High-Level Expression of *Aspergillus niger* β-Galactosidase in *Ashbya gossypii*

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Ashbya gossypii has been recently considered as a host for the expression of recombinant proteins. The production levels achieved thus far were similar to those obtained with Saccharomyces cerevisiae for the same proteins. Here, the β-galactosidase from Aspergillus niger was successfully expressed and secreted by A. gossypii from 2-μm plasmids carrying the native signal sequence at higher levels than those secreted by S. cerevisiae laboratorial strains. Four different constitutive promoters were used to regulate the expression of β-galactosidase: A. gossypii AgTEF and AgGPD promoters, and S. cerevisiae ScADH1 and ScPGK1 promoters. The native AgTEF promoter drove the highest expression levels of recombinant β-galactosidase in A. gossypii, leading to 2- and 8-fold higher extracellular activity than the AgGPD promoter and the heterologous promoters, respectively. In similar production conditions, the levels of active β-galactosidase secreted by A. gossypii were up to 37 times higher than those secreted by recombinant S. cerevisiae and ~2.5 times higher than those previously reported for the β-galactosidase-high producing S. cerevisiae NCYC869-A3/pVK1.1. The substitution of glucose by glycerol in the production medium led to a 1.5-fold increase in the secretion of active β-galactosidase by A. gossypii. Recombinant β-galactosidase secreted by A. gossypii was extensively glycosylated, as are the native A. niger β-galactosidase and recombinant β-galactosidase produced by yeast. These results highlight the potential of A. gossypii as a recombinant protein producer and open new perspectives to further optimize recombinant protein secretion in this fungus. © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:261–268, 2014
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Introduction

Ashbya gossypii is a filamentous hemiascomycete[1] of important biotechnological interest due to its natural ability to overproduce riboflavin/vitamin B2.[2] Furthermore, *A. gossypii* presents several unique characteristics that have expanded the interest in this organism beyond riboflavin production, such as one of the smallest eukaryotic genomes sequenced, which shares high homology and gene order conservation with that of the yeast *Saccharomyces cerevisiae*.[3] It possesses haploid nuclei and exhibits high homologous recombination efficiency, thereby allowing for the manipulation of genes by simple PCR-based gene targeting techniques.[4,5] Additionally, it has the capacity to freely replicate plasmids harboring *S. cerevisiae* autonomous replicating sequence (ARS) elements.[6] These features, combined with the *A. gossypii* ability to grow in inexpensive media to high cell densities,[7,8] to perform protein post-translation modifications[9,10] and to natively secrete few proteins to the culture medium[9–12] have brought attention to this fungus as a potential host for the production of heterologous proteins.

The expression and secretion of recombinant proteins by *A. gossypii* have been minimally explored. Two cellulases from *Trichoderma reesei*, endoglucanase I (EGI) and cellobiohydrolase I (CBHI), were successfully secreted by *A. gossypii* into the culture medium.[9] Recombinant EGI was expressed as a functional enzyme and at similar levels to those obtained with the closely related host *S. cerevisiae*, whereas recombinant CBHI was presumably inactive and only detectable by Western blot using monoclonal antibodies.[9] These cellulases have been reported as challenging enzymes to be produced by recombinant microbial hosts, both as functional proteins and at reasonable yields, especially CBHI.[9] Therefore, to further assess *A. gossypii* as a recombinant host, the expression in this organism of recombinant proteins which have been produced biologically-active and at high levels by other model hosts is of interest.

The β-galactosidase from the filamentous fungus *Aspergillus niger* is an extracellular enzyme of easy detection that has been efficiently produced by recombinant *S. cerevisiae* strains in high amounts.[13,14] Furthermore, this β-

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galactosidase has a high biotechnological interest, namely for the hydrolysis of lactose from acid cheese whey.[15] For these reasons, this enzyme was chosen as a model protein to be recombinantly expressed in *A. gossypii*, aiming at further investigating the potential of this fungus as a recombinant protein producer.

Several approaches can be carried out to improve the productivity of recombinant hosts. Among them, random mutagenesis and targeted modification of the cell wall permeability have been previously attempted in *A. gossypii*, but with no relevant effect on the production levels of the recombinant protein.[16] Another strategy commonly used involves finding an appropriate promoter to drive the overexpression of recombinant proteins. The *S. cerevisiae ScPGK1* constitutive promoter was previously used to drive the expression of the *T. reesei* EGI and CBHI in *A. gossypii*.[9] The *A. gossypii AgTEF* and *AgGPD* constitutive promoters have been frequently used to drive the overexpression of genes associated with riboflavin biosynthesis, resulting in increased riboflavin production,[17–19] and for the expression of intracellular heterologous proteins.[20] The *AgTEF* promoter has been shown to be a strong promoter, leading to higher expression of proteins in *A. gossypii* than the *A. gossypii AgMET3* or *S. cerevisiae ScTHI13* inducible promoters,[21,22] but its strength has never been directly compared with that of the *AgGPD* or *ScPGK1* promoters. Thus, this work also aimed at studying the influence of different homologous (*A. gossypii AgGPD* and *AgTEF*) and heterologous (*S. cerevisiae ScPGK1* and *ScADH1*) constitutive promoters on the expression of recombinant proteins in *A. gossypii* using β-galactosidase as a model protein.

Materials and Methods

Strains

A. gossypii ADH1lacA (pFMAlacA), PGK1lacA (pFMPlacA), GPDlacA (pFMGlacA), and TEFlacA (pFMTlacA) strains were obtained by transformation of *A. gossypii* ATCC10895 (provided by Prof. P. Philippsen from Basel University) as described below. *S. cerevisiae* CEN.PK 113-7D (MATα, MAL2–8^c SUC2) and NCYC869-wt (MATα FLO1) were used as hosts to obtain comparable *S. cerevisiae* transformant strains: *S. cerevisiae* ADH1lacA (pFMAlacA), PGK1lacA (pFMPlacA), GPDlacA (pFMGlacA), and TEFlacA (pFMTlacA). *S. cerevisiae* NCYC869-A3/pVK1.1[13] was used to produce recombinant β-galactosidase for zymogram analysis. *Escherichia coli* NZ5α (NZYTech; fhuA2-(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was used as the recipient for all cloning experiments.

Culture media

A. gossypii strains were maintained on solidified agar (15 g L^{-1} agar) *Ashbya* full medium (AFM; 10 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 1 g L^{-1} myo-inositol, 20 g L^{-1} glucose), supplemented with 150 μg mL^{-1} G418 (Sigma-Aldrich) for selection of transformants. When indicated, 20 g L^{-1} glycerol were used instead of glucose. Spore suspensions were prepared by collecting 7–10-day-old mycelium from solidified agar plates, digesting the mycelia with 4.5 mg mL^{-1} Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich) 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. *S. cerevisiae* strains were maintained on solidified agar (15 g L^{-1} agar) YPD (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract), supplemented with 150 μg mL^{-1} G418 for selection. Submerged cultures of *A. gossypii* and *S. cerevisiae* transformants were made in selective AFM and YPD, respectively. LB medium (10 g L^{-1} sodium chloride, 10 g L^{-1} yeast extract, 5 g L^{-1} tryptone, pH adjusted to 7.5) supplemented with 15 g L^{-1} agar and 100 μg mL^{-1} ampicillin was used for *E. coli* cultivations.

Plasmids construction

The plasmid pMI516 used by Ribeiro et al.[9] to express recombinant proteins in *A. gossypii* was modified as follows. A multiple cloning site (MCS) containing restriction sites for *Eco*RI, *Bam*HI, *Nhe*I, *Eag*I, *Not*I, *Sac*II, *Kpn*I, *Sal*I, and *Xho*I was introduced between the *ScPGK1* promoter and terminator, generating plasmid pMI516MCS. This was accomplished by amplifying the *ScPGK1* promoter (*ScPGK1p*) from pMI516 with primers pMI-MCS_FW and pMI-MCS_RV (Table 1), digesting the amplified fragment with *Sac*I and *Xho*I (New England Biolabs) and cloning it between the *Sac*I and *Xho*I sites in pMI516. The *kanMX* and *S. cerevisiae URA3* expression modules in pMI516MCS were then exchanged with the *GEN3* module from pGEN3[5] using the *Bgl*II/*Bgl*II sites, generating plasmid pMIGEN3.

Plasmids pFMA, pFMG, and pFMT were obtained by exchanging the *ScPGK1p* in pMIGEN3 with the *ScADH1*, *AgGPD*, and *AgTEF* promoters, respectively. The 700 bp *ScADH1p*[23] was amplified by PCR from *S. cerevisiae* CEN.PK 113-7D genomic DNA using the primers ScADH1p-SacI_FW and ScADH1p-EcoRI_RV (Table 1), the *AgGPDp*[24]

Table 1. Oligonucleotides Used for the Construction of Plasmids and Strains Screening

Oligonucleotide	Sequence
pMI-MCS_FW	5′ CCAGTGAATTGGCCGATGC 3′
pMI-MCS_RV	5′GCC**CTCGAGGTCGACGGTACCCCGCGGGCGGCCGCGCTAGCGGATCC**GAATTCGGCGCGTAAGTT 3′
ScADH1p-SacI_FW	5′ TCTATC**GAGCTC**CGGGTGTACAATATGGACT 3′
ScADH1p-EcoRI _RV	5′ CG**GAATTC**TGAGATAGTTGATTGTATGCTTGGT 3′
AgGPDp-SacI_FW	5′ TCTATC**GAGCTC**TGTCTGGGTGCACGACAC 3′
AgGPDp-EcoRI_RV	5′ CG**GAATTC**GGTGTGTATGTGTGGACTGAA 3′
AgTEFp-SacI_FW	5′ TCTATC**GAGCTC**GATCTGTTTAGCTTGCCTCG 3′
AgTEFp-EcoRI_RV	5′ CG**GAATTC**TTGTTTATGTTCGGATGTGATG 3′
LacA_KpnI_FW	5′ CGG**GGTACC**ATGAAGCTTTCCTCCGCTTG 3′
LacA_XhoI_RV	5′ CCG**CTCGAG**CTAGTATGCACCCTTCCGCTTC 3′

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

was amplified from *A. gossypii* ATCC10895 genomic DNA with primers AgGPDp-SacI_FW and AgGPDp-EcoRI_RV (Table 1) and the *AgTEFp*[25] was amplified by PCR from pUG66[26] with primers AgTEFp-SacI_FW and AgTEFp-EcoRI_RV (Table 1). The amplified promoters were digested with *Sac*I and *Eco*RI (New England Biolabs) and cloned between the *Sac*I and *Eco*RI sites in pMIGEN3, replacing the *ScPGK1p*. The *A. niger lacA* gene was amplified from pVK1.1 vector[27] with primers LacA_KpnI_FW and LacA_XhoI_RV (Table 1), digested with *Kpn*I and *Xho*I (New England Biolabs) and inserted into the *Kpn*I/*Xho*I sites of pFMA, pMIGEN3, pFMG, and pFMT, in frame with the different promoters. The resulting plasmids were named pFMAlacA (Figure 1A), pFMPlacA (Figure 1B), pFMGlacA (Figure 1C), and pFMTlacA (Figure 1D), respectively. The constructions were verified by sequencing.

All PCR reactions were carried out with Taq DNA polymerase 5 U μL^{-1} (NZYTech), with the exception of *lacA* gene amplification, which was done using Phusion® High-fidelity DNA polymerase 2 U μL^{-1} (Finnzymes). PCR conditions for amplification with Taq DNA polymerase were: 5 min at 95°C, 35 cycles of 45 s at 95°C, 45 s at 50°C (53°C for amplification of the MCS), and 1 min at 72°C, with a final extension at 72°C for 10 min. For *lacA* amplification, PCR conditions were: 1 min at 98°C, 35 cycles of 10 s at 98°C, 30 s at 68°C, and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min.

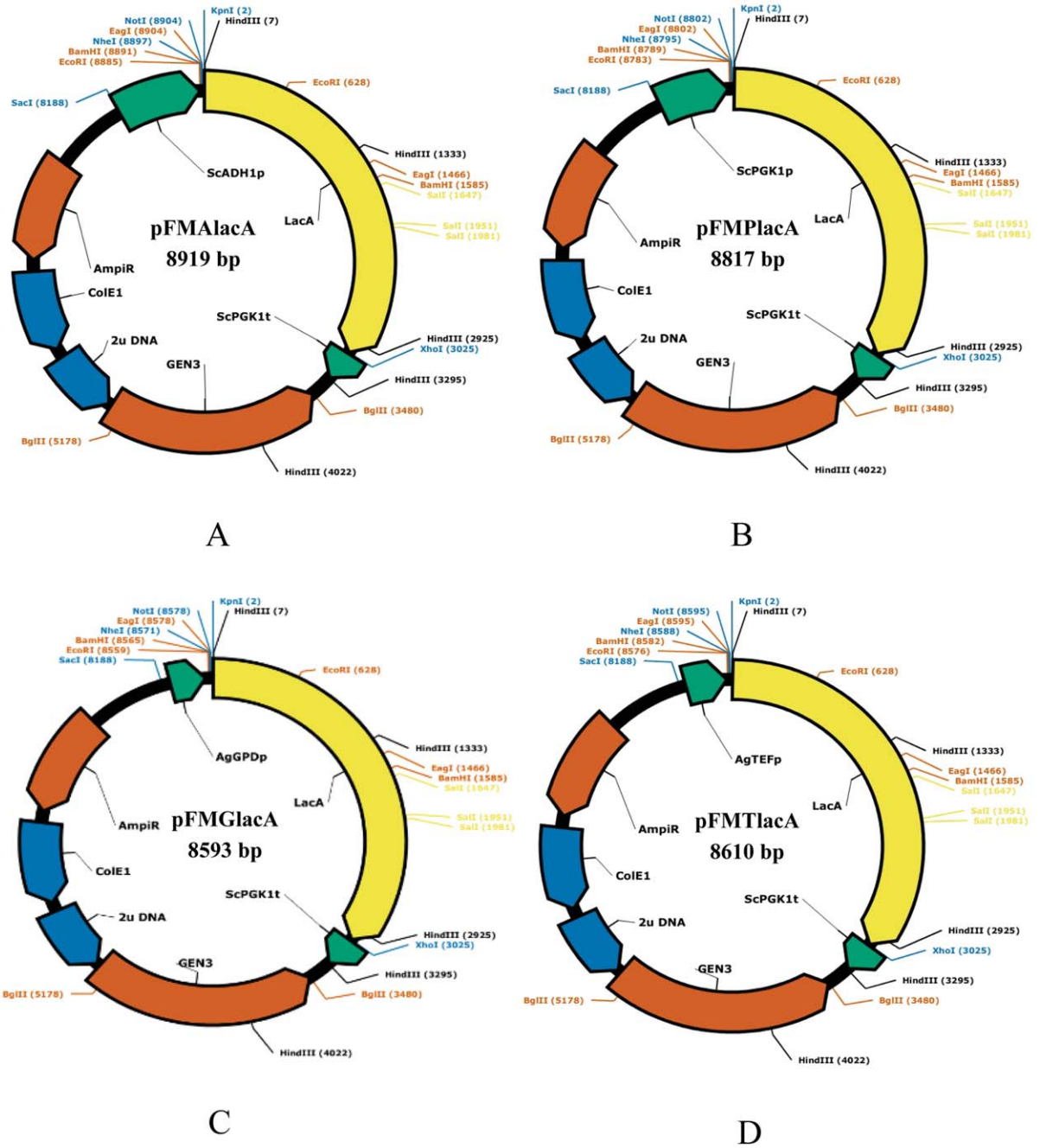

Figure 1. Plasmids constructed for the expression of *A. niger* β-galactosidase in *A. gossypii* under the regulation of different promoters: (A) *S. cerevisiae ADH1* promoter, (B) *S. cerevisiae PGK1* promoter, (C) *A. gossypii GPD* promoter, and (D) *A. gossypii TEF* promoter.

A.gossypii transformation and screening of transformants

A. gossypii ATCC10895 was transformed by electroporation as described by Wendland et al.[5] with 5 μg of plasmid DNA (pFMAlacA, pFMPlacA, pFMGlacA, pFMTlacA, and pFMT as a negative control). Transformant colonies were selected on selective AFM plates, transferred to fresh selective AFM plates, and grown for 5 days. For the initial β-galactosidase activity screenings, a small patch of mycelium from the edge of 10 newly grown transformant colonies was used to inoculate 10 mL test tubes containing 5 mL of selective AFM. After 2 days of growth at 30°C and 200 rpm, β-galactosidase activity was measured from culture supernatants and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal; NZYTech) was added to the tubes to a final concentration of 0.04 mg mL^{-1}. Hydrolysis of the X-gal by the produced β-galactosidase turned the supernatants blue. Based on the β-galactosidase assays and on the intensity of the blue color in the different tubes, four transformant colonies were selected for further study: *A. gossypii* ADH1lacA, *A. gossypii* PGK1lacA, *A. gossypii* GPDlacA, and *A. gossypii* TEFlacA.

S.cerevisiae transformation and selection of transformants

S. cerevisiae strains were transformed by the Lithium Acetate method[28] with the same plasmids as *A. gossypii*. Transformant colonies were selected on selective YPD medium and expression of β-galactosidase was confirmed in X-gal plates. After 3 days of growth at 30°C four transformant colonies were randomly chosen for further study: *S. cerevisiae* ADH1lacA, *S. cerevisiae* PGK1lacA, *S. cerevisiae* GPDlacA, and *S. cerevisiae* TEFlacA.

Expression of recombinant β*-galactosidase*

Recombinant *A. gossypii* and *S. cerevisiae* strains were grown at 30°C and 200 rpm in 250 mL Erlenmeyer flasks containing 50 mL of selective AFM and YPD, respectively. An inoculum of 10^7 spores was used for *A. gossypii* growths. For *S. cerevisiae*, pre-cultures grown for 12 h were used to inoculate production cultures to an initial absorbance at 600 nm (OD_{600}) of 0.1. Samples were taken to determine cell concentration (OD_{600}) and to collect culture supernatants by centrifugation at 5,000 rpm for 10 min, at 4°C. The OD_{600} was converted to cell dry weight (DW) using a standard curve. Total reducing sugar concentration in the culture supernatants was determined by the dinitrosalicylic acid method.[29]

β*-Galactosidase assay*

β-Galactosidase activity was measured from culture supernatants through the quantification of *p*-nitrophenol released from *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG), as described in Domingues et al.[30] Briefly, the samples were incubated for 10 min at 65°C in 1.7 mM of *p*NPG in 0.075 M acetate buffer (pH 4.5). The reaction was stopped by raising the pH to 10 with 1 M Na_2CO_3 and the absorbance was measured at 405 nm. One enzyme unit (U) was defined as the amount of enzyme that hydrolyzed 1 nmol of *p*NPG per min at 65°C.

β*-Galactosidase zymogram*

The supernatant of a shake-flask culture of recombinant *A. gossypii* TEFlacA and *S. cerevisiae* NCYC869-A3/pVK1.1 was concentrated in Amicon® Ultra-15 30,000 MWCO centrifugal filter devices (Millipore) according to the manufacturer's instructions. Concentrated supernatants were treated with Endoglycosidase H (New England Biolabs) according to the manufacturer's instructions, with the exception of the denaturing step. For in situ detection of β-galactosidase activity, the concentrated samples (with a normalized β-galactosidase activity of ~40,000 U mL^{-1}) were separated by polyacrylamide gel electrophoresis (PAGE) in an 8% native polyacrylamide gel. β-Galactosidase activity staining was carried out as described in O'Connell and Walsh[31] by incubating the native gel in a solution of 0.02% (w/v) X-gal in 0.2 M sodium acetate buffer (pH 4.5) for 5 h at 45°C, with agitation (100 rpm).

Results

Expression of A. niger β*-galactosidase in A. gossypii under the regulation of different promoters*

Four different promoters were used to drive the expression of *A. niger* β-galactosidase in *A. gossypii*: two *A. gossypii* promoters (*AgGPDp* and *AgTEFp*) and two *S. cerevisiae* promoters (*ScADH1p* and *ScPGK1p*). As described in the "Materials and Methods" section, each promoter was cloned in frame with the *lac*A gene (coding for secreted *A. niger* β-galactosidase) in the modified pMI516 plasmid,[9] resulting in four expression vectors: pFMAlacA, pFMPlacA, pFMGlacA, and pFMTlacA (Figure 1). *A. gossypii* mycelium was transformed by electroporation with each expression vector constructed, with transformation efficiencies of 50–100 colonies per μg of plasmid DNA. In the screening assays, it was already possible to see that the native promoters (*AgTEFp* and *AgGPDp*) led to a stronger expression of recombinant β-galactosidase than the *S. cerevisiae* promoters (*ScADH1p* and *ScPGK1p*) (Figure 2).

The β-galactosidase activity secreted by the selected recombinant *A. gossypii* strains under the control of each promoter was measured in the culture supernatants of shake-flasks fermentations. No differences in the specific growth rate of the recombinant strains were observed when compared with the parental strain transformed with the empty vector, indicating that the secretion of recombinant β-galactosidase did not impose significant metabolic load in this host (not shown). In all strains, the specific activity of secreted β-galactosidase (U mg^{-1} DW) increased along the fermentation time and reached the maximum values when all glucose was consumed and cells entered the stationary phase (at about 87 h) (Figure 3). Similar activity levels were obtained with both *S. cerevisiae* promoters (*ScADH1p* and *ScPGK1p*), as observed in the preliminary screening, and higher levels were obtained with the native promoters. The native *AgTEFp* led to 2-fold higher recombinant β-galactosidase activity than the native *AgGPDp* and 8-fold higher than the *S. cerevisiae* promoters (Table 2).

Recombinant *A. gossypii* TEFlacA grown in AFM containing glycerol instead of glucose reached a specific activity of secreted β-galactosidase of 137 ± 13 U mg^{-1} DW (corresponding to a volumetric activity of $1{,}127 \pm 67$ U mL^{-1}) 80 h after inoculation. At this time point, cells entered the stationary phase, but a glycerol concentration of ~8 g L^{-1} was still present in the medium. These levels of specific activity are ~1.5-fold higher than those obtained with the same strain grown until the beginning of the stationary phase in standard AFM with glucose as primary carbon source (Figure 3B).

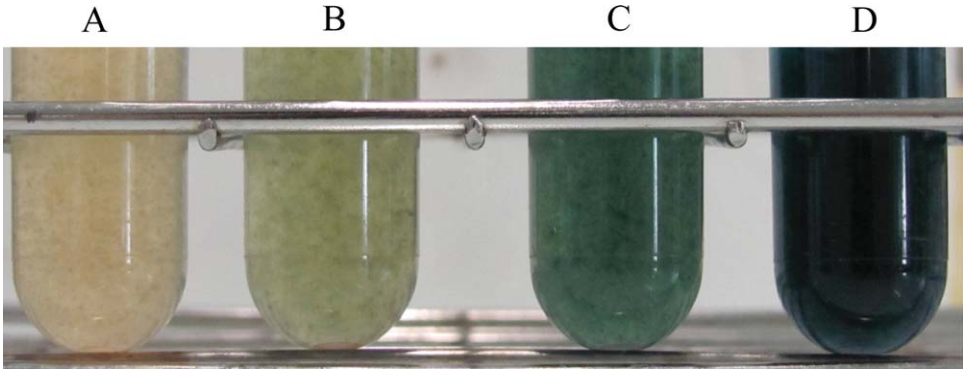

Figure 2. Test tubes for β-galactosidase activity screening containing *A. gossypii* ADH1lacA (A), PGK1lacA (B), GPDlacA (C), and TEFlacA (D) grown for 2 days in 5 mL of selective AFM supplemented with 0.04 mg mL^{-1} X-gal.

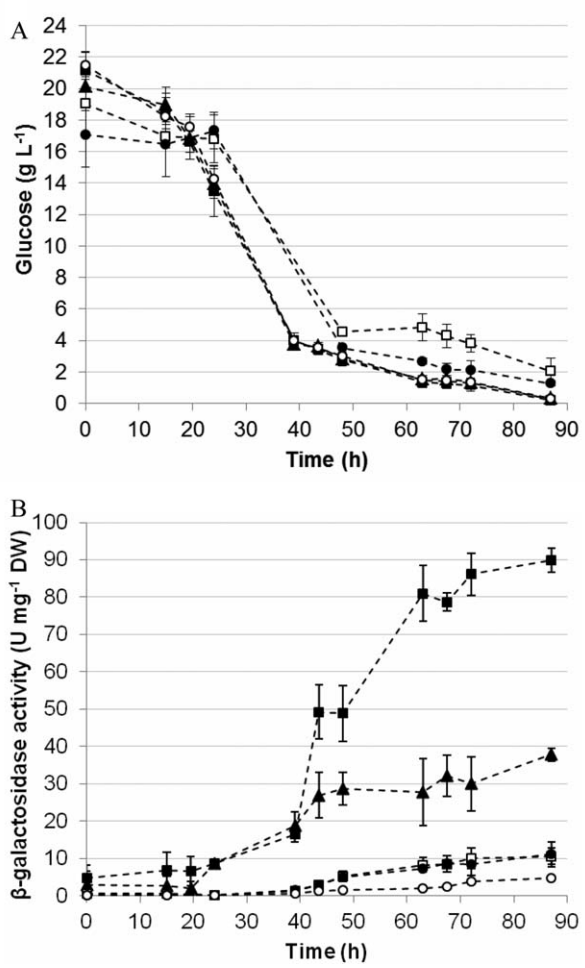

Figure 3. Glucose consumption (A) and specific β-galactosidase activity (B) for the *A. gossypii* recombinant strains growing at 30°C and 200 rpm in 250 mL shake-flasks containing 50 mL of selective AFM: *A. gossypii* TEFlacA (solid square), GPDlacA (solid triangle), ADH1lacA (open square), PGK1lacA (solid circle), and empty vector (open circle).

Error bars represent the standard deviation of three biological replicates.

Expression of A. niger β-*galactosidase in S. cerevisiae under the regulation of different promoters*

To compare the levels of recombinant β-galactosidase activity obtained in *A. gossypii* with a yeast host, the *S. cerevisiae* strains CEN.PK 113-7D and NCYC869-wt were transformed with the same expression vectors used to transform *A. gossypii*. The β-galactosidase activity secreted by the selected yeast recombinants was determined as mentioned above for the *A. gossypii* recombinants. Identical results were obtained for the two *S. cerevisiae* strains used (Table 2). The levels of recombinant β-galactosidase activity secreted by *S. cerevisiae* were considerably lower than those obtained in *A. gossypii*, independently of the promoter used (Table 2). The yeast secreted nearly 40 times less recombinant β-galactosidase activity than *A. gossypii* in similar production conditions (Table 2). As in the case of *A. gossypii*, the *AgTEF* promoter also led to the highest levels of recombinant β-galactosidase activity in *S. cerevisiae* (Table 2).

Partial characterization of the recombinant β-*galactosidase secreted by A. gossypii*

The recombinant β-galactosidase secreted by *A. gossypii* was analyzed in a NATIVE-PAGE gel stained for β-galactosidase activity with X-gal (Figure 4). The *A. niger* β-galactosidase produced by recombinant *S. cerevisiae*[13] was included for comparison. Recombinant β-galactosidase produced by *A. gossypii* was seen in the gel as a diffuse high molecular weight band (Figure 4, lane A). This was more heterogeneous than the one produced by *S. cerevisiae* (Figure 4, lane B), judging by its larger smear. After digestion with Endoglycosidase H that heavy band was resolved from the zymogram, giving place to a much lower molecular weight band, thus confirming that the recombinant β-galactosidase secreted by *A. gossypii* is highly N-glycosylated (Figure 4, lane C), as are the β-galactosidase secreted by *A. niger* and recombinant *S. cerevisiae*.[27]

Discussion

A. gossypii presents several features that suggest it can be a good host for recombinant protein production, but until now only two heterologous proteins had been reported to be secreted by this fungus and at modest levels.[9] In this work, the β-galactosidase from *A. niger* was successfully expressed and secreted by the *A. gossypii* ATCC10895 strain from 2-μm plasmids under the control of the native promoters *AgGPDp* and *AgTEFp*, and of the *S. cerevisiae* promoters *ScPGK1p* and *ScADH1p* (Figures 2 and 3). This is the first time that *A. niger* β-galactosidase is expressed in this fungus, expanding the number of recombinant proteins of different origins to be effectively secreted by *A. gossypii*.

Table 2. Maximum β-Galactosidase Activity Secreted by *A. gossypii* and *S. cerevisiae* Recombinant Strains Under the Regulation of Different Promoters

	Maximum volumetric (U mL^{-1}) and specific (U mg^{-1} DW)* β-galactosidase activity		
Promoter	*A. gossypii*	*S. cerevisiae* CEN.PK 113-7D	*S. cerevisiae* NCYC869
ScADH1	72 ± 18 (10.7 ± 2.7)	2 ± 1 (0.4 ± 0.1)	5 ± 1 (0.8 ± 0.2)
ScPGK1	70 ± 16 (10.9 ± 2.6)	8 ± 1 (1.2 ± 0.2)	9 ± 1 (1.5 ± 0.2)
AgGPD	248 ± 14 (37.8 ± 2.1)	10 ± 2 (1.8 ± 0.3)	13 ± 1 (2.1 ± 0.2)
AgTEF	523 ± 30 (89.6 ± 5.2)	14 ± 2 (2.4 ± 0.4)	16 ± 2 (2.4 ± 0.3)

The values represent the average ± standard deviation of the β-galactosidase activity measured at the glucose depletion time-point in the supernatants of three independent shake-flask cultures performed at 30°C and 200 rpm in 50 mL of selective AFM (for *A. gossypii*) and YPD (for *S. cerevisiae*).
*Specific activity is represented between brackets.

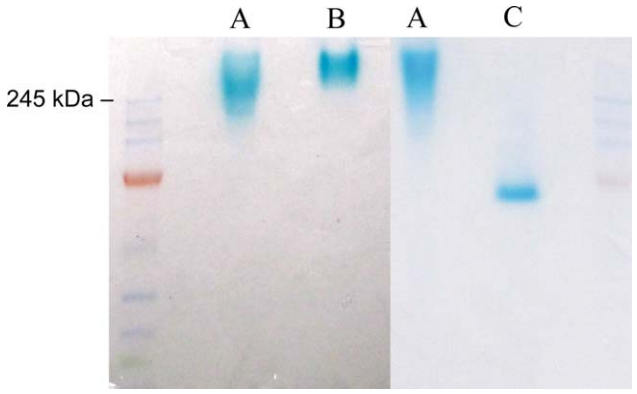

Figure 4. β-Galactosidase zymogram. *A. niger* β-galactosidase produced by *A. gossypii* TEFlacA (lane A) and *S. cerevisiae* NCYC869-A3/pVK1.1 (lane B).

Lane C corresponds to the *A. gossypii* recombinant β-galactosidase digested with Endoglycosidase H. The recombinant β-galactosidase samples applied to the gel had an activity of ~600 U.

The level of recombinant β-galactosidase secreted by *A. gossypii* was strictly dependent on the promoter used. The highest extracellular β-galactosidase activity was obtained with the native *AgTEFp*, followed by the other native promoter, the *AgGPDp* (Table 2). The *AgTEFp* presented the double driving force of the *AgGPDp*. The *S. cerevisiae* promoters *ScPGK1p* and *ScADH1p* functioned in a similar way in *A. gossypii*, but led to lower protein expression levels than the native promoters, as commonly happens in other filamentous fungi[32] (Table 2). A better performance of native promoters over *S. cerevisiae* promoters in *A. gossypii* has also been previously reported, namely using the *AgTEFp*.[22] The *ScPGK1p* has already been employed once in the expression of heterologous proteins in *A. gossypii*,[9] but to our knowledge, this is the first time that the *ScADH1p* has been used. The plasmids constructed in this work, especially that carrying the native *AgTEFp*, thus revealed to be suitable protein expression vehicles for *A. gossypii*.

Laboratorial *S. cerevisiae* strains transformed with the same plasmids secreted lower levels of recombinant β-galactosidase than *A. gossypii* (up to 16 U mL^{-1}; Table 2). This result contrasts with previous reports, where the levels of recombinant proteins secreted by both hosts were comparable, using a 2-μm based plasmid identical to that used in this work.[9] However, this does not directly imply that *A. gossypii* is a better *A. niger* β-galactosidase producer than *S. cerevisiae*, because the efficient expression of this enzyme in *S. cerevisiae* seems to be strain and/or plasmid dependent. In the first attempts to produce *A. niger* β-galactosidase in *S. cerevisiae*, very low activity levels were obtained. A Mauri distiller's yeast transformed with plasmid pVK1.1, harboring the *URA3* selective marker and the *A. niger* β-galactosidase under the control of the *S. cerevisiae ADH1* promoter and terminator (*lacA* cassette), expressed 10 U mL^{-1} of extracellular recombinant β-galactosidase.[33] Similarly, a flocculent brewer's yeast transformed with a plasmid having as selective marker the yeast *CUP1* gene, conferring resistance to copper (pET13.1 plus *lacA* cassette), expressed 17 U mL^{-1} of extracellular recombinant β-galactosidase.[34] These activity levels are of the same order of magnitude as those obtained in this work for *S. cerevisiae* transformed with the plasmids here described, even though different media and carbon sources were used (in this work, glucose was the primary carbon source used, while lactose has been used in the previous studies). However, very high yields of recombinant β-galactosidase activity were reported for the *S. cerevisiae* NCYC869-A3 strain transformed with the plasmid pVK1.1.[13] Unfortunately, it is not possible to transform *A. gossypii* with the pVK1.1 plasmid for direct comparison with *S. cerevisiae*, because the *S. cerevisiae URA3* does not complement uracil auxotrophy in *A. gossypii Agura3* auxotrophes[35] and also the *ScADH1* terminator shows ARS activity in *A. gossypii*.[36] Nevertheless, in similar culture conditions, the recombinant *A. gossypii* strain carrying the plasmid pFMTlacA (where the *lacA* gene is under the regulation of the native *AgTEFp*) secreted ~2.5 times higher levels of recombinant β-galactosidase activity than those reported for the recombinant high-level producer *S. cerevisiae* NCYC869-A3/pVK1.1 strain (212 U mL^{-1}).[14] This result highlights the suitability of *A. gossypii* as an alternative recombinant β-galactosidase producer with still plenty of room for improvement. A change in the primary carbon source used (from glucose to glycerol) already improved ~1.5-fold the productivity of the best recombinant *A. gossypii* β-galactosidase-producing strain (1,127 U mL^{-1}). In *S. cerevisiae*, optimization of the operation and culture conditions resulted in a 21-fold increase in the recombinant β-galactosidase production, when compared with the values described for flask cultivations,[15] approaching the extracellular activity levels obtained in nonmodified *A. niger* strains (152–3,000 U mL^{-1}).[31,37,38]

Besides good expression capacity, *A. gossypii* also presented in this work other desirable features for a recombinant protein producer. The native signal peptide of the *A. niger* β-galactosidase was recognized by *A. gossypii* as a secretion signal, directing the production of this protein into the culture medium, which facilitates its downstream processing. *A. gossypii* seems to be able to recognize a wide range of signal sequences, which is advantageous in a recombinant host. In previous works, *A. gossypii* recognized the native signal sequences of *T. reesei* cellulases EGI and CBHI[9] and

S. cerevisiae invertase (*SUC2* gene) (our unpublished results). Furthermore, recombinant β-galactosidase secreted by *A. gossypii* was N-glycosylated, as shown by the reduction of its molecular weight in a native gel after deglycosylation (Figure 4). The native *A. niger* β-galactosidase is a highly glycosylated enzyme, with several potential sites for N-linked (12 sites) and O-linked glycosylation.[27] This enzyme presented an apparent size of 130 kDa in Western blots, migrating with a diffuse aspect, which was attributed to heterogeneous glycosylation.[27] Heterogeneous and extensive N-glycosylation has also been reported for recombinant β-galactosidase produced in *S. cerevisiae*.[27] Here, the β-galactosidase secreted by *A. gossypii* displayed a more diffuse pattern in the native gel than that secreted by *S. cerevisiae*, possibly as a result of higher glycosylation heterogeneity (Figure 4). Previously, recombinant *T. reesei* EGI and CBHI secreted by *A. gossypii* were observed to be less extensively glycosylated than the same cellulases secreted by *S. cerevisiae*.[9] Despite its higher heterogeneity, the same tendency to a less extensive hyperglycosylation of the recombinant β-galactosidase produced by *A. gossypii* in comparison with that produced by *S. cerevisiae* was also apparent in this work (Figure 4).

Conclusion

In conclusion, β-galactosidase from *A. niger* was for the first time produced and secreted by *A. gossypii* at higher levels than those obtained with recombinant *S. cerevisiae* laboratorial strains producing this enzyme in similar culture conditions. Moreover, the *AgTEFp* revealed to be the best promoter for its overexpression. The results obtained herein demonstrate the potential of *A. gossypii* as a new recombinant protein production platform. Further optimization of expression plasmids, culture conditions or strain tailoring can be now envisaged, aiming at improving the productivity of the non-engineered laboratorial strain here used.

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