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BIOTECHNOLOGICAL VERSATILITY OF RIBOFLAVIN PRODUCER *Ashbya gossypii* - EXPRESSION OF *Trichoderma reesei* CELLULASES CBHI AND EGI

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ABSTRACT

The filamentous fungus *Ashbya gossypii* shows the potential for the production of, yet unexploited, valuable compounds other than riboflavin. To explore the ability of *A. gossypii* as a host for the expression of recombinant proteins, endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from the fungus *Trichoderma reesei* were expressed in *A. gossypii* under *Saccharomyces cerevisiae PGK1* promoter. The proteins were secreted into the culture medium, but there were differences in the amount or activity of the protein being produced. In one hand, CBHI activity was not detected using 4-methylumbelliferyl-β-D-lactoside as substrate, being only detected by Western blot. On the other hand, EGI activity was detectable, the level of activity being comparable to that produced by a *S. cerevisiae* strain containing the same plasmid. Thus 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 to the native *T. reesei* proteins, but the glycosylation was less extensive than on cellulases expressed in *S. cerevisiae*.

INTRODUCTION

The filamentous hemiascomycete *A. gossypii* is a plant pathogen that mainly attacks cotton or citrus fruits and was first described in 1926 by Ashby and Nowel. *A. gossypii* does not develop specialized infection structures such as penetration hyphae, rather than that, the spores or mycelia fragments are dispersed by insects.

The ability of *A. gossypii* to produce riboflavin (vitamin B2), responsible for its yellow colour, was soon recognized, being the first organism used in industrial large-scale production of this vitamin (Stahmann et al. 2000).

Based on rDNA sequences, *A. gossypii* is more closely related to *Saccharomyces cerevisiae* than *Neurospora crassa* or *Aspergillus nidulans* (Wendland et al. 1999). The two species diverged more than 100 million years ago, however, 95% of the 4,718 *A. gossypii* protein-coding genes were found to have an homolog in *S. cerevisiae*. *A. gossypii* genome has been extremely useful for refining the annotation of the budding yeast genome and has provided the definitive proof that *S. cerevisiae* has undergone a whole-genome duplication event. *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 Philipsen 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, the areas of study related to this microorganism, are mainly focused on riboflavin production and polar growth, which makes the information available concerning recombinant protein production by *A. gossypii* very limited or even unexistent. So far, an 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).

Preliminary results obtained in our laboratory, supported the hypothesis, that *Ashbya* was a good candidate to develop as a protein expression host, since it demonstrated both that *A. gossypii* can secrete homologous protein into the extracellular medium and that low levels of extracellular proteases were secreted. Production of extracellular proteases is one of the major drawbacks of heterologous protein production, since degradation of the protein generally leads to a considerable yield reduction. Thus limited protease production is a major advantage in a protein producing host. Contrary to most filamentous fungi, *A. gossypii* grows at a neutral-high optimal pH range. This could be an additional advantage for the production of proteins that are stable at higher pH values, where protease production may be a problem with e.g. *Aspergilli.* Another benefit considered when developing *A. gossypii* as a recombinant protein production platform is the fact that, few extracellular proteins other than the product of interest are secreted into the extracellular medium, which represents a benefit in terms of product recovery and purification.

Since *A. gossypii* is a filamentous fungus it can be expected to have efficient protein secretion, but its close relationship to *S. cerevisiae* may suggest a limited secretion ability. We therefore expressed two cellulase genes from the filamentous fungus *T. 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 (CBH; EC 3.2.1.91), which act as exoenzymes and release cellobiose as a main product from crystalline cellulose, endoglucanases (EG; 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 (Fig. 1). The homolog genes of these enzymes are not present in *A. gossypii* genome.

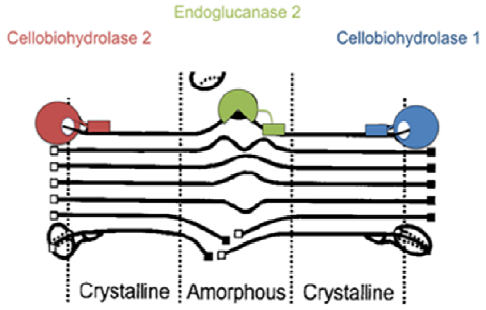


Fig. 1 Mode of action of cellulases

T. reesei CBHI and EGI are useful as model proteins for recombinant protein production, since they have been extensively studied in several expression systems, as well as in *T. reesei*, and are considered a challenge when produced by other organisms. Expression of CBHI in *Escherichia coli* (Laymon et al. 1996; Teeri 1987a) 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 endoglucanase I (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.

Hereby, we present some results from a study recently published (Ribeiro et al. 2010) with the goal to 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 hosts. In order to estimate the secretion levels of recombinant proteins by *Ashbya*, a comparison between *A. gossypii* and *S. cerevisiae* was done, keeping in mind the close genetic relationship between the two hosts.

MATERIALS AND METHODS

Strains

A. gossypii ATCC10895 was obtained from Prof. P. Philippsen (Basel University) and maintained on solidified *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, 20 g l^{-1} agar). *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*.

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 (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, yeast nitrogen base (Difco), 20 g l^{-1} glucose, 1g l^{-1} $CaCo_3$ and 1g l^{-1} agar.

Plasmid construction

Plasmid pMI519 contained *T. reesei egl1* cDNA and plasmid pMI514 *T. reesei cbh1* cDNA. Plasmids pMI519 and pMI514 were constructed from plasmids pTTc11 (Penttilä et al. 1987b) and pTTc1 (Teeri et al. 1987b), respectively, together with B609 (Ruohonen et al. 1995). 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 to confer to the transformants resistance to the aminoglycoside antibiotic G418 (Jimenez and Davies 1980) was derived from a modified pUG6 vector (Güldener et al. 1996).
The control vector pMI516 without a cellulase insert was also constructed.

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 done on medium containing G418.

Transformants were screened in two ways. Initial screening was performed by PCR using specific primers. Template DNA was extracted by a rapid-miniprep method. 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 AFM supplemented with 200 µg ml^{-1} G418 and extracellular EGI or CBHI enzymatic activities were determined by using 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma) as substrate. Based on these results, one strain expressing EGI and another expressing CBHI were selected for further study.

S. cerevisiae H81 was transformed and screened in the same way as *A. gossypii* transformants.

Culture conditions

Transformed strains of *A. gossypii* were grown at 30ºC or 24ºC in flasks (250 ml flasks containing 50 ml medium) at 200 r.p.m. on AFM or SCD medium with 20 g l^{-1} glucose as carbon source and 200 µg ml^{-1} G418.

Cellulase activity on soluble substrates and insoluble substrates

Cellulase activities were determined using soluble MULac as substrate. Liberation of 4-methylumbelliferone (MU) was detected by fluorescence measurement (excitation wavelength = 355 nm and emission wavelength = 460 nm). Volumetric enzyme activity was measured as µmol of MU formed per minute per liter under the assay conditions. Protein concentrations in the cell-free broth were measured with Bio-Rad protein reagent, using bovine serum albumin (BSA) as standard.

Enzymatic deglycosylation

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. The separating gel contained 0.1% (w/v) CMC (Sigma) added prior to polymerization.

SDS-PAGE and Western blot analyses

Proteins were separated by 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 labelled 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.

RESULTS AND DISCUSSION

Heterologous expression of EGI and CBHI in *A. gossypii*

T. reesei cellulases CBHI and EGI where expressed in *A. gossypii*, after transformation of protoplasts with expression plasmids pMI514, pMI519 and pMI516.

Transformants with the highest cellulase activity were selected for batch cultures in rich AFM and SCD medium at 30ºC or 24ºC at 200 r.p.m. Similarly, to what have been observed in *S. cerevisiae* (Penttila et al., 1987 b), the specific growth rate of the recombinant strains was not affected by the production of cellulases. Figure 2 shows the growth of strains expressing CBHI or EGI and the negative control containing the empty plasmid, pMI516. *A. gossypii* CBHI had a specific growth rate of 0.12 h^{-1} ± 0.01, *A. gossypii* EGI of 0.11 h^{-1} ± 0.01 and the negative control of 0.09 h^{-1} ± 0.00.

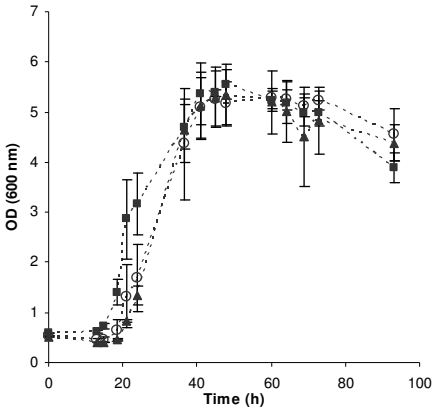
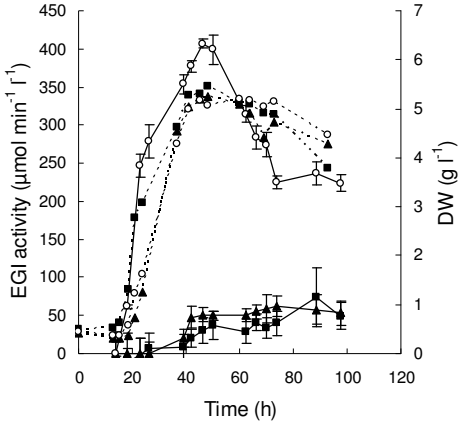


Fig. 2 Growth of *A. gossypii* recombinant strains secreting (▲) CBHI or (○) EGI and the (■) negative control with an empty vector. Cells were grown in flasks in AFM with glucose as carbon source at 30ºC, 200 r.p.m. Growth was measured as OD at 600 nm. Data represents the mean ± standard error of the mean of 3 independent cultures.

Heterologous expression of CBHI and EGI was analyzed by measuring the activity on the soluble substrate MULac (Fig. 3). EGI activity in *A. gossypii* supernatant was maximal (400 µmol min^{-1} l^{-1}) after 50 h growth in batch culture (Fig. 3a), at the end of the decelerating growth phase. In *S. cerevisiae*, maximal activity of 1000 µmol min^{-1} l^{-1} was detected after 60 h (Fig. 3b), also as the cells entered stationary phase. CBHI activity was not detectable in *A. gossypii* cultures with the MULac assay (Fig. 3a), but *S. cerevisae* produced a maximum of 348 µmol min^{-1} l^{-1} (Fig. 3b). One of the major challenges for the production of recombinant proteins, is that the secreted protein is often degraded by proteases produced by the fungal host. This was the case of recombinant cellulases expressed in *A. oryzae*, especially in the late stage of cultivation (Takashima et al. 1998). 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. 3), probably when cell lysis occurred and proteases were released into the culture medium.

A



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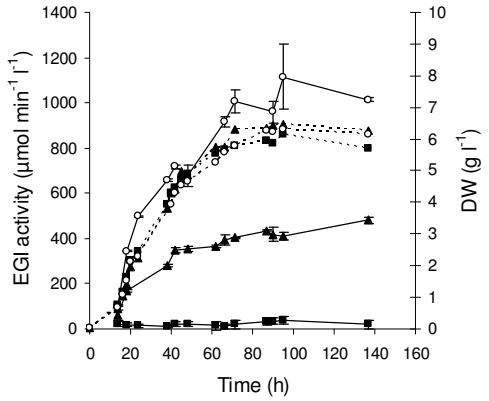

Fig. 3 Average volumetric cellulase activities and dry weight (right axis) from supernatant of *A. gossypii* (a) and *S. cerevisiae* (b) grown in AFM at 30ºC, 200 r.p.m. Activities were measured with the MULAc as substrate, described in materials and methods. Data represent average ± standard error of the mean obtained from three independent cultures. Strains were transformed with plasmids expressing (▲) CBHI; (○) EGI or (■) an empty vector as the negative control. Dashed lines show dry weight (g l^{-1}) for the corresponding strain. (Adapted from: Ribeiro et al. 2010)

The cellobiohydrolase and endoglucanase from *T. reesei* were secreted by *A. gossypii* ATCC10895 using the native signal peptide and expressed for the first time under *S. cerevisiae PGK1* promoter. The housekeeping enzyme phosphoglycerate kinase (PGK) catalyses a key reaction within the glycolytic pathway to generate ATP and also operates in gluconeogenesis. Carbon source regulation of *PGK* genes is common, with glucose induction having been observed for *Aspergillus orzyae*, *Rhizopus niveus* and *S. cerevisiae* (Maitra and Lobo 1971; Nakajima et al., 2000). In the present work, both glucose and fructose were used as C-source for EGI production, without significative difference in the amount of protein activity produced, revealing that fructose is equivalent to glucose as an inducer of the *PGK1* promoter for heterologous protein production in *A. gossypii* (data not shown).

The CBHI and EGI native signal sequences from *T. reese*i were able to direct the proteins for secretion in *A. gossypii*. Western blot analyses (Fig. 4 and Fig. 5) using monoclonal antibodies raised against the catalytic domain of the CBHI or EGI protein confirmed that *T. reesei* EGI and CBHI 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. 4), demonstrating that the amount of secreted protein was less than the amount of EGI.

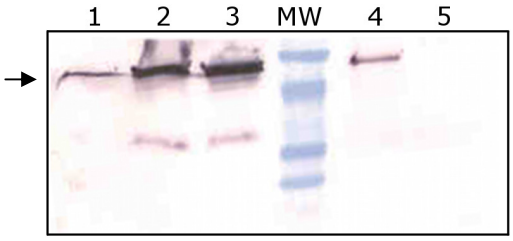

Fig. 4 Western blot analysis of recombinant CBHI treated with PNGase F. Legend: lane 1, *T. reesei* CBHI without treatment; lane 2 and 3, *T. reesei* CBHI after PNGaseF treatment or incubated with water instead of PNGaseF, respectively; lane 4 and 5, *A. gossypii* CBHI after PNGaseF treatment and without PNGaseF treatment, respectively. (Adapted from: Ribeiro et al. 2010)

EGI produced in *A. gossypii* had been correctly processed by the fungus since it was able to bind to Avicel (data not shown). Thus the recombinant enzyme had both the cellulose binding domain and the active catalytic domain.

Zymograms containing 0.1% (w/v) CMC in the resolving gel confirmed that EGI secreted by *A. gossypii* is active against CMC (Fig. 5).

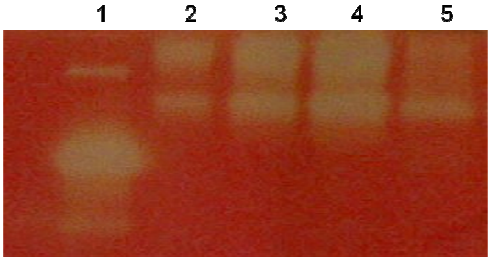

Fig. 5 - Zymogram with 0.1% (w/v) CMC incorporated in the SDS-PAG gel and stained with Congo red. Legend: lane 1, purified *T. reesei* EG I; lane 2 to 4, *S. cerevisiae* recombinant EGI with sequential concentration; lane 5, *A. gossypii* recombinant EGI. (Ribeiro et al. 2010)

CBHI activity was not detected in zymograms containing 0.1% (w/v) Avicel, indicating that less CBHI was secreted than EGI or that the secreted protein was not active.

Growth temperature may affect both expression levels and protein solubility, such that expression at a lower temperature increases the yield of the recombinant protein. This may result from enhanced protein folding and thus more efficient secretion, reduced protease activity or increased cell viability at lower temperature (Li et al., 2001; Shi et al., 2003). In *A. gossypii*, cellulase expression was not enhanced when the temperature was lowered from 30 ºC to 24 ºC (data not shown) either in rich AFM or in SCD medium.

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

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. The choice of *A. gossypii* as a recombinant protein production host was also based on the fact that being a eukaryotic microorganism it has the ability to perform post-translation modifications by adding glycans to the protein structure. Glycosylation of cellulases is important not only because provides the optimal distance between the core and carbon binding domain but also protects the linker peptide from proteolytic attack (Clarke, 1997; Srisodsuk et al. 1993). Similar to what has been observed in other host microorganisms, the EGI secreted by *A. gossypii* had a higher molecular weight than the native enzyme from *T. reesei* (Fig. 5 and Fig. 6). The higher molecular weight indicated that the protein secreted by *A. gossypii* was glycosylated. After treating EGI with Endo H, a minor band shift occurred (Fig. 6). When compared to *S. cerevisiae*, the deglycosylated protein produced by *A. gossypii* had a molecular weight closer to the native protein from *T. reesei*.

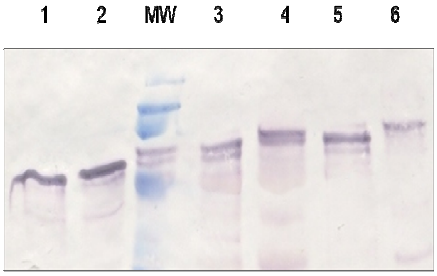

Fig. 6 – Western blot analysis of recombinant EGI treated with Endo H. Legend: lane 1 and 2, *T. reesei* EGI after and before Endo H treatment; MW, molecular weight standard; lane 3 and 4, *A. gossypii* recombinant EGI after and before Endo H; lane 5 and 6, *S. cerevisiae*

recombinant EGI after and before Endo H treatment (respectively).

The CBHI produced by *A. gossypii* was also hyperglycosylated, but less then CBHI produced by *S. cerevisiae* (Fig. 4). Both PNGaseF 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 (Fig. 4). In contrast, Endo-H treatment only partially deglycosylated recombinant CBHI produced by *S. cerevisiae* (data not shown).

Although *A. gossypii* glycosylated both EGI and CBHI, the glycosylation was less extensive than in *S. cerevisiae*. A reduction on 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 decorate proteins with mannose rich glycans, but of more limited size than *S. cerevisiae* (Desphande 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 present differences in what concerns the extent of protein glycosylation by adding less or smaller glycans. Further analysis would be required to unveil the glycosylation pattern and degree of glycosylation performed by *A. gossypii*.

CONCLUSION

A host for recombinant protein production should have the ability to produce and secrete high levels of recombinant protein, introduce post-translational modifications, and correctly fold the protein (Rose and Van Zyl 2002; Takashima et al. 1998). In this study, the expression and secretion of heterologous cellulases under the *S. cerevisiae PGK1* promoter with *T. reesei* native signal peptides was successfully achieved in *A. gossypii*. The recombinant proteins carrying the native signal sequence entered the secretory pathway of *A. gossypii*. Enzymatic removal of the carbohydrate residues demonstrated that the secreted recombinant enzymes were glycosylated. EGI activity was detectable against both soluble and insoluble substrates. In comparison with the yeast *S. cerevisiae*, *A. gossypii* secreted similar amounts of EGI, but less CBHI protein. However, *Ashbya* did not hyper-glycosylate the proteins to the same extent as *S. cerevisiae*. This analysis of cellulase expression in *A. gossypii* opens new biotechnological perspectives in the development of the riboflavin producer *A. gossypii* as a heterologous protein production host.

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