

## Characterization of *Aspergillus niger* endo-1,4- $\beta$ -glucanase ENG1 secreted from *Saccharomyces cerevisiae* using different expression vectors

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**ABSTRACT.** Heterologous expression of *Aspergillus niger* endo-1,4- $\beta$ -glucanase (ENG1) in *Saccharomyces cerevisiae* was tested both with an episomal plasmid vector (YEGAp/*eng1*) and a yeast vector capable of integration into the *HO* locus of the *S. cerevisiae* chromosome (pHO-GAPDH-*eng1*-KanMX4-*HO*). In both cases, *eng1* gene expression in yeast, with its native signal sequence for secretion, was under the control of the strong glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter. We aimed to verify how each expression system affects protein expression, posttranslational modification, and biochemical properties. Expression of *eng1* from the episomal plasmid vector YEGAp/*eng1* significantly slowed the growth of a yeast cell culture. However, expression of *eng1* from the vector integrated into the *HO* locus of the chromosome did not cause growth suppression, and the enzyme activity in a culture supernatant was maintained throughout the incubation time. ENG1 has optimum catalytic activity at pH 6.0, and

is stable in the pH range 5.0-9.0. The enzyme's optimum temperature for catalytic activity at pH 6.0 is 70°C; importantly, more than 95% of the enzyme's initial activity remained after a 2-h incubation at 60°C. The biochemical characterization of ENG1 confirmed the correct expression of the protein and showed that ENG1 expressed by the *pHO-GAPDH-eng1-KanMX4-HO* vector, in addition to its N-linked sites, is overglycosylated at its O-glycosylation sites compared with ENG1 expressed by the *YEGAp/eng1* vector. It is likely that the O-glycosylated form of the *A. niger* ENG1 retains more stable activity during continuous cultivation of recombinant yeasts than the form that is only N-glycosylated.

**Key words:** Endo-1,4- $\beta$ -glucanase; *Aspergillus niger*; *HO* locus; *Saccharomyces cerevisiae*; Episomal plasmid vector; Glycosylation

## INTRODUCTION

Cellulose is the most abundant biological polymer and has a linear structure composed of unbranched D-glucopyranose residues that are joined via 1,4- $\beta$ -glucosidic bonds, allowing the molecule to form long, straight chains. Depending on the source, native cellulose can have a very high degree of polymerization: around 10,000 glucose units in wood and 15,000 in cotton (Azizi Samir et al., 2005). Biodegradation of cellulose with the formation of soluble sugars is catalyzed by a specific multi-enzyme cellulase system composed of endo-1,4- $\beta$ -glucanase (EC 3.2.1.4), exo-1,4- $\beta$ -glucanase (EC 3.2.1.91), exo-1,4- $\beta$ -glucosidase (EC 3.2.1.74), and cellobiase (EC 3.2.1.21) (Turon et al., 2008; Horn et al., 2012). Properties of individual enzymes and their interactions in these cellulase complexes determine the efficiency of the hydrolytic degradation of cellulose substrates to soluble oligomeric and monomeric sugars. Glucose, and other sugars produced by the enzymatic hydrolysis of lignocellulosic biomass, can be further converted by certain microorganisms into bioethanol and/or biobutanol.

Among producers of microbial cellulases, the filamentous fungus *Aspergillus niger* plays a leading role. This fungus secretes a complex set of cellulolytic enzymes, which are utilized in numerous commercial applications such as food, textile, and pharmaceutical industries (De Vries, 2003). *A. niger* endo-1,4- $\beta$ -glucanase, the main component of the cellulases, plays a critical role in the action of the multi-enzyme system. This enzyme is the first to attack the cellulose, hydrolyzing internal  $\beta$ -1,4-glycosidic bonds at sites remote from the ends of the polymer chain to form cello-oligosaccharides such as mono-, di-, and tri-saccharides (Li et al., 2012).

The yeast episomal plasmid (YE<sub>p</sub>) has been used to express the *A. niger* endo-1,4- $\beta$ -glucanase gene (*eng1*) in *Saccharomyces cerevisiae* to study the physical and chemical properties of the enzyme (Hong et al., 2001). However, in the absence of selective pressure, YE<sub>p</sub> vectors are difficult to maintain stably within cells, and are often lost from the transformed cells during culture growth under non-selective conditions (Yamada et al., 2013). From a practical point of view, yeast-integrated plasmid systems might be more suitable for the stability of the protein, and can also prevent the loss of the recombinant gene in the absence of selective pressure. Recently,  $\delta$ -integration vectors have been used for stably enhancing target gene copy numbers in *S. cerevisiae*. A multi-copy gene has been constructed comprising endoglucanase, cellobiohydrolase, and  $\beta$ -glucosidase genes integrated into the *S. cerevisiae* genome (Yamada

et al., 2010a,b). This method drastically increased the cellulolytic activity of the strain. However, the integration vectors employed auxotrophic markers such as LEU2-d, HIS3, TRP1, and URA3, and these selective markers differentially influenced the expression of heterologous cellulase genes (Liu et al., 2013). Moreover, these yeast strains might not be suitable for industrial applications, since the industrial yeast strains do not contain internal genetic markers that can identify the need for amino or nucleic acids, for example.

Voth et al. (2001) have reported the establishment of several integral yeast vectors that can be inserted in the yeast chromosome by gene replacement using the homologous sequence portion of the *HO* gene. The *HO* gene encodes an endonuclease responsible for initiating mating type switching and initiation of diploidization of heterothallic yeast (Bakhrat et al., 2004). It was shown that deletion of the *HO* locus does not affect the growth of yeast, and that nearly all industrial and laboratory strains of *S. cerevisiae* have a mutation at the *HO* locus (Baganz et al., 1997; Walker et al., 2003). Moreover, a marker gene for resistance to the antibiotic geneticin (G418), contained in the expression cassette, is dominant and may provide a means for the selection of transformants, regardless of the genotype of the host cell.

In this paper, we present a comparative biochemical analysis of the ENG1 protein expressed using an episomal plasmid vector and a yeast vector that is capable of integration into the *HO* locus of the *S. cerevisiae* chromosome.

## MATERIAL AND METHODS

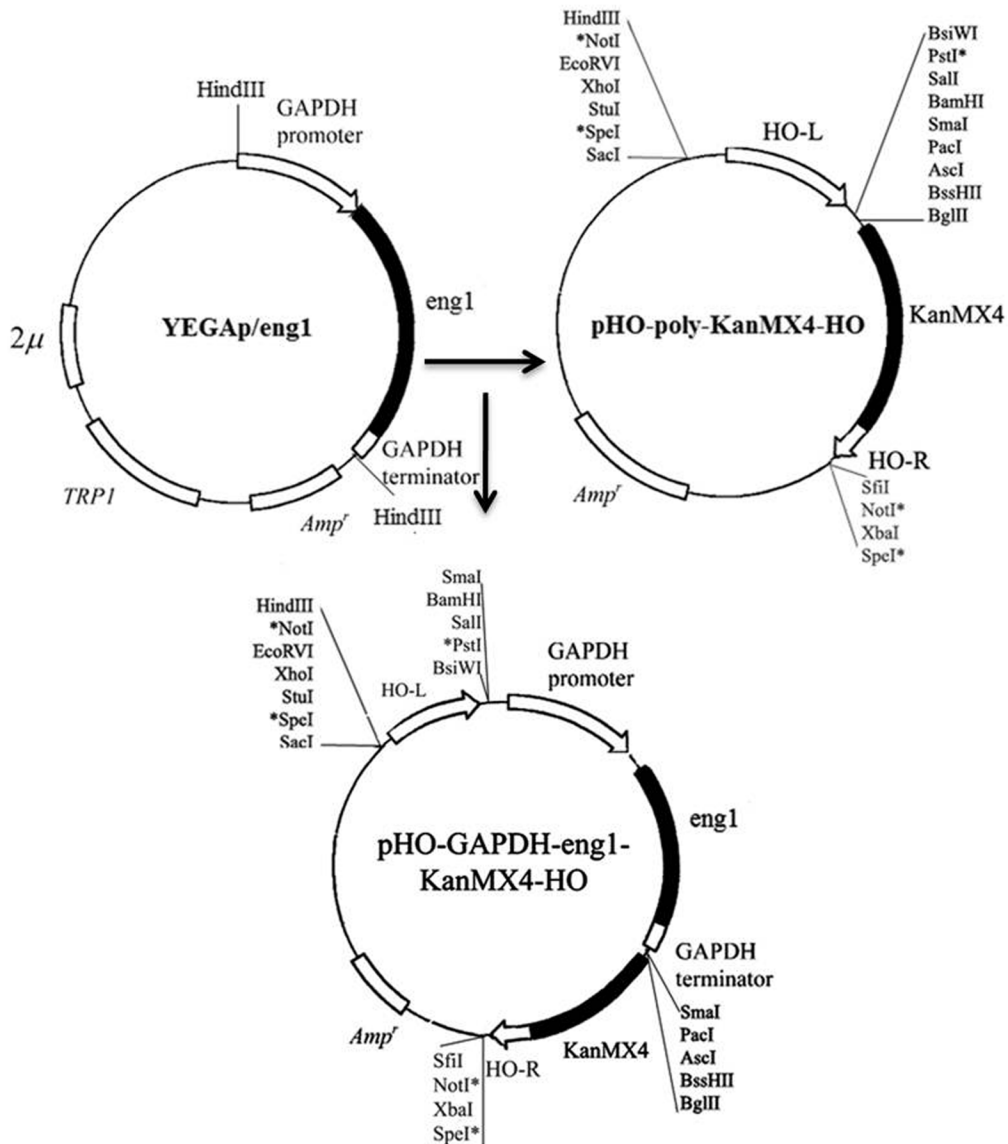
### Strains and cultivation conditions

*Escherichia coli* DH5 $\alpha$  was used for plasmid maintenance and construction. *S. cerevisiae* strain FF18733 (*MATa his7-2 leu2-3,112 lys1-1 trp1-289 ura3-52*) was used as a recipient strain for transformation. *E. coli* were grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100  $\mu$ g/mL) when necessary. Yeast strains were grown at 30°C in YPD medium [1% yeast extract (w/v), 2% peptone (w/v), 2% glucose (w/v)]. Screening the transformed yeast cells was performed in YPD medium with G418 at a final concentration of 100  $\mu$ g/mL.

### Construction of *pHO-GAPDH-eng1-KanMX4-HO*

The *HO*-poly-KanMX4-*HO* plasmid, kindly provided by David J. Stillman, University of Utah, USA (Voth et al., 2001), carries the KanMX4 selectable marker and two fragments of the *HO* gene for integration at the *HO* locus, and was used to construct the yeast expression strain. The cassette containing the *A. niger eng1* gene under the control of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter and *GAPDH* terminator was obtained from plasmid YEGAp/*eng1* (Hong et al., 2001), which was kindly provided by Dr. Hidehiko Kumagai, Kyoto University, Japan. To create a vector expressing ENG1, the *Hind*III fragment from YEGAp/*eng1*, containing the *GAPDH* promoter, the *A. niger eng1* gene, and the *GAPDH* terminator, was blunt-ended and cloned into the blunt and dephosphorylated *Sma*I site of the *HO*-poly-KanMX4-*HO* plasmid, creating the *HO*-GAPDH-*eng1*-KanMX4-*HO* expression plasmid vector with flanking homology to target the genes to the *HO* endonuclease locus (Figure 1). *pHO*-GAPDH-*eng1*-KanMX4-*HO* DNA was linearized by treatment with restriction enzymes *Xho*I and *Xba*I and transformed into yeast cells using a standard lithium acetate method (Gietz and Woods, 2002). YPD medium supplemented

with filter-sterilized G418 (100 µg/mL) was used for selection and maintenance of the yeast transformants harboring the G418 resistance gene. Yeast transformants displaying ENG1 activity were selected on a 0.5% carboxymethylcellulose (CMC) YPD medium supplemented with G418 (100 µg/mL) agar plate by the Congo Red halo test. The resulting recombinant strain was designated as FF18733/pHO-GAPDH-*eng1*-KanMX4-HO. Integration of pHO-GAPDH-*eng1*-KanMX4-HO DNA was confirmed by polymerase chain reaction (PCR) using primers pr1-pr5 (Table 1).



**Figure 1.** Construction of the integrative vector for the expression of *Aspergillus niger* endo-1,4-β-glucanase in yeast.

**Table 1.** Oligonucleotide primers used to analyze the efficiency of integration of the integrative vector into the yeast chromosome.

Primers	DNA sequence (5'-3')
Pr-1	ATTTTAGCAGATGCGCGCACC
Pr-2	GTTTTTCTCAACATTCAAGGAAAG
Pr-3	GGAGGCCCAAGAATACCCTCCTTGA
Pr-4	ATGAAGTTTCAGAGCACTCT
Pr-5	TCAAAGATATGCCTCCAGGA

Yeast genomic DNA of FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* recombinant strains were isolated according to the method of Harju et al. (2004) and used as a template for amplification by PCR.

Transformation of YEGAp/*eng1*, a multi-copy shuttle plasmid harboring the *TRPI* gene, as a selection marker into *S. cerevisiae* strain FF18733 was carried out by the lithium acetate method. Colonies of transformants showing a phenotype of the prototroph for tryptophan were selected on SD [0.67% yeast nitrogen base without amino acids (Difco Laboratories, MI, USA), 2% glucose] agar plates supplemented with appropriate amino acids. Expression of ENG1 was measured by Congo Red staining on culture plates (Teather and Wood, 1982). *S. cerevisiae* harbored multiple copies of the expression cassette, which was designated as FF18733/YEGAp-*eng1*.

### Protein extraction

Transformed yeast cells were cultured in YPD medium containing 0.1% CMC, and if necessary supplemented with G418 at a final concentration of 100 µg/mL in a shaker at 30°C, 200 rpm for 24, 48, 72, 96, and 120 h. Samples were collected at different time points after inoculation, unless specified otherwise. The culture supernatants were obtained by centrifugation (5 min at 3000 g) and filtered through a 0.22-µm sterile Millipore filter. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The presence of ENG1 in the culture supernatants of the transformants was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by activity staining. For SDS-PAGE, the culture supernatant was mixed with a saturated solution of ammonium sulfate to 60% saturation. The suspension was stirred for 12-16 h and maintained at 4°C, then centrifuged at 19,500 g for 30 min. The supernatant was removed and the precipitate was dissolved in 50 mM sodium acetate buffer (pH 6.0). Cell growth was monitored by measuring optical density at 600 nm using a spectrophotometer PD-303UV (Apel Co., Ltd., Japan).

### Determination of ENG1 activity

Enzyme activity was assayed with CMC as a substrate by measuring the amount of reducing sugar according to Miller (1959) with some modifications. The culture supernatant (1 mL) was pre-warmed at 60°C for 5 min and added to 1 mL substrate solution (2% CMC in 100 mM sodium acetate buffer, pH 6.0), and the mixture was incubated at 60°C for exactly 2 h. The reaction was stopped by adding 3 mL dinitrosalicylic acid solution and boiling for 15 min. The mixture was then cooled immediately and 0.2 mL reaction solution was diluted with 2.5 mL distilled water. The amount of reducing sugar was calculated by measuring the absorp-

tion value at 540 nm. One unit of activity was defined as the amount of enzyme capable of producing 1  $\mu$ M reducing sugar per minute (in 1 mg total protein) under the above conditions.

The optimal pH for enzyme activity was determined at 60°C with CMC. The following buffers used were: sodium acetate, pH 4.0-6.0; phosphate, pH 7.0-8.0; and NaOH/glycine, pH 9.0. The optimal temperature was determined by performing the standard assay at various temperatures from 40 to 80°C. Thermal stability of the enzyme was estimated by determining residual enzyme activity after incubation at 40°-80°C for 2 h.

### SDS-PAGE and activity staining

SDS-PAGE was carried out according to the method of Laemmli (1970). SDS-PAGE was performed on gels containing 12% acrylamide and 0.1% SDS using a discontinuous Tris-glycine buffer system. For detection of ENG1 activity, the proteins were separated on a 12% polyacrylamide gel with 0.2% CMC. After SDS-PAGE, the gel was washed twice in a solution containing 50 mM sodium acetate buffer, pH 6.0, and 25% isopropanol at room temperature to remove the sodium dodecyl sulfate. The proteins were renatured in a 50 mM sodium acetate buffer, pH 6.0, containing 5 mM  $\beta$ -mercaptoethanol by rocking the gel for 2 h at room temperature. The gel was then transferred to a 50 mM sodium acetate buffer, pH 6.0, and incubated at 70°C for another 2 h. The gel was stained with 0.5% Congo Red for 1 h and destained in 1 M NaCl. Clear bands on the red background indicated the breakdown of CMC.

### Deglycosylation of ENG1

The ENG1 enzymes were deglycosylated using Endoglycosidase H (Endo H; New England Biolabs, Inc., Ipswich, MA, USA) under denaturing conditions according to manufacturer recommendations. Endo H (500 U) was used to treat 0.02 mL of concentrated culture medium, estimated at approximately 1 mg/mL protein. The reactants were incubated overnight at 37°C and investigated by SDS-PAGE and enzyme activity staining.

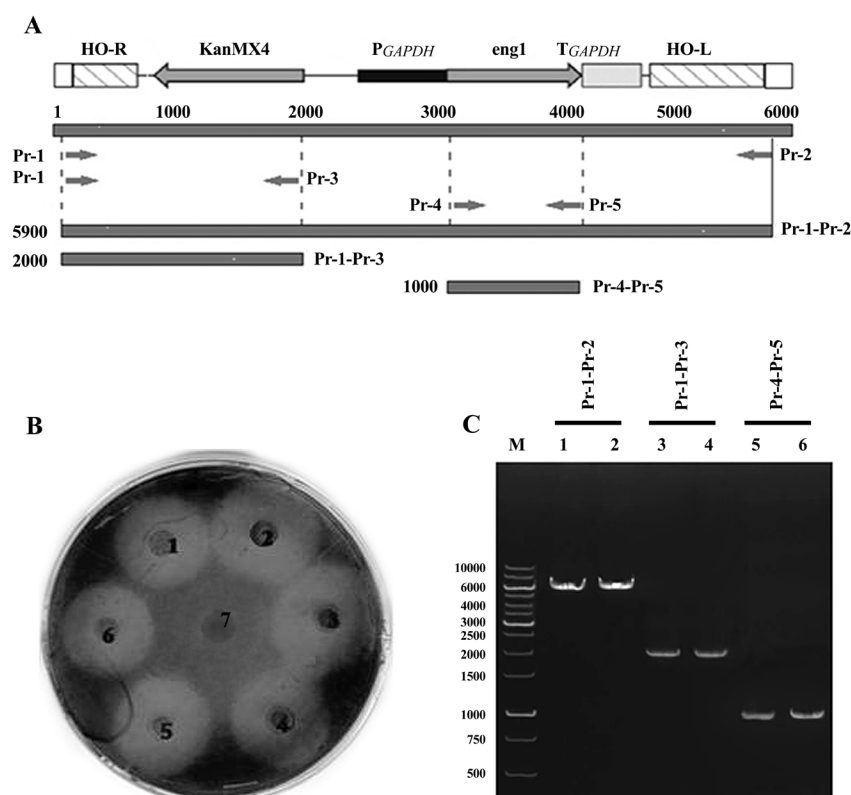
## RESULTS

### Transformation and screening of recombinant strains

To construct a stably expressing yeast strain, we first inserted cDNA of the *A. niger* ENG1 into the *HO*-poly-KanMX4-*HO* cassette under the control of the constitutive yeast *GAPDH* promoter and *GAPDH* terminator (Figure 1). The resulting expression vector *pHO-GAPDH-eng1-KanMX4-HO* (Figure 2A) harboring the gene encoding ENG1 with the native signal peptide for secretion was transformed into *S. cerevisiae*. Selection of the yeast transformants was performed using YPD agar medium containing the antibiotic G418. In addition, the recombinant clones of *S. cerevisiae* expressing ENG1 were detected by staining on CMC-supplemented agar plates with 0.5% Congo Red dye. As shown in Figure 2B, the Congo Red staining of the CMC plates revealed a visible clear zone around the yeast colonies transformed with the *HO-GAPDH-eng1-KanMX4-HO* plasmid. Remarkably, no clear zones were visible around the yeast colonies transformed with *HO*-poly-KanMX4-*HO* plasmid. These data indicate that ENG1 is expressed in an active form and that the native signal peptide attached to the enzyme is recognized by the secretory system of *S. cerevisiae*. Next, we verified the chromosomal integration of the *eng1*



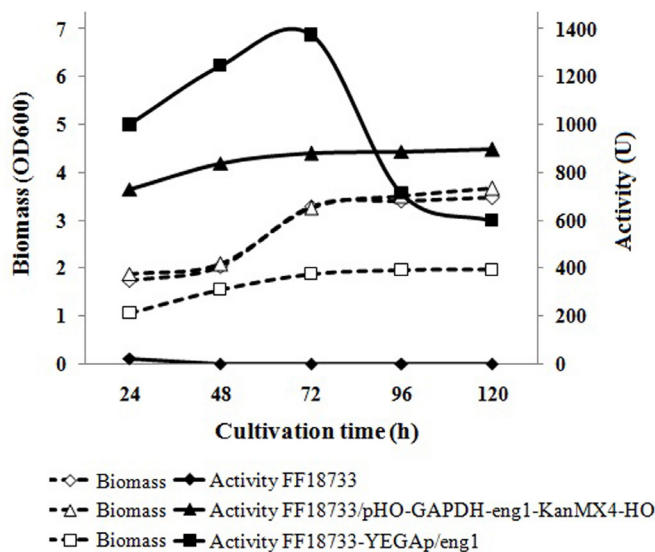
gene into the *HO* locus of the yeast chromosome using PCR. For this, the chromosomal DNA from the positive G418-resistant yeast clones was amplified using the primers shown in Table 1, and the reaction products were analyzed by agarose gel electrophoresis. As shown in Figure 2C (lanes 1-2), amplification of the portion of chromosome flanking the *HO* locus using Pr1 and Pr2 primers gave rise to a DNA fragment of the expected size [around 6000 base pairs (bp)], whereas amplification with Pr1 (homologous to the portion of the *HO* gene) and Pr3 (homologous to the portion of the *KanMX4* gene) primers gave rise to the expected DNA fragment of 2000 bp (Figure 2C, lanes 3-4). Finally, the presence of the *A. niger eng1* gene encoding ENG1 in the yeast chromosome was confirmed by amplification of yeast DNA using gene-specific Pr4 and Pr5 primers (Figure 2C, lanes 5-6). Taken together, these results confirm the integration of the cassette *pHO-GAPDH-eng1-KanMX4-HO* into the *HO* locus of the genome of *S. cerevisiae*.



**Figure 2.** Genomic localization and assay of endo-1,4- $\beta$ -glucanase activity on agar plates. **A.** Integrating vector *pHO-GAPDH-eng1-KanMX4-HO* contained the *Aspergillus niger* endo-1,4- $\beta$ -glucanase gene fused to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter. The expression cassette shown was flanked by homology (*HO-R* and *HO-L*) regions of the *Saccharomyces cerevisiae HO* endonuclease gene, which targets integration to the *HO* locus on chromosome IV. Integration of the *pHO-GAPDH-eng1-KanMX4-HO* cassette was confirmed by polymerase chain reaction (PCR) using primers Pr1-Pr5. The locations and sizes of the PCR products amplified by Pr1-Pr5 are indicated. **B.** Detection of endo-1,4- $\beta$ -glucanase activity in *S. cerevisiae* transformants by cleavage of carboxymethyl cellulose. FF18733/*pHO-GAPDH-eng1-KanMX4-HO* recombinant yeast cells (1-6) and untransformed yeast cells (7) were spotted on a carboxymethyl cellulose-supplemented agar plate, and incubated at 30°C for 3 days. The plate was then stained with Congo Red. **C.** Agarose gel electrophoresis of PCR products amplified by Pr1-Pr5 primers. Lane M = GeneRuler 1-kb DNA ladder.

### Expression of the *A. niger eng1* gene

The ability of FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strains to secrete recombinant ENG1 was determined in culture fluids after 24-120 h of incubation (Figure 3).



**Figure 3.** Growth profile of the recombinant *Saccharomyces cerevisiae* strains and enzyme activity of the secreted endo-1,4- $\beta$ -glucanase.

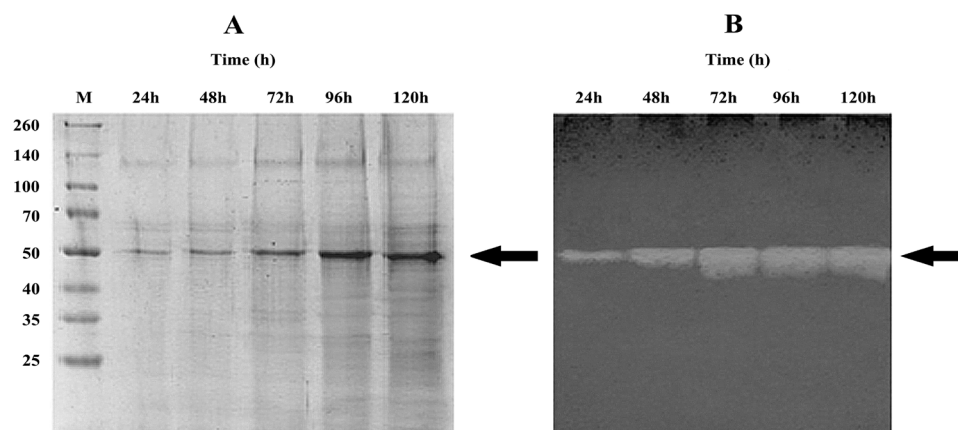
After 48 h of culture, the activity of the secreted ENG1 rose to 840 units. After 72 h of culture, the enzyme activity was maintained at 880 units. The culture medium of the control recipient *S. cerevisiae* FF18733 strain showed no ENG1 activity. Importantly, the recombinant strain showed no significant difference in growth rate and biomass yield from the control parent strain, indicating the absence of any negative influence of the heterologous protein production in the yeast FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strain. We also determined the biomass yield and the activity of the secreted recombinant enzyme in the yeast FF18733 strain transformed with the multi-copy shuttle plasmid vector YEGAp/*eng1* that contained the gene encoding *A. niger* ENG1. As shown in Figure 3, the activity of the enzyme secreted by the cultured FF18733/YEGAp-*eng1* strain increased significantly within 72 h of incubation, and was more than 1.5-fold higher than that of the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strain. A further increase of incubation time of the FF18733/YEGAp-*eng1* culture led to a significant decrease in the activity of the enzyme. Moreover, the FF18733/YEGAp-*eng1* strain grew more slowly and gave a lower yield of biomass, as compared with the parental and FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* yeast strains.

Analysis by SDS-PAGE of the cell-free culture broth from the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strain revealed a protein band with a molecular weight of approximately 50 kDa (Figure 4A).

Measurement of ENG1 activity in the SDS-PAGE gel using CMC as the substrate showed good agreement between the enzyme activity and the 50-kDa protein, indicating the presence of the secreted *A. niger* ENG1 in the culture broth (Figure 4B). It should be noted



that in the yeast cells transformed with the empty control plasmid *HO*-poly-KanMX4-*HO*, no active protein band was detected (data not shown). These data indicate that the major secretory protein in the transformed *S. cerevisiae* cells was the *A. niger* ENG1.



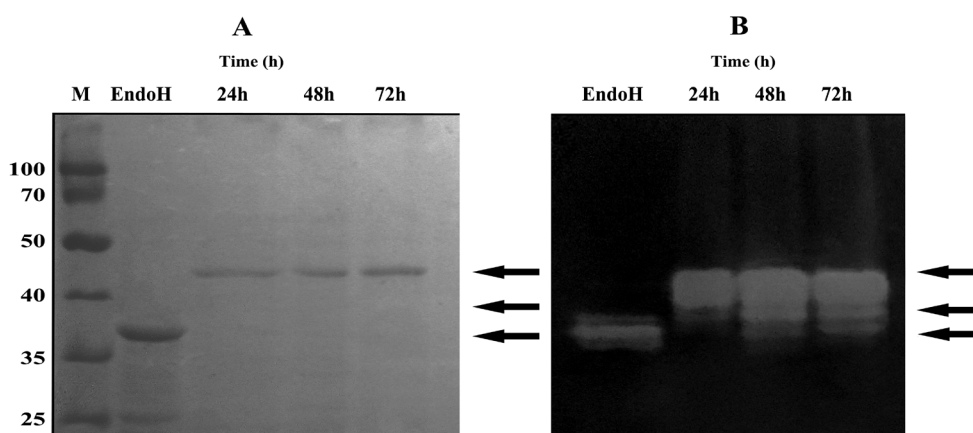
**Figure 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram of the proteins from the FF18733/*pHO*-GAPDH-*eng1*-KanMX4-*HO* strain culture supernatant. **A.** Concentrated protein from culture supernatant of FF18733/*pHO*-GAPDH-*eng1*-KanMX4-*HO* on 12% SDS-PAGE visualized by Coomassie blue staining. Lane *M* = Spectra Multicolor Broad Range Protein Ladder. **B.** Zymogram analysis of the SDS-PAGE gel using 0.2% carboxymethyl cellulose incorporated into the polyacrylamide. Protein samples were taken from the culture after 24-72 h of incubation at 30°C.

In subsequent experiments, we studied the secretion of recombinant ENG1 depending on the time of cultivation of FF18733/YEGAp-*eng1* yeast strains. Samples for SDS-PAGE and activity staining were obtained every 24 h from recombinant FF18733/YEGAp-*eng1* *S. cerevisiae* grown in YPD. Interestingly, in contrast to the FF18733/*pHO*-GAPDH-*eng1*-KanMX4-*HO* yeast strains, the multi-copy shuttle plasmid vector YEGAp/*eng1*-harboring yeast strains produced a protein band at 44 kDa (Figure 5A). Moreover, variation in molecular weight and heterogeneity of the ENG1 protein was seen in the activity staining experiment (Figure 5B). The ENG1 from the cultivation of FF18733/YEGAp-*eng1* in YPD shifted gradually from a more homogeneous protein in the beginning to a smaller and heterogeneous protein with increasing incubation time. At 72 h, the secreted ENG1 mainly consisted of three active isoforms with molecular weights of 37, 39, and 44 kDa. The proteins with molecular weights of 37 and 39 kDa did not show clear bands in the Coomassie-stained gels, indicating that these proteins were present at low concentrations.

### N-glycosylation of the secreted enzyme

The cDNA of the *eng1* gene of *A. niger* fungus strain IF031125 has been cloned and characterized previously (Hong et al., 2001). The gene encodes a protein consisting of 332 amino acids with a molecular weight of 37 kDa. As mentioned above, SDS-PAGE analysis of the ENG1 from the FF18733/YEGAp-*eng1* strain revealed the presence of three enzymatically active protein bands with apparent molecular weights of 37, 39, and 44 kDa. Interestingly, the molecular weight of the ENG1 secreted by the yeast FF18733/*pHO*-GAPDH-*eng1*-KanMX4-*HO* strain based on the SDS-PAGE analysis (50 kDa) was much higher than the calculated

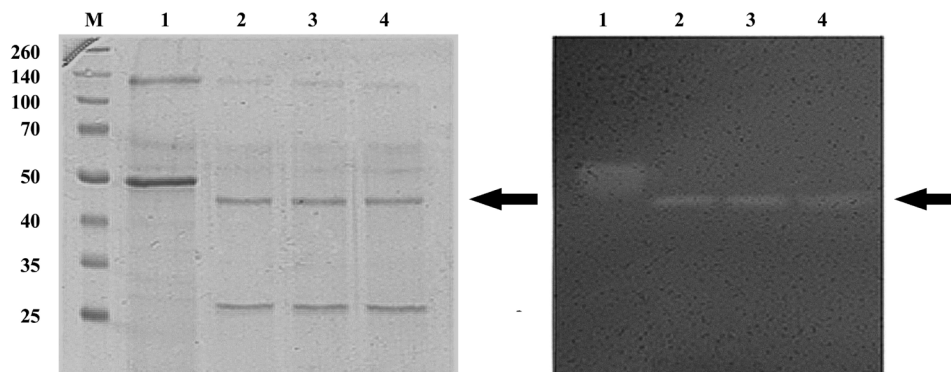
molecular weight of the enzyme (37 kDa). These observations indicate that the ENG1 protein when expressed in yeast cells has a higher molecular weight than expected. Such an increase in molecular weight can be attributed to protein glycosylation. Glycosylated proteins are often presented in the form of a collection of different molecular weight glycoforms differing in length and composition of the oligosaccharide chains covalently attached to the protein (Gao et al., 2012). To verify this assumption, the amino acid sequence of ENG1 was analyzed for the presence of N-linked glycosylation sites using the National Center for Biotechnology Information database. Based on the analysis of the primary structure of ENG1, we found four potential N-linked and three O-linked glycosylation sites (data not shown).



**Figure 5.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram of the proteins from the FF18733/YEGAp-*eng1* strain culture supernatant treated by Endo H. The recombinant endo-1,4- $\beta$ -glucanase from the culture supernatant of FF18733/YEGAp-*eng1* was deglycosylated by incubation with Endo H protein and then analyzed by SDS-PAGE followed by an enzyme activity test. **A.** SDS-PAGE. **B.** Zymogram. Lane *Endo H* = recombinant endo-1,4- $\beta$ -glucanase digested by Endo H; lanes 24-72 h = concentrated protein samples from the culture supernatant of FF18733/YEGAp-*eng1* were taken after 24-72 h of incubation; lane *M* = spectra Multicolor Broad Range Protein Ladder.

The endo- $\beta$ -N-acetylglucosaminidase H (Endo H, EC 3.2.1.96) is a highly specific endoglycosidase that cleaves asparagine-linked oligosaccharides (N-glycosylation), but not O-linked oligosaccharides (Maley et al., 1989). Therefore, recombinant *eng1* from a culture supernatant of FF18733/YEGAp-*eng1* was deglycosylated by incubation with Endo H and then analyzed by SDS-PAGE followed by an enzyme activity test. After digestion with Endo H, a single band with a molecular weight corresponding to the expected 37 kDa was observed (Figure 5B). These results indicate that differently glycosylated forms of ENG1 were present in the FF18733/YEGAp-*eng1* yeast strain.

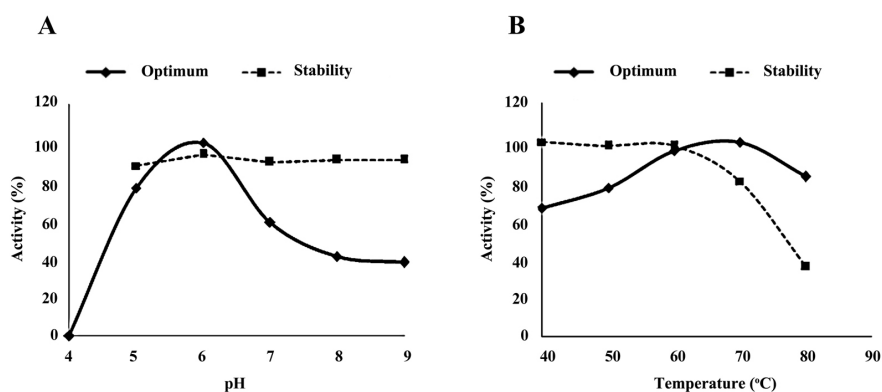
Consequently, the extracellular proteins secreted by the FF18733/pHO-GAPDH-*eng1*-KanMX4-*HO* strain were incubated with 500 U Endo H for 2, 6, and 12 h, and then the products of reaction were analyzed by SDS-PAGE. As shown in Figure 6, the Endo H-catalyzed deglycosylation led to a decrease in the molecular weight of recombinant ENG1 until about 45 kDa, which is larger than the calculated molecular weight (37 kDa). Furthermore, the enzymatic activity measurements using SDS-PAGE showed similar results, indicating the presence of partially deglycosylated ENG1.



**Figure 6.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymogram of the proteins from the FF18733/*pHO-GAPDH-eng1-KanMX4-HO* strain culture supernatant treated by Endo H. Lane 1 = recombinant endo-1,4- $\beta$ -glucanase without Endo H digestion; lanes 2-4 = endo-1,4- $\beta$ -glucanase digested by Endo H; lane M = spectra Multicolor Broad Range Protein Ladder.

### Determination of the reaction conditions for the recombinant *A. niger* ENG1

Characterization of the recombinant enzyme was carried out using CMC as the substrate. To determine the effect of pH on the stability of the enzyme, the enzyme preparation was incubated in buffers at 60°C for 2 h at various pH values. The recombinant enzyme demonstrated stability over a broad pH range, and more than 90% of total activity remained after incubation in the pH range of 5.0-9.0 (Figure 7A). Study of the influence of elevated temperature over time on the activity of recombinant ENG1 was performed at pH 6.0. The recombinant enzyme showed high thermostability; 100% of total activity remained after incubation of the protein preparation at 60°C for 2 h, and 80% activity remained after incubation at 70°C (Figure 7B). Taken together, these results indicate that the recombinant enzyme is very resistant to high temperatures.



**Figure 7.** Activity of *Aspergillus niger* recombinant endo-1,4- $\beta$ -glucanase on carboxymethylcellulose as a function of temperature and pH. **A.** Optimal and pH stability of endo-1,4- $\beta$ -glucanase. To determine pH stability of the recombinant endo-1,4- $\beta$ -glucanase, the protein was incubated at the indicated pH for 2 h and the enzyme activity was measured at 60°C. **B.** Optimal temperature and temperature stability. To determine temperature stability of the recombinant endo-1,4- $\beta$ -glucanase, the protein was incubated at the indicated temperatures for 2 h and the enzyme activity was measured at 70°C.

## DISCUSSION

Since *A. niger* ENG1 has four potential sites for N-glycosylation and three proposed O-glycosylation sites, this enzyme also becomes suitable for a comparison of glycosylation levels when expressed in the integrative and episomal constructs. Moreover, it allows for an evaluation of how different glycosylation levels influence enzyme performance, mainly its activity and other biochemical properties such as stability. For this purpose, we cloned *A. niger eng1* cDNA in the episomal plasmid vector YEGAp and a yeast vector capable of integration into the *HO* locus of the *S. cerevisiae* chromosome. In both cases, *eng1* gene expression in yeast, with its native signal sequence for *secretion*, was under the control of the strong *GAPDH* promoter. The *HO* endonuclease is not expressed in yeast with high ploidy, and is active only in haploid organisms (Hector et al., 2011); deletion of the *HO* gene does not affect the growth rate and viability of yeast (Baganz et al., 1997; Voth et al., 2001).

When both episomal and integrative expression systems were compared in *S. cerevisiae*, the activity of ENG1 was higher in the episomal than in the integrative construct at the beginning of cultivation. However, further increase of incubation time led to a significant decrease in the activity of the enzyme and in slower growth of the culture compared to the control strain. In contrast, in the case of the recombinant strain FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO*, we did not observe suppression of growth, and the ENG1 activity was maintained throughout the incubation time.

Yeast cells transformed with autonomously replicating vector DNAs can contain up to 40 plasmid copies per cell (Ghosh et al., 2007; Jeon et al., 2009). Therefore, continuous expression of a large number of the heterologous proteins in FF18733/YEGAp-*eng1* strains might lead to the depletion of energy resources and the disruption of cell metabolism. In addition, the autonomously replicating plasmid is generally difficult to maintain stably in the cells and, as a general rule, a relatively high number of cells lose the plasmid, especially in the absence of selective pressure for the plasmid markers (Yamada et al., 2013).

The biochemical characterization of ENG1 performed here, in terms of molecular weight estimation using denaturing gels, activity at different pH values, and stability with regards to pH and temperature, confirms that both yeasts were able to express and secrete the expected heterologous protein.

The pH for maximal ENG1 activity expressed in the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strain was 6.0, and the enzyme was stable over a wide pH range (5.0-9.0). The optimum temperature at pH 6.0 was 70°C, and the initial activity of the recombinant ENG1 remained stable during incubation at 60°C for 2 h. These data are in agreement with the results reported by Hong et al. (2001), indicating that the stable expression does not alter the properties of the fungal enzyme. *A. niger* ENG1 secreted by FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* recombinant strains was represented by a single band with a molecular weight of 50 kDa. Endo H-catalyzed protein deglycosylation led to a reduction of molecular weight but not to 37 kDa. Activity staining of SDS-PAGE revealed at least three active protein bands (37, 39, and 44 kDa) from the culture supernatants of FF18733/YEGAp-*eng1* strains according to molecular weight. Furthermore, the Endo H-catalyzed protein deglycosylation resulted in the reduction of molecular weight to the expected 37 kDa (Figure 5A and B), suggesting that a different glycosylated form of the ENG1 enzyme is secreted when it is expressed in episomal constructs.

It seems that the heterologous ENG1 was more hyperglycosylated in the integrative construct than in the episomal construct, and that hyperglycosylation did not negatively affect enzyme activity or stability. We may speculate that the ENG1 expressed in the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strain is extensively glycosylated at its N-glycosylation sites compared with the enzyme expressed in the FF18733/YEGAp-*eng1* yeast strain. To validate our suggestion, the extracellular proteins secreted by the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* strains were incubated with 500 U Endo H for 2, 6, and 12 h, and then the products of reaction were analyzed by SDS-PAGE. Interestingly, the prolonged treatment of the glycosylated ENG1 by Endo H did not lead to formation of a completely deglycosylated 37-kDa protein, but only to a relatively small decrease in molecular weight down to 45 kDa, estimated from the mobility of the protein in SDS-PAGE. These results indicate that the increased molecular weight of the deglycosylated recombinant *A. niger* ENG1 could be due to additional O-linked glycosylation of the enzyme in the FF18733/p*HO*-GAPDH-*eng1*-KanMX4-*HO* yeast host. Consequently, we propose that the observed differences in the glycosylation pattern of the *A. niger* ENG1 are due to a difference in the posttranslational modifications of the recombinant protein when it is expressed from the chromosomally integrated genes compared to the autonomously replicating and plasmid vectors.

Glycosylation is an important posttranslational modification of proteins that influences their stability, conformation, secretion, and biological activity (Zhang et al., 2012). O-linked glycosylation is important for enzyme stabilization (Chen et al., 2014). It is likely that the O-glycosylated form of ENG1 retains more stable activity during continuous cultivation of recombinant yeasts than the form that is only N-glycosylated.

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