of total protein were loaded in small wells and stained with Congo red after 20 h incubation at 30°C. **a** Wells contained *1* supernatant from *A. gossypii* expressing EGI grown in AFM; *2* supernatant from *S. cerevisiae* expressing EGI; *3* supernatant from the *A. gossypii* negative control strain; *4* native *T. reesei* EGI purified protein (5 μg). Or **b** wells contained *1* supernatant from *A. gossypii* expressing EGI grown in SCD medium; *2* supernatant from *A. gossypii* expressing EGI grown in AFM; *3* and *4* supernatant from two clones of *S. cerevisiae* expressing EGI; *5* native *T. reesei* EGI purified protein (5 μg)

Hyperglycosylation was not as extensive as that observed in CBHI produced by *S. cerevisiae*, since both PNGaseF (Fig. 2b) and Endo H were able to deglycosylate the *A. gossypii* recombinant protein, resulting in a protein of similar molecular weight to the native *T. reesei* CBHI. In contrast, Endo H was only able to partially deglycosylate recombinant CBHI produced by *S. cerevisiae* (Fig. 2a, lane 4).

Discussion

The filamentous fungus *A. gossypii* has been used in the industrial production of the vitamin riboflavin. The experimental tools available for use in *A. gossypii* together with its small genome and the regularity of filamentous growth and hyphal branching make it suitable for extensive study of polar (filamentous) growth. In this study, we take advantage of the same distinctive features to express and secrete heterologous cellulases under the *S. cerevisiae PGK1* promoter with *T. reesei* native signal peptides in *A. gossypii*. The transformation method used here was efficient, compared with that described by Kato and Park (2005), in which only ten to 20 transformants per microgram of plasmid DNA were obtained. Plasmids containing the 2-μm replication origin were also used. Plasmids containing autonomously replicating sequence (ARS) such as the 2-micron plasmid replication origin and chromosomal replication origin (ARS1) from *S. cerevisiae* replicate freely in *A. gossypii* in contrast to lack of replication in other filamentous ascomycetes (Wright and Philippsen 1991), resulting in maintenance of higher copy number of the recombinant DNA than is generally obtained by integration.

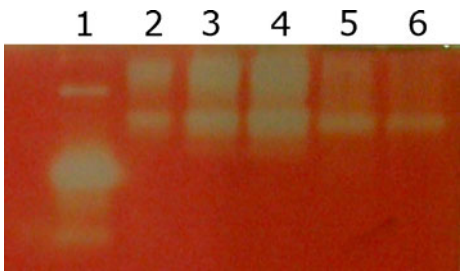

Fig. 5 Zymogram containing 0.1% (*w*/*v*) CMC incorporated in the SDS-PAGE gel, stained with Congo red, and washed with 1 M NaCl. Legend: *lane 1*, 80 ng of purified *T. reesei* EGI; *lane 2*, *S. cerevisiae* recombinant EGI, 70 h after inoculation three times concentrated (1.2 μg total protein); *lane 3*, *S. cerevisiae* recombinant EGI 15 times concentrated (6 μg total protein); *lane 4*, *S. cerevisiae* recombinant EGI 30 times concentrated (12 μg total protein); *lane 5*, supernatant from a culture of *A. gossypii* expressing EGI, 62 h after inoculation (11 μg total protein); and *lane 6*, supernatant from an *A. gossypii* EGI producing culture, 43 h after inoculation (6 μg total protein). *A. gossypii* samples were concentrated thirty-fold

Several aspects should be taken into account in recombinant proteins production, namely the promoter, signal sequence, culture conditions, and host genetic background. The most commonly used promoters in *A. gossypii* are AgGPD (Jiménez et al. 2005) and AgTEF (Kanamasa et al. 2007; Kato and Park 2005). Recently, Dünkler and Wendland (2007) reported the use of *S. cerevisiae* and *A. gossypii MET3* promoters (regulated by methionine), contributing to the molecular toolbox available for *A. gossypii*. To our knowledge, this is the first time that the *S. cerevisiae PGK1* promoter has been used in *A. gossypii*. The PGK promoter is growth-related, and thus,

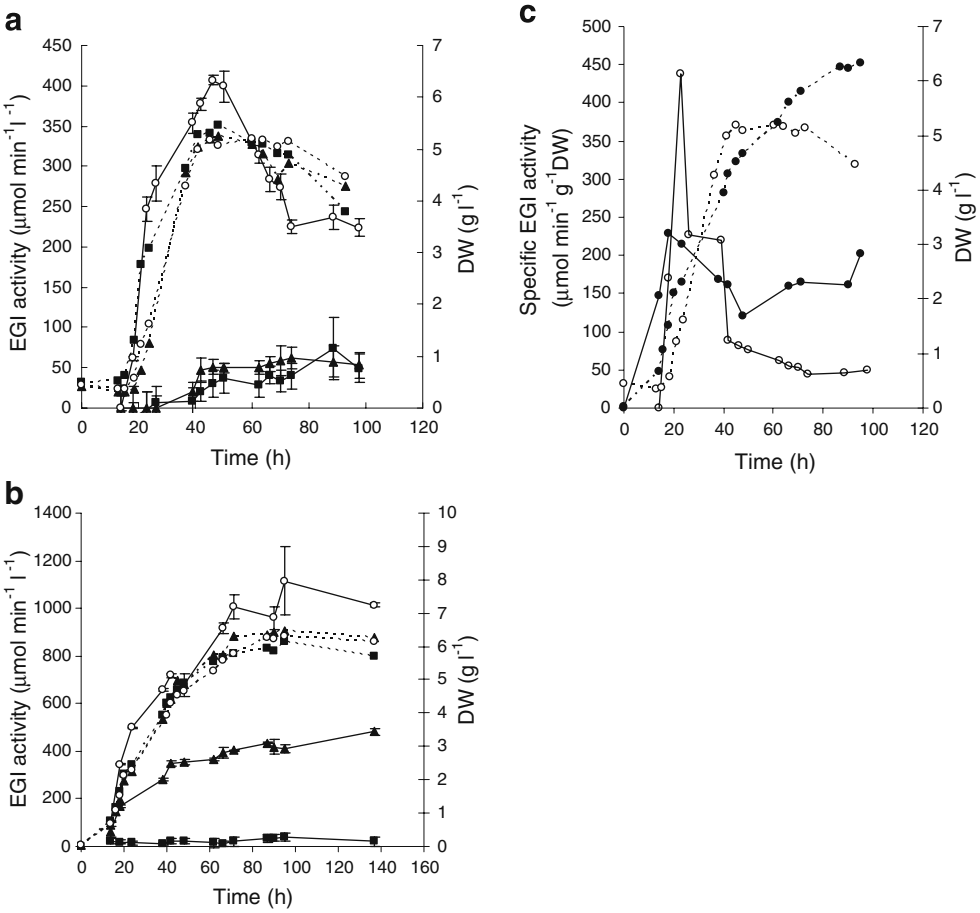

Fig. 6 Average volumetric cellulase activities and dry weight (*right axis*) from supernatant of *A. gossypii* (**a**) and *S. cerevisiae* (**b**) growing in flasks in AFM at 30°C, 200 rpm. Activities were measured with the MULAc assay, described in Materials and methods. Data represent average ± standard error of the mean obtained from three independent cultures. Strains were transformed with plasmids with genes for (*solid triangle*) CBHI, (*open circle*) EGI, or (*solid square*) an empty vector as the negative control. *Dashed lines* show dry weight (grams per liter) for the corresponding strain. **c** *A. gossypii* (*open circle*) and *S. cerevisiae* (*solid circle*) EGI specific activity per gram biomass during growth, represented as dry weight (*dashed line*)

protein production would be unlikely to be affected by riboflavin production, which occurs primarily after substrate depletion (Karos et al. 2004). The recombinant EGI and CBHI, carrying the heterologous, native signal sequences, entered the secretory pathway of *A. gossypii*, and functional EGI was found in the extracellular medium. EGI activity was detectable against both soluble and insoluble substrates. This was comparable to the endoglucanase activity against CMC illustrated by Althöefer et al. (2001) for *A. gossypii* expressing the endo-β-1,4-glucanase from *S. halstedii*. *A. gossypii* was not able to secrete CBHI protein to the same extent as EGI. CBHI production is always lower than other cellulases, including CBHII (Penttilä et al. 1988), possibly because the formation of disulfide bridges is important for correct folding (Boer et al. 2000). These results demonstrated the protein secretion by *A. gossypii* is comparable to *S. cerevisiae* and that a filamentous growth form is not sufficient to ensure high levels of protein secretion.

Both CBHs and EGs are glycoproteins containing heterogeneous *N*- and/or *O*-linked glycans (Kubicek 1992) which may result in multiple protein bands in Western analysis. Glycosylation plays a role in the secretion of these cellulolytic enzymes, providing not only the optimal distance between the core and CBD but protecting the linker peptide from proteolytic attack (Clarke 1997; Srisodsuk et al. 1993). Similar to what has been observed in other host microorganisms, both EGI and CBHI were glycosylated by *A. gossypii*. The glycosylation was less extensive than in *S. cerevisiae*. A limitation in the extent of glycosylation is an advantage in the production of heterologous proteins whose properties may be adversely affected by extensive glycosylation. *S. cerevisiae*, in particular, forms high-mannose glycoproteins (Dean 1999; Gemmill and Trimble 1999). Filamentous fungi also have mannose rich glycans, but of more limited size than *S. cerevisiae* (Deshpande et al. 2008; Maras et al. 1997; Stals et al. 2004). In addition, filamentous fungi are known to secrete different glycosidases to the extracellular medium that may trim protein-linked glycans, adding heterogeneity to the glycan structure (Maras et al. 1999). Although *A. gossypii* is more closely related to *S. cerevisiae* than to other filamentous fungi, it appears to be able to limit the extent of protein glycosylation. Further analysis would be required to unveil the glycosylation pattern and degree of glycosylation performed by *A. gossypii*.

Although high levels of protein secretion are generally associated with filamentous growth, *A. gossypii* was more similar to the closely related yeast *S. cerevisiae* than to other filamentous fungi in its ability to produce and secrete

EGI, demonstrating that filamentous growth alone is not sufficient to ensure good protein secretion. In comparison with the yeast *S. cerevisiae*, *A. gossypii* showed similar specific production of EGI, but less of CBHI. The expression of recombinant cellulases in *A. gossypii* provides opportunity for additional insights on the relationship between protein secretion and the filamentous growth form, while, as with *S. cerevisiae*, the low levels of protein secretion observed here do not necessarily limit the future development of *A. gossypii* as a heterologous protein production host.

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The authors declare that they have no conflict of interest.

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