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IMPROVED CELLULOLYTIC EFFICACY IN *PENICILLIUM DECUMBENS* VIA HETEROLOGOUS EXPRESSION OF *HYPOCREA JECORINA* ENDOGLUCANASE II

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Abstract - *Hypocrea jecorina* endoglucanase II (*Hjegl2*) was heterologously expressed in *Penicillium decumbens* (yielding strain *Pd*::*Hjegl2*). After induction in cellulose containing media, strain *Pd::Hjeg2* displayed increased carboxymethylcellulase activity (CMCase, 5.77 IU/ml, representing a 21% increase) and cellulose degradation determined with a filter paper assay (FPA, 0.40 IU/ml, 67% increase), as compared to the parent strain. In media supplemented with glucose (2%), *Pd::Hjegl2*, displayed 51.2-fold and 3-fold higher CMCase and FPA activities, respectively, as compared to the parent strain. No changes in the expression levels of the four main native cellulase genes of *P. decumbens* (*Pdegl1*, *Pdegl2*, *Pdcbh1*, and *Pdcbh2*) were noted between the transformant and wild-type strains. These data support the idea that *Hjegl2* cleaves both internal and terminal glycosidic residues, in a relatively random and processive manner. *In situ* polyacrylamide gel-activity staining of extracts derived from wild-type and *Pd::Hjegl2* revealed two additional active fractions in the latter strain; one with a molecular mass ~50-65 KDa and another ~80-116 kDa.

Key words: Penicillium decumbens, endoglucanase II, cellulose degradation, heterologous expression

INTRODUCTION

Lignocellulosic biomass represents an abundant, renewable, and exploitable global carbon resource. Cellulose can be efficiently degraded through the synergistic action of cellulolytic enzymes that contain three major groups based on their amino acid sequence and substrate specificity: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). Endoglucanases act randomly against the amorphous structure of cellulose chains producing both reducing and non-reducing ends, substrates for cellobiohydrolases that produce cellobiose. Cellulose chains are thus efficiently degraded to soluble cellobiose and cello-oligosaccharides by the endo-exo synergism of these two enzymes. In the last step of enzymatic cellulose degradation, cello-oligosaccharides are hydrolyzed to glucose by β-glucosidases (Lynd et al., 2002).

Among the cellulolytic bacteria and fungi studied, *Hypocrea jecorina* (previous *Trichoderma reesei*) represents one of the best-studied cellulase-producing organisms (Martinez et al., 2008). In *H. jecorina*, two main endoglucanases (*Hjegl1* and *Hjegl2*) have been characterized. *Hjegl2* appears to be the preponderant endoglucanase accounting for 5-10% of the total cellulase and >60% of the endoglucanase activity produced by *H. jecorina* (Medve et al., 1998a, Fujita et al., 2004). Lack of *Hjegl2* production reduces the endoglucanase activity in the culture supernatant by as much as 55% (Karlsson et al., 2002). Recently, *Hjegl2* in combination with other cellulolytic enzymes was used for saccharification and fermentation of cellulose to ethanol (du Plessis et al., 2010; Apiwatanapiwat et al., 2011).

Several *Penicillium* species have been reported in recent years with the ability to produce high cellulase and hemicellulase specific activities and are therefore being considered as valuable additional strains for industrial applications (Krogh et al., 2004; Jørgensen et al., 2005; Dutta et al., 2008; Singhvi et al., 2011; Dillon et al., 2011). *Penicillium decumbens* 114-2 isolated in our laboratory is a fast-growing filamentous fungus that secretes a complete extracellular cellulolytic enzyme system capable of breaking down cellulose to glucose (Sun et al., 2008). A modified *P. decumbens* mutant, resulting in a strain with 24-fold higher cellulase activity, has been used in industrial applications for cellulase production and biomass hydrolysis (Cheng et al., 2009, Liu, 2010). As in *H. jecorina*, *P. decumbens* also possesses two main endoglucanases (*Pdegl1* and *Pdegl2*). However, while the orthologous enzymes from these two fungi are similar in amino acid sequence, *Hjegl2* displays a 12-fold higher specific activity than *Pdegl2*, using amorphous cellulose as a substrate (Wei et al., 2010). We sought, therefore, to test the hypothesis that heterologous expression of *Hjeg2* in *P. decumbens* would be an effective approach to improve the cellulolytic ability of *P. decumbens*.

In this report, we show that heterologous expression of *Hjegl2* in *P decumbens* increases the cellulytic potential of *P decumbens* as examined using both carboxymethylcellulase (CMCase) and filter paper assays (FPA).

MATERIALS AND METHODS

Isolation and analysis of P. decumbens 5-FOA resistant (uracil auxotrophs) mutants

P. decumbens strain 114-2 was maintained on 10% wheat bran agar medium for sporulation (100 g wheat bran, boiled in 1 l distilled water for 30 min, and filtered through eight layers of gauze). Approximately $1x10^8$ conidia from *P. decumbens* 114-2 were spread on SDC agar (6.7 g l^{-1} yeast nitrogen base without amino acid [Difco laboratories, MI], 20 g l^{-1} glucose, and 21 mg l^{-1} Adenine, 85.6 mg l^{-1} L-Alanine, 85.6 mg l^{-1} L-Arginine HCl, 85.6 mg l^{-1} L-Asparagine, 85.6 mg l^{-1} L-Cysteine HCl, 85.6 mg l^{-1} L-Glutamine, 85.6 mg l^{-1} Glycine, 85.6 mg l^{-1} L-Histidine HCl, 85.6 mg l^{-1} myo-Inositol, 85.6 mg l^{-1} L-Isoleucine, 173.4 mg l^{-1} L-Leucine, 85.6 mg l^{-1} L-Lysine HCl, 85.6 mg l^{-1} L-Methionine, 8.6 mg l^{-1} para-Aminobenzoic Acid, 85.6 mg l^{-1} L-Phenylalanine 85.6 mg l^{-1} L-Proline, 85.6 mg l^{-1} L-Serine, 85.6 mg l^{-1} L-Threonine, 85.6 mg l^{-1} L-Tryptophan, 85.6 mg l^{-1} L-Tyrosine, 85.6 mg l^{-1} L-Valine) containing 5- fluoroortic acid (5-FOA, 500 mg l^{-1}) and uracil (500 mg l^{-1}). Plates were incubated for 5 days at 28°C. Colonies (putative mutants) growing on the SDC/FOA/uracil medium were picked and their phenotypes were further evaluated by growing them on SDC and SDC supplemented with uracil and/or 5-FOA. Colonies resistant to 5-FOA and requiring uracil for growth were considered as uracil auxotrophs.

Genomic DNA of one of the uracil auxotrophs (strain *P. decumbens* M12) was isolated from mycelia cultured in SDC for 2 days (30°C) as described (Garber and Yoder, 1983). The resultant genomic DNA was used as the template in PCR reactions for the cloning of OMP decarboxylase (*pyrG*). Two primers Pu-F and Pu-R (Table 1) were designed according to the *P. decumbens* 114-2 *pyrG* nucleotide sequence (GenBank GU574814). Reactions were performed using Tgradient PCR system (Biometra, Germany) under the following conditions: 1 cycle at 94°C (2 min), 30 cycles of 94°C (1 min), 60 °C (1 min), 72°C (1 min) and 1 cycle at 72°C (10 min). The *pryG* sequence determined from the mutant (*P. decumbens* M12) was sequenced and compared to that of the wild type parent strain (*P. decumbens* 114-2).

Construction of Hjegl2 expression vector and transformation into P. decumbens

Escherichia coli DH5α (*supE*44, *ΔlacU*169 *(φ80lacZΔM15), hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi-1, relA*1) was used as the host strain for the recombinant DNA manipulations. Plasmid ANIp8 harboring the *pyr*G gene (kind gift from Adrian Tsang, Concordia University, Canada) was used as the expression vector. Full-length *Hjegl2* derived from fun-

Table 1. Primers used in this work.

Primers	Sequences
Pu-F	5'-ATGTCTTCCAAATCCCAATTGACAT-3'
Pu-R	5'-TTACTGGCCTCCGACACGAGCAAGA-3'
*Peg-F	5'-GAAGTTCGGCTAGCGGCACG-3'
*Peg-R	5'-GTTGGCGAGCTAGCCTAGTGGTGGTGGTGGTGCTTTCTTG-3'
Pz-F	5'-GCTCATCAACTCCACCCA
Pz-R	5'-CCAACATAGCCAAGATAGACA
act-F	CTCCATCCAGGCCGTTCTG
act-R	CATGAGGTAGTCGGTCAAGTCAC
eg1-F	AACCTGGAAGAACGGCACC
eg1-R	CCTTGTCACAGTCATCGGAGC
eg2-F	AACGCCGACGCTTTCAA
eg2-R	CACGAGGTCCATCCAAGGTAA
cbh1-F	GTACTTGCGATCCTGATGGG
cbh1-R	CCACGGTGAAGGGAGACTTG
cbh2-F	CTCCACCTGCCCATCTTACAC
cbh2-R	CCCAGGCTTGTTGCTTAGTG

*: with an NheI restriction site is underlined and a six histidine tag is shown in bold.

gal cDNA was amplified by PCR using primers Peg-F and Peg-R (Table 1), designed to add a six amino acid His-tag to the carboxy terminus of the protein. The resultant product was digested with *Nhe*I and ligated into the *Nhe*I site of ANIp8 to yield ANIp8-Hjegl2, where the Pki promoter from *Aspergillus niger* present on ANIp8 would drive expression of the insert. The ANIp8-*Hjegl2* expression cassette and the empty vector (ANIp8, control) were transformed into the uracil-auxotrophic mutant of *P. decumbens* (M12) as described previously (Koukaki et al., 2003). Transformants were selected on SDC medium without uracil and were purified through single spore isolation and culturing for further analysis.

Genomic DNA of putative transformants was isolated and used as a template for PCR reactions using primer designed to the expression cassettes (Pz-F and Pz-R, Table 1) under the following reaction conditions: 1 cycle at 94°C (2 min), 30 cycles of 94°C (1 min), 56°C (1 min), 72°C (2 min) and 1 cycle at 72°C (10 min). The PCR products were purified by E.Z.N.A. Gel Extraction Kit (OMEGA, U.S.A.) and cloned into the pEASY-T1 cloning vector (TransGene Biotech, China). The integrity of all constructs was verified by sequencing.

Enzyme activity measurements

P. decumbens M12::ANIp8-*Hjegl2* (*Pd::Hjegl2*) and an empty vector transformant (*Pd::ANIp8*) were grown in modified Mandels' solution containing (per liter): 3 g KH_2PO_4, 2.6 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2$, 0.5 g urea, 7.5 mg $FeSO_4 \cdot 7H_2O$, 2.5 mg $MnSO_4 \cdot H_2O$, 3.6 mg $ZnSO_4 \cdot 7H_2O$, 3.7 mg $CoCl_2 \cdot 6H_2O$ and 1 g peptone, supplemented with either 2% w/v of glucose, OR 1% w/v of wheat bran + 1% w/v of microcrystalline cellulose. The pH of all media was adjusted to 5.5. Cultures were cultivated in 1 l baffled shaker-flasks containing 200 ml medium and were grown at 29°C, 180 rpm. Cultures were harvested after 1 day, and the cells removed by centrifugation (10000g, 10 min). Aliquots of supernatants were diluted to assay the enzyme activities. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of product per minute under the standard assay conditions. The reaction system was 0.2 mol l^{-1} acetate buffer (pH 4.8) with different substrates at the reaction temperature of 50°C. Endoglucanase activity (CMCase activity) was measured using 1% (w/v) sodium carboxymethyl cellulose for 30 min. Filter paper activity (FPase activity) was measured using Whatman No.1 filter

paper (1 × 6 cm, 50 mg) for 60 min; the amount of reducing sugar released from the enzyme reaction was estimated by the DNS method with glucose as the standard (Sun et al., 2008; Cheng et al., 2009)

Quantitative real time PCR

The expression levels of *Pd*egl1, *Pd*egl2, *Pd*cbh1, and *Pd*cbh2 were determined using quantitative real-time PCR. Mycelia derived from *Pd::Hjegl2* and *Pd::ANIp8* were harvested after 48 h growth in the modified Mandels' glucose media as described above. Total RNA was prepared from the mycelia by grinding under liquid nitrogen with a mortar and pestle, followed by extraction using the RNAiso™ reagent (TaKaRa, Japan) according to manufacturer's protocol. Total RNA was treated with DNase I (RNase free) in order to remove any contaminating genome DNA. First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa, Japan) using oligo-dT and random 6-mers as primers. PCR amplification was performed using SYBR® *Premix Ex Taq*™ Kit (TaKaRa) with primer sets for *actin* (accession no. EU855739), *Pdegl1* (accession no. EU339127), *Pdegl2* (accession no. EU315320), *Pdcbh1* (accession no. EF397602), and *Pdcbh2* (accession no. HQ286637) (Table 1). Gene expression copy numbers were calculated using standard curves constructed for each gene, and the data were then normalized to the expression levels of the *actin* gene. Data processing and statistical analyses were performed using Microsoft Excel. The average values of at least three determinations for each gene were considered as its expression level, with standard deviations below 10%.

SDS-PAGE with activity staining and Western blot

Proteins in the culture supernatant were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by activity staining. Carboxymethylcellulase (CMCase) activity was visualized in the polyacrylamide gels after renaturation as described (Medve et al., 1998b) with the following modifications. Proteins were electrophoretically separated on a 10% polyacrylamide gel containing 0.15% sodium carboxymethyl cellulose (CMC-Na). After electrophoresis, the gel was washed at room temperature in two changes of washing solution: 0.5 mol l^{-1} NaAc and 25% isopropanol, to remove the SDS. Proteins were renaturated in a 50 mmol l^{-1} acetate buffer (pH 5.0) containing 5 mmol l^{-1} β-mercaptoethanol by rocking the gel overnight at 4°C. The gel was then placed in 50 mmol l^{-1} NaAc-HAc buffer (pH 5.0) for 2 h and incubated at 50°C for another 2 h. For detection of activity, the gel was stained in 0.2% Congo Red for 1 h and destained in 1 mol l^{-1} NaCl. Clear bands on the red background indicated the CMC digestion.

For Western blot analyses, culture supernatants derived from *Pd::Hjegl2* and *Pd::ANIp8* were separated by SDS-PAGE and electroblotted onto PVDF membranes. Membranes were blocked for 1 h with 5% nonfat dry milk powder in Tris-buffered saline containing 0.05% Tween 20 and then immunoblotted with a mouse monoclonal His antibody (Beyotime, China) for 2 h. After washing the membranes six times with TBST, they were incubated with mouse peroxidase-conjugated secondary antibody for 1 h (Beyotime, China). Finally, the blots were washed and developed by enhanced chemiluminescent staining using ECL reagents (Amersham Buchler).

RESULTS

Isolation and selection of pyrG-auxotrophic mutants

In order to use the ANIp8 vector that carries the pryG selection marker, a *P. decumbens* uracil auxotroph was isolated. Approximately $1x10^8$ spores of *P. decumbens* were spread on SDC medium supplemented with uracil and 5-FOA as described in the Methods section. A total of 44 5-FOA resistant colonies were isolated and further evaluated via replica plating on SDC and SDC supplemented with uracil and/or 5-FOA. A collection of eighteen mutants displaying a consistent phenotype was further tested for the ability to maintain uracil auxotrophy in rich medium (wheat bran agar supplemented with glucose and uracil) over several generations. Of these, thirteen of the mutants reverted to uracil prototrophy, whereas the remaining five were unable to grow on

the minimal-medium plates containing either orotic acid or orotidine. These latter mutants represented a class of uracil auxotrophs likely due to deficiencies in either OMP decarboxylase (*pyrG*) and/or OPRTase. One such mutant designated as *P. decumbens* M12 was chosen for further study.

DNA analysis of uracil auxotroph P. decumbens-M12 and subsequent transformation of the M12

In order to verify the nature of the mutation in M12 that resulted in uracil auxotrophy, the nucleotide sequence the *pyrG* gene was isolated and sequenced. Comparison of the mutant (M12) sequence to the wild-type *P. decumbens pyrG* indicated the presence of a nonsense mutation, most likely as a consequence of a C-T transversion at nucleotide 676 that resulted in the conversion of the codon for glutamine 226 to a *stop codon*. These data supported the conclusion that *P. decumbens* M12 could be defined as an OMP decarboxylase deficient (*pyrG*$^-$) mutant.

P. decumbens strain M12 was then used as the host for transformation via complementation of the *pyrG* mutation by the appropriate selection marker on plasmid ANIp8. A construct bearing the *H. jecorina egl2* gene driven by an *A. nidulans* P*ki* promoter and containing a 6-His tag carboxy terminus fusion to the protein product (ANIp8-*Hjegl2*), was made and transformed into M12 as described in the Methods section, yielding strain *Pd::Hjegl2*. A control strain transformed with the empty vector, *Pd::ANIp8*, was also constructed. Transformants bearing the ANIp8-*Hjegl2* construct were verified by PCR using primers designed to *Hjegl2*. PCR products corresponding to the predicted (898 bp) fragment were obtained from *Pd::Hjegl2* strains and not from any wild-type parent or *Pd::ANIp8* strains. Sequencing of the PCR products obtained from the former cells confirmed its identity to *Hjegl2*.

Increased cellulase activity in strain Pd::Hjegl2

Two assays were used to measure overall cellulytic activity: (1) endoglucanase activity using carboxymethylcellulose as the substrate (CMCase), and (2) broad cellulase measurement using a filter paper activity assay (FPA). The CMCase and FPA of strains *Pd::Hjegl2* and *Pd::ANIp8* were measured after cultivation for 1 to 5 days. In culture media supplemented with glucose, the glucose was rapidly taken up by the cells (Fig 1a, open symbols). Under these growth conditions, little to no CMCase activity was observed in *Pd::ANIp8*, whereas 0.4-0.5 IU/ml of CMCase activity was produced by *Pd::Hjegl2* on day 1: this increased two fold (representing a >50-fold higher level of CMCase activity than the control) and remained constant for the rest of the time period examined (up to five days, Fig. 1a, closed symbols). These data confirmed the catabolite repressive effect of glucose on CMCase activity and the constitutive nature of CMCase activity in the *Hjegl2* transformant. FPA activity was also higher in *Pd::Hjegl2* than in *Pd::ANIp8*, reaching a maximum difference of ~ 3-fold on day 3 (Fig. 1 b).

In culture media supplemented with cellulose, CMCase activity increased to ~4 and 5.5 IU/ml for *Pd::ANIp8* and *Pd::Hjegl2*, respectively, representing a 21% increase for *Pd::Hjegl2* as compared to the control strain (Fig. 2a). Maximum FPA (0.40 IU/ml) was seen for *Pd::Hjegl2* on day 3, representing a 67% increase in activity as compared to the control strain, *Pd::ANIp8* (Fig. 2 b). In all cases, both CMCase and FPA were elevated in *Pd::Hjegl2* as compared to the control strain when grown in either glucose or cellulose containing media.

The expression level of four native cellulase genes (Pdegl1, Pdegl2, Pdcbh1, and Pdcbh2) of in Pd::Hjegl2.

Real-time quantitative PCR was used to examine the expression patterns of the four major *P. decumbens* cellulose genes, *Pdegl1, Pdegl2, Pdcbh1,* and *Pdcbh2*, in strains *Pd::Hjegl2* and *Pd::ANIp8*. Transcript levels of the four genes were quantified in cells grown in glucose or cellulose containing media for 24 and 48 h (Fig. 3). A small increase in *Pdegl2* transcript levels was seen after growth in glucose for 48 h as compared to the 24 h time-point, but a dramatic increase was seen (>100-fold) when the cells were grown on

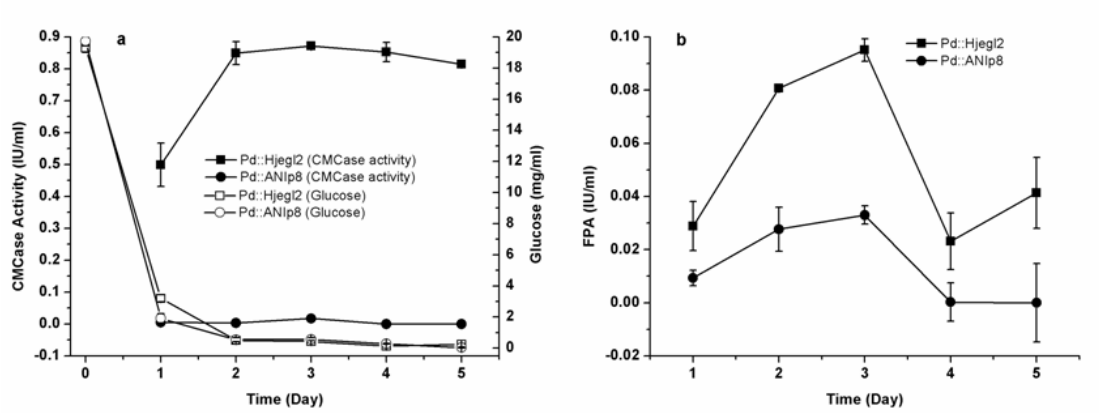

Fig. 1. Glucose consumption and endoglucanase activity (a) and filter paper activity (b) of *Pd::Hjegl2* and *Pd::ANIp8* cultured in media containing glucose as the main carbon source.

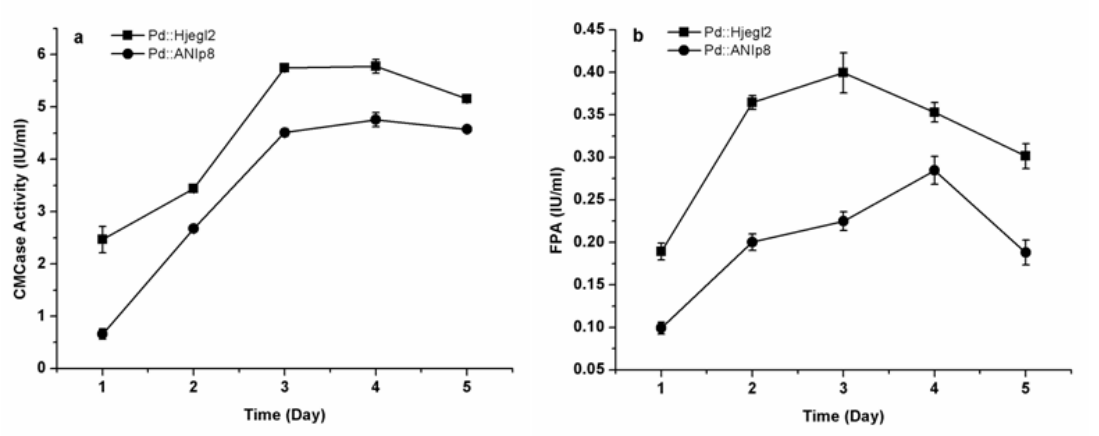

Fig. 2. Endoglucanase activity (a) and filter paper activity (b) of *Pd::Hjegl2* and *Pd::ANIp8* cultured in media containing microcrystalline cellulose as the main carbon source.

cellulose. No significant differences, however, were observed between strains *Pd::Hjegl2* and *Pd::ANIp8* in transcript levels of *Pdegl2* or any of the other genes (*Pdegl1*, *Pdchb1*, and *Pdchb2*) in cells grown on either glucose or cellulose for either 24 or 48 h. *Pdegl2* levels (in both *Pd::Hjegl2* and *Pd::ANIp8*) increased ~10-fold after 48 h of growth in glucose and was approximately another 10-fold higher in cells grown on cellulose for 24 h, remaining level after 48 h. *Pdchb1*. *Pdchb2* levels also increased ~10-fold from 24 to 48 h of growth in glucose, but the transcript levels of these two genes was more than 1,000-fold higher when grown on cellulose (whether at 24 or 48 h) as compared to the glucose 48 h levels.

The molecular mass and in situ activity staining of P. decumbens expressed Hjegl2

Cell-free culture supernatants derived from *Pd::Hjegl2* and *Pd::ANIp8* grown in either wheat-bran microcrystalline cellulose or glucose-containing media were analyzed by SDS-PAGE, followed by in-gel renaturation of the proteins and *in situ* CMCase activity staining as described in the Methods section. At least five bands of CMCase activity, of approximate molecular weights of 25, 31, 35, 45, and 60 kDa, with the latter two displaying the largest zones of clearing of the substrate, could be detected in the control strain when grown in the cellulose-containing media

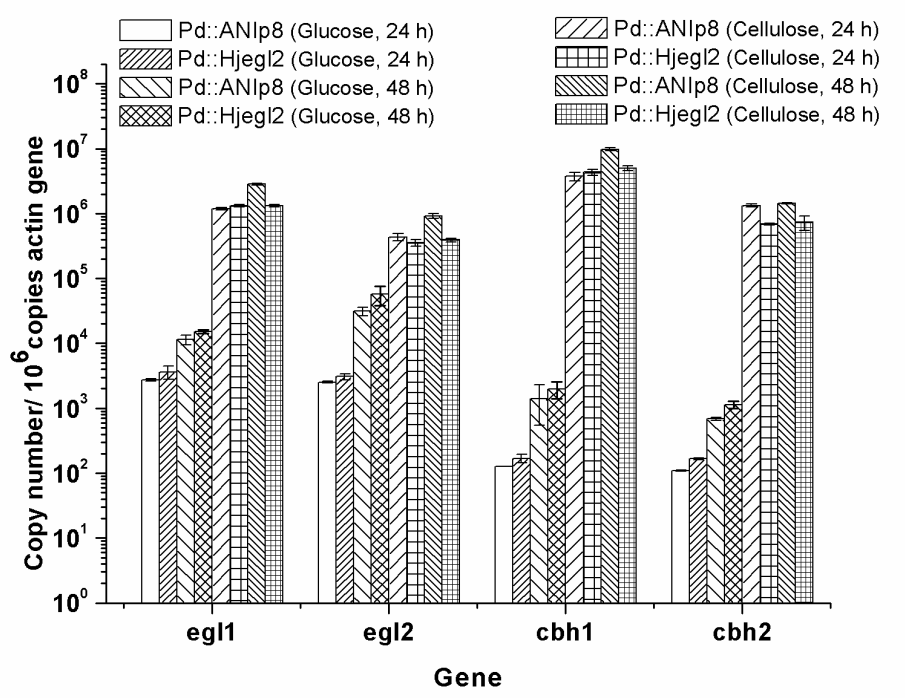

Fig. 3. Gene expression of the four native *P. decumbens* cellulases genes (*Pdegl1*, *Pdegl2*, *Pdcbh1*, and *Pdcbh2*) in strains *Pd::Hjegl2* and *Pd::ANIp8* cultured in media containing glucose or wheat bran-microcrystalline cellulose as carbon sources, respectively. Expression of the actin gene was analyzed as a control. Copy number was normalized using actin expression for the same sample.

(*Pd::ANIp8*, Fig. 4, lane 1). These same bands could be seen in the Pd::Hjegl2 strain as well as two additional somewhat diffuse bands between ~50-65 and ~80-116 kDa (Fig. 4, lane 2). The two diffuse sets of CMCase activity bands were evident in the culture extracts derived from *Pd::Hjegl2* grown in glucose, whereas no CMCase activity could be seen in extracts from *Pd::ANIp8* grown under identical conditions (Fig. 4, lanes 3 and 4). Western blots probed with an anti-His tag monoclonal antibody revealed the presence of two proteins in strain *Pd::Hjegl2*, one with a molecular mass of approximately 22 kDa and another ~60-65 kDa (Fig. 4, lane 5).

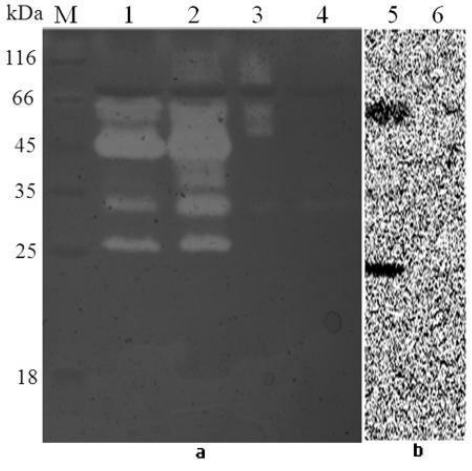

Fig. 4. SDS-PAGE followed by *in situ* CMCase activity staining (a) and Western-blot (b) analysis of endoglucanase expression in strain *Pd::Hjegl2* and *Pd::ANIp8*. Lanes 1 and 2, *Pd::ANIp8* and *Pd::Hjegl2* cultured in wheat bran-microcrystalline cellulose media for 3 days, respectively; Lanes 3 and 4, *Pd::Hjegl2* and *Pd::ANIp8* cultured in glucose containing media for 3 days, respectively; Lanes 5 and 6, Western-blot analysis of *Pd::Hjegl2* and *Pd::ANIp8*, respectively, probed with an anti-His monoclonal antibody.

DISCUSSION

Improvements in the catalytic efficiency of cellulose degradation by microbes can have tremendous impact in increasing the potential of industrial applications. Our data show that cellulose degradation, as determined by both CMCase activity and FPA of

the host strain *P. decumbens*, could be improved by heterologous expression of endoglucanase II from *H. jecorina*. Expression of *Hjegl2* in *P. decumbens* was constitutive. The transformed strain, *Pd::Hjegl2*, showed significant CMCase and FPA activity levels, even in glucose-containing catabolite-repressed culture conditions, an important industrial problem leading to the inhibition of the synthesis of native cellulases (Calza 1991; Alriksson et al., 2009). Expression analysis of the four main cellulose genes of *P. decumbens* revealed no changes in their transcript levels between the wild-type and *Hjegl2*-transformed strains, supporting the conclusion that the observed increase in cellulytic activity was due to the heterologous expression of the *Hjegl2* gene. The expression studies did, however, confirm the inductive nature of the presence of cellulose on the transcript levels of the genes that comprise the endogenous cellulytic pathway of *P. decumbens*.

As expected, expression of *Hjegl2* in *P. decumbens* led to increased CMCase activity. However, since the filter paper assay is an indication of what can be considered total cellulase activity in the sense that it requires the functions of three kinds of enzyme activities (endoglucanases, cellobiohydrolases and β-glucosidases), the question arises as to why the expression of *Hjegl2* would lead to an increase in this activity. There are several possible explanations for this result. First, it is conceivable that the endoglucanases activity is the rate-limiting step, thus an increase in this enzyme and its products would lead to an increase in apparent total cellulose activity. Alternatively, according to the most widely accepted hypothesis, a strict endoglucanase cleaves the cellulose substrate internally, thus disrupting the crystalline structure of the cellulose and generating new free ends (Beguin and Aubert, 1994). However, although endoglucanase II has been labeled as an endo-glucanase, its enzymatic reactivity could be broader, producing glucose, cellobiose, and/or cellotriose during its hydrolysis of cellulose. The production of cellobiose in particular would be important since this is not an expected product of a typical endoglucanase (Nakazawa et al., 2008; Qin et al., 2008a). Indeed, it has been hypothesized that *H. jecorina* endoglucanase II may be partially processive due to its extended substrate-binding site and the presence of a cellulose-binding domain (Medve et al., 1998a, Qin et al., 2008b). Similarly, in some organisms, such as *Gloeophyllum trabeum*, *Fomitopsis pinicola*, and *Clostridium thermocellum*, processive endoglucanases are produced that cleave cellulose internally but also release soluble oligosaccharides before detaching from the polysaccharide substrate (Cohen et al., 2005; Zverlov et al., 2005; Yoon et al., 2008).

In situ activity staining of the heterologous-expressed *Hjegl2* in *P. decumbens* revealed the presence of two rather diffuse activity bands (between ~50-65 and ~80-116 kDa), not found in the control strain. These were especially evident in extracts derived from cells grown in glucose-containing media. Unexpectedly however, probing of the extract in Western blots using an anti-His tag antibody revealed the presence of two bands not found in controls corresponding to 25 and 60-65 kDa, respectively. Although the latter His-antibody identified band matches the corresponding activity bands, it is unclear why the higher molecular weight activity bands (80-116 kDa) did not react with the antibody, or what the 25 kDa His-antibody reactive band may correspond to. The possibility exists that the lower band may correspond to a proteolytic cleavage product of *Hjegl2*, and the upper bands failed to react with the antibody due to steric hindrance derived from a post-translational modification (e.g. glycosylation or phosphorylation). Further experiments examining these possibilities may shed light on this issue, and precedent for such modification occurring exists. Homologous expression of the *H. jecorina* Cel7B (endoglucanase I) catalytic module revealed the production of several isoforms of the protein in the recombinant strain, and that this heterogeneity was considerably reduced after enzymatic N-deglycosylation (Eriksson et al., 2004; Hui et al., 2002). Besides N-glycan heterogeneity, O-linked glycosylation and/or protein oligomerization have also been shown to result in protein production heterogeneity. The linker region of CBH I from *H. jecorina* was shown to be extensively glycosylated with di-, and tri-saccharides at Thr and Ser residues (Hui et al., 2001), and furthermore, a band around 20

kDa (apparently inactive) was also observed in this case via the Western blot analysis; the authors suggest that this band may be due to proteolytic cleavage of the heterologous product.

In conclusion, *Hjegl2* was expressed in *P. decumbens*, increasing its cellulose degrading potential. Expression of the enzyme was constitutive and two diffuse bands of activity could be visualized. Western blot analysis matched one of the activity band regions with the predicted product, although the other active band did not appear to react with the antibody, and an additional smaller molecular-weight (inactive) band anti-His antibody reactive band was visualized. Future work aimed at further maximizing the cellulytic potential of *P. decumbens* is underway.

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REFERENCES

Alriksson, B. Rose, S.H., van Zyl, W.H., Sjöde, A., Nilvebrant, N.-O. and *L.J. Jönsson* (2009) Cellulase production from spent lignocellulose hydrolysates by recombinant *Aspergillus niger*. *Appl Environ Microbiol*. **75**, 2366-2374.

Apiwatanapiwat W, Murata Y, Kosugi A, Yamada R, Kondo A, Arai T, Rugthaworn P, and *Y. Mori* (2011) Direct ethanol production from cassava pulp using a surface-engineered yeast strain co-displaying two amylases, two cellulases, and β-glucosidase. *Appl Microbiol Biotechnol*. **90**, 377-384.

Beguin, P. and *J. P. Aubert* (1994) The biological degradation of cellulose. *FEMS Microbiol Rev.* **13**, 25-58.

Calza, R.E. (1991) Carbon source, cyclic nucleotide, and protein inhibitor effects on protein and cellulase secretions in *Neocallimastix frontalis* EB188. *Curr Microbiol*. **22**, 213-219.

Cheng, Y.F., Song, X., Qin, Y.Q. and *Y.B. Qu* (2009) Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. *J Appl Microbiol*. **107**, 1837-1846.

Cohen, R., Suzuki, M.R. and *K.E. Hammel* (2005) Processive endoglucanase active in crystalline cellulose hydrolysis by the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol*. **71**, 2412-2417.

Dillon, A.J., Bettio, M., Pozzan, F.G., Andrighetti, T., and *M. Camassola* (2011) A new *Penicillium echinulatum* strain with faster cellulase secretion obtained using hydrogen peroxide mutagenesis and screening with 2-deoxyglucose. *J Appl Microbiol.* **111**, 48-53.

du Plessis, L., Rose, S.H., and *W.H. van Zyl (*2010) Exploring improved endoglucanase expression in Saccharomyces cerevisiae strains. *Appl Microbiol Biotechnol*. **86**, 1503-1511.

Dutta, T., Sahoo, R., Sengupta, R., Ray, S.S., Bhattacharjee, A. and *S. Ghosh* (2008) Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. *J Ind Microbiol Biotechnol*. **35**, 275-282

Eriksson, T., Stals, I., Collén, A., Tjerneld, F., Claeyssens, M., Stålbrand, H. and *H. Brumer* (2004) Heterogeneity of homologously expressed *Hypocrea jecorina* (*Trichoderma reesei*) Cel7B catalytic module. *Eur J Biochem*. **271**, 1266-1276.

Fujita, Y., Ito, J., Ueda, M., Fukuda, H., and *A. Kondo* (2004) Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. *Appl Environ Microbiol.* **70**, 1207-1212.

Garber, R.C. and *O.C. Yoder* (1983) Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. *Anal Biochem*. **135**, 416-422.

Hui, J.P.M., Lanthier, P., White, T.C., McHugh, S.G.,Yaguchi, M., Roy, R. and *P. Thibault* (2001) Characterization of cellobiohydrolase I (Cel7A) glycoforms from extracts of *Trichoderma reesei* using capillary isoelectric focusing and electrospray mass spectrometry. *J Chromatogr B*. **752**, 349–368.

Hui, J.P., White, T.C. and *P. Thibault* (2002) Identification of glycan structure and glycosylation sites in cellobiohydrolase II and endoglucanases I and II from *Trichoderma reesei*. *Glycobiology* **12**, 837-849.

Jørgensen, H., Kristian, A., Krogh, B.R. and *L. Olsson* (2005) Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis. *Enzyme Microb Technol.* **36**, 42-48.

Karlsson, J., Siika-aho, M., Tenkanen, M. and *F. Tjerneld* (2002) Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. *J Biotechnol*. **99**, 63-78.

Koukaki, M., Giannoutsou, E., Karagouni, A. and *G. Diallinas* (2003) A novel improved method for *Aspergillus nidulans* transformation. *J Microbiol Methods* **55**, 687-695.

Krogh, K.B.R., Mørkeberg, A., Jørgensen, H., Frisvad, J.C. and *L. Olsson* (2004) Screening genus *Penicillium* for producers of cellulolytic and xylanolytic *enzymes. Appl Biochem Biotechnol.* 113-116, 389-401.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qu, Y., and *L. Xiao* (2010) High concentration ethanol production from corncob residues by fed-batch strategy. *Bioresour Technol.* **101**, 4952-4958.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., and *I.S. Pretorius* (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev*. **66**, 506-577.

Martinez D, Berka RM, Henrissat B, et al., (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol*. **26**, 553-560.

Medve, J., Karlsson, J., Lee, D. and *F. Tjerneld* (1998a) Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol Bioeng.* **59**, 621-634.

Medve, J., Lee, D. and *F. Tjerneld* (1998b). Ion-exchange chromatographic purification and quantitative analysis of *Trichoderma reesei* cellulase cellobiohydrolase I, II and endoglucanase by fast protein liquid chromatography. *J Chromatogr A*. **808**, 153-156.

Nakashima, A., Yoshida, M., Nakayama, K., Kato-Furuno, A., Ueno, M., Ushimaru, T., and *M. Uritani* (2002) Genes for a nuclease and a protease are involved in the drastic decrease in cellular RNA amount in fission yeast cells during nitrogen starvation. *J Biochem.* **131**, 391-398.

Nakazawa, H., Okada, K., Kobayashi, R., Kubota, T., Onodera, T., Ochiai, N., Omata, N., Ogasawara, W., Okada, H., and *Y. Morikawa* (2008) Characterization of the catalytic domains of *Trichoderma reesei* endoglucanase I, II, and III, expressed in Escherichia coli. *Appl Microbiol Biotechnol*. **81**, 681-689.

Qin, Y.Q., Wei, X.M., Liu, X.M., Wang T.H. and *Y.B. Qu* (2008a) Purification and characterization of recombinant endoglucanase of *Trichoderma reesei* expressed in *Saccharomyces cerevisiae* with higher glycosylation and stability. *Protein Expr Purif*. **58**, 162-167.

Qin, Y.Q., Wei, X.M., Song, X. and *Y.B. Qu* (2008b) Engineering of endoglucanase II from *Trichoderma reesei* towards improved catalytic efficiency with higher pH optimum. *J. Biotechnol*. **135**, 190-195.

Qu, Y.B., Zhao, X., Gao, P.J. and *Z.N. Wang* (1991) Cellulase production from spent sulfite liquor and paper-mill waste fiber. *Appl Biochem Biotechnol*. 28-29, 363-368.

Singhvi, M.S., Adsul, M.G., and *D.V. Gokhale* (2011) Comparative production of cellulases by mutants of *Penicillium janthinellum* NCIM 1171 and its application in hydrolysis of Avicel and cellulose. *Bioresour Technol*. **102**, 6569-6572.

Sun, X.Y., Liu, Z.Y., Zheng, K., Song, X., and *Y.B. Qu* (2008) The composition of basal and induced cellulase systems in *Penicillium decumbens* under induction or repression conditions. *Enzyme Microb Technol*. **42**, 560-567

Wei, X.M., Qin, Y.Q. and *Y.B. Qu* (2010) Molecular cloning and characterization of two major endoglucanases from *Penicillium decumbens*. *J Microbiol Biotechnol.* **20**, 265-270.

Yoon. J.J., Cha, C.J., Kim, Y.S. and *W. Kim* (2008) Degradation of cellulose by the major endoglucanase produced from the brown-rot fungus Fomitopsis pinicola. *Biotech Lett.* **30**, 1373-1378.

Zverlov, V.V., Schantz, N. and *W.H. Schwarz* (2005) A major new component in the cellulosome of *Clostridium thermocellum* is a processive endo-β-1, 4-glucanase producing cellotetraose. *FEMS Microbiol Lett*. **249**, 353-358.