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Full Length Article

Screening and Identification of Anti-Glucose Repression Proteins in Cellulase Production Fungus *Trichoderma orientalis* EU7-22

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Abstract

The Cys_2His_2-type transcription factor *CreI*/*CreA* mediates carbon catabolite repression by binding to the promoter region of target genes to inhibit protein expression. The aim of this study was to identify anti-glucose repression proteins from cellulase producing fungus *Trichoderma orientalis* having the potential to influence the promoter-mediated expression of industrially useful proteins in a glucose-rich medium. Among the four cellulose inducers, wheat bran, pretreated *Miscanthus* cellulose, Avicel, and sodium carboxymethyl cellulose, pretreated *Miscanthus* cellulose was the most effective inducer for cellulase production, based on the results of the cellulase activity and the secreted proteins concentration. Conversely, glucose mixed with pretreated *Miscanthus* cellulose obviously repressed cellulase production in *T. orientalis*; the higher the concentration of glucose, the lower the cellulase activity and the concentration of secreted proteins. The cellulase activity and secreted protein content was reduced by 96.9 and 71.95%, respectively, with 3% glucose as inhibitor compared to when glucose was absent. Moreover, SDS-PAGE analysis revealed that certain proteins were stably secreted, indicating anti-glucose repression. These proteins were identified by MALDI-TOF. One was an aspartic protease while two were predicted proteins. The gene encoding the aspartic protease was cloned by PCR and analyzed. This showed an efficient method to obtain the target protein.

Keywords: Anti-glucose repression protein; Aspartic protease; Cellulase; *Trichoderma orientalis*

Introduction

The depletion of fossil fuels is an impending problem for the world economy, and the combustion of fossil fuels has been recognized as the major cause of global warming. To reduce fossil fuel dependence, scientists have been exploring alternative energy sources. Cellulosic biomass has been considered as an attractive resource for fuels, chemicals, polymers, and materials because it is renewable, abundant, and does not compete with food crops for land (Cherubini 2010; Luo *et al*. 2010). Cellulolytic enzymes are important for converting cellulosic biomass to sugars, which can be further converted to other bioproducts (Banerjee *et al*. 2010; Silva *et al*. 2015). Filamentous fungi like *Trichoderma*, *Penicillium*, and *Aspergillus* are commonly used in the fermentation industry for large-scale production of industrial cellulase enzymes that can be used in hydrolyzing biomass for fermentable sugars, which are subsequently converted to bio-fuels like ethanol (Banerjee *et al*. 2010; Luo *et al*. 2010; Silva *et al*. 2015). These fungi have been developed as hosts for the production of both homologous and heterologous gene products (Nevalainen *et al*. 2005; Liu *et al*. 2008; Lubertozzi and Keasling 2009; Sukumaran *et al*. 2009). When fungi produced abundant secreted protein in culture, the production is regulated by the Cre I/CreA carbon catabolite repressor that mainly controls carbon assimilation (Portnoy *et al*. 2011). Cre I/CreA binds to the promoters of the target genes *via* the consensus motif 5′-SYGGRG-3′, whose function *in vivo* has been shown in both *Trichoderma reesei* (Takashima *et al*. 1996; Portnoy *et al*. 2011) and *Aspergillus nidulans* (Cubero and Scazzocchio 1994).

Strong promoters like the *cbh1* promoter from *T. reesei* is used to construct high-efficiency expression vectors and drive protein expression (Nyyssönen and Keränen 1995; Liu *et al*. 2008). However, the *cbh1* promoter is repressed by glucose and negatively regulated by Cre I/CreA (Takashima *et al*. 1996; Kubicek *et al*. 2009). Nakari-Setala and Penttila (1995) made use of two promoters (one from translation elongation factor 1a, *tef1*, and the other from an unidentified gene for cDNA1) to efficiently express homologous cellobiohydrolase I and the catalytic core domain of endoglucanase I in *T. reesei*

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in a glucose medium. Bando *et al*. (2011) found a novel promoter from a hemolysin-like gene, *hlyA*, which was characterized for protein over-expression in *A. oryzae* grown in solid-state culture. Harnpicharnchai *et al*. (2014) reported that the *GAP* (glyceraldehyde-3-phosphate dehydrogenase) promoter from the thermotolerant yeast strain *Pichia thermomethanolica* BCC16875 was shown to be able to function at higher temperatures up to 42°C and could be useful for large-scale protein production in glucose or other carbon sources medium.

Glucose is a cheap material for industrial-scale fermentation to manufacture useful products. The development of recombinant DNA technology has allowed the large-scale production of medically and industrially useful proteins, because promoter sequences can regulate the expression of target proteins. Unlike inducible promoters, which are repressed by glucose, most constitutive promoters are active in a glucose-rich medium. Therefore, although promoters have the potential to efficiently drive protein expression, identification of candidate proteins is a difficult and serious problem. Han *et al*. (2010) showed that proteomic approaches are valuable tools for discovering such proteins.

Previously we reported the promoter *proA* drove the over-expression of *Bgl1* gene in *Hypocrea orientalis* (*T. orientalis*), as evidenced by 22.2% enhancement of filter paper activity and 700% enhancement of β-glucosidase activity under 2% glucose repression condition, compared to the wild type strain (Long *et al*. 2013).

In this study, it was reported that the cellulase producion in *T. orientalis* EU7-22, was efficiently induced by pretreated *Miscanthus* cellulose and was strongly repressed by glucose. And there was an interesting finding that four specific proteins were stably secreted by SDS-PAGE analysis in the glucose rich medium. Thus we described this effective method to screen and identify these anti-glucose repression proteins by MALDI-TOF mass spectrometry.

Material and Methods

Fungal strains and culture conditions

The cellulase producing strain *T. orientalis* EU7-22 (originally named *Aspergillus glaucus* EU7-22) was created by mutating *T. orientalis* XC-9 (originally named *A. glaucus* XC-9), which was preserved in our laboratory (Xu *et al*. 2006). It was maintained on potato dextrose agar (PDA) slants at 4°C, and was transferred to a new agar slants every two weeks.

Cellulase production

Experiments were conducted in 250 mL Erlenmeyer flasks. The volume of fermentation medium (pH 5.2) was 50 mL. The medium contained cellulose inducer substrate (2%, w/v), wheat bran (1%, w/v), 0.5% peptone, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 0.4% Tween-80, and 0.25% KH_2PO_4.

Cellulose inducers including pretreated *Miscanthus* (*Erianthus arundinaceus*; soaked in NaOH (2%, w/v) and H_2O_2 (1%, v/v) for 2 h at 60°C, washed free of the inducers, dried, and milled to 40 mesh powder), Avicel (PH101) and sodium carboxymethyl cellulose (CMC-Na, 300–800 mPa.s; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were investigated for cellulase production. Medium containing only wheat bran was used as the control.

Different concentrations of glucose (0, 0.4, 1.2, 2.5 and 3.0%) as a repressor were supplemented with pretreated *Miscanthus* cellulose.

The fungi spores (1×10^6 mL^{-1}) were inoculated in the potato dextrose liquid medium, and were incubated for 34 h (180 rpm, 30°C). Then it transferred to cellulase production medium with a 10% inoculum for 4d (180 rpm, 30°C).

Cellulase activity assay and SDS-PAGE analysis

The filter paper activity (FPA) was measured as described by Ghose (1987) and Long *et al.* (2013). The protein concentration was determined with a Bradford protein assay kit (Sangon Biotech Co. Ltd., Shanghai, China). The secreted proteins were further analyzed by SDS-PAGE on a 13% polyacrylamide gel (Long *et al*. 2013).

Protein identification

SDS-PAGE revealed that some proteins were stably secreted under repression conditions (Fig. 4). Samples from the four bands were manually excised and digested with trypsin using the method of Shevchenko *et al*. (1996). Extracted peptides were identified on an Ultra flex I MALDI-TOF/TOF device using flex Control 3.0 for data collection and flex Analysis 3.0 for spectral analysis/peak list generation (Bruker Daltonics, Billerica, M.A.). Peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) spectra were submitted to the MASCOT server (www.matrixscience.com) for an NCBI database search limited to the taxon Fungi. With respect to the sample preparation, fixed modification of cysteine thiols to S-carbamidomethyl derivatives and variable methionine oxidation were defined for the database search. Furthermore, MS tolerance of 100 ppm, MS/MS tolerance of 0.6 Da, and up to one missed cleavage was allowed. Results were regarded as significant with an allowed likelihood for a random hit of $P \leq 0.05$, according to the MASCOT score.

Genomic DNA isolation

Genomic DNA was extracted from all available mycelia according to the method of Penttilä *et al*. (1987).

Gene cloning and analysis

Primers sample I-F (ATGCAGACCTTTGGAGCTTTTC) and sample I-R (TTATTTCTGAGCCCAGCCCAG), sample

III-F (ATGGATGCTATCCGAGCCAGGAGTGCTG) and sample III-R (CTATTCATACTCAACAGTCACAGTGCTGCCA), sample IV -F (ATGCAACTGTCCAACCTCTTC) and sample IV -R (TTAGAGACCGCAGTTGCTGAC) were designed to amplify the target gene by PCR according to the corresponding nucleotide sequences of the identified proteins. The PCR products were first electrophoretically resolved and cloned into the pMD19-T vector (TaKaRa, Shiga, Japan), then transformed into *Escherichia coli* DH5α competent cells. The white colonies were picked, characterized by PCR, and sequenced by BGI (Beijing, China).

The nucleic acid and deduced amino acid sequences were analyzed by an NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST/). The proteins were analyzed by ProtParam (http://web.expasy.org/protparam/).

Result

Effects of cellulose inducer on cellulase production and secreted extracellular proteins

The effect of different cellulose (2%) inducers (wheat bran, pretreated *Miscanthus* cellulose, Avicel, and CMC-Na) on cellulase production was investigated. FPA activity was summarized in Fig. 1. Wheat bran and CMC-Na were poor inducers of cellulase production. *Miscanthus* and Avicel were the strong inducers for cellulase production. The activity of FPA induced by *Miscanthus* and Avicel were at an equal level up to 0.86 IU mL^{-1}, respectively.

Secreted extracellular proteins analyzed by SDS-PAGE from the control without cellulose and CMC-Na were limited, with only one clear band at about 33 kDa (lanes 1–3 and 10–12, respectively; Fig. 2). The protein concentrations of these samples were 0.24 mg mL^{-1} and 0.35 mg mL^{-1}, respectively. Abundant secreted extracellular protein was obtained from the pretreated *Miscanthus* cellulose and Avicel samples, with bands distributed between 14 ~ 116 kDa (lanes 4–6 and 7–9; Fig. 2), with protein concentrations of 0.78 mg mL^{-1} and 0.71 mg mL^{-1}, respectively.

Effects of glucose plus *Miscanthus* on cellulase production and secreted extracellular proteins

The effects of the different concentrations of glucose mixed with 2% pretreated *Miscanthus* cellulose on cellulase production were investigated. As expected, as the glucose concentration increased, the FPA activity was markedly repressed. When glucose concentration exceeded 2.5%, *T. orientalis* EU7-22 yielded almost no cellulase. The FPA activity was reduced by 96.9% for 3% glucose (Fig. 3).

SDS-PAGE analysis of secreted extracellular proteins at different glucose concentrations was shown in Fig. 4. The color of the interzone protein bands declined as glucose concentration increased (lanes 1–10). The protein concentration decreased correspondingly. At glucose

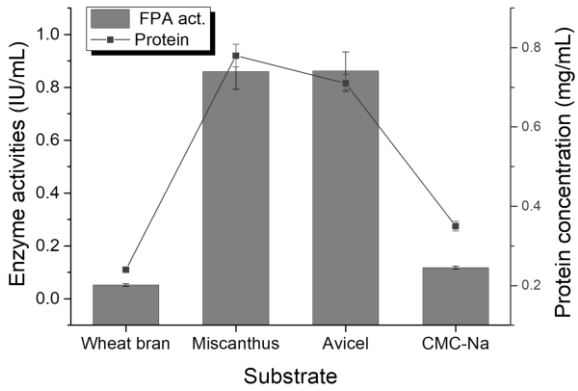

Fig. 1: Effects of various cellulose substrates on cellulase production

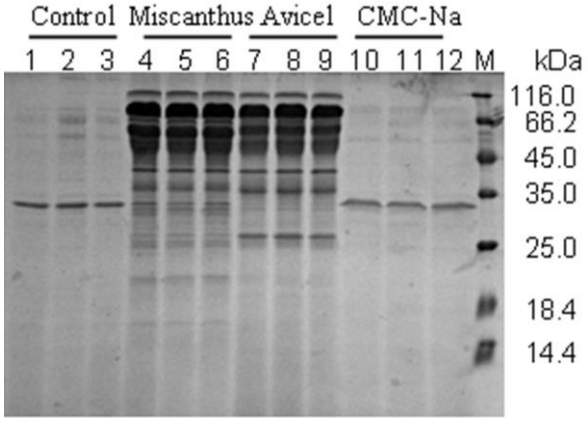

Fig. 2: SDS-PAGE analysis of secreted extracellular protein from *T. orientalis* EU7-22 on various cellulose substrates. Lanes 1–3 contain control without cellulose, lanes 4–6 contain 2% pretreated *Miscanthus*, lanes 7–9 contain 2% Avicel, and lanes 10–12 contain 2% CMC-Na

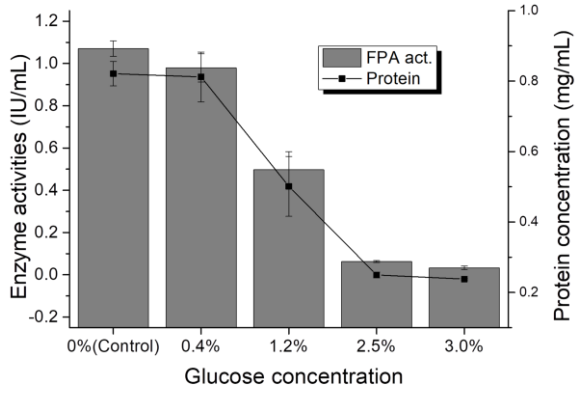

Fig. 3: Effects of glucose concentration plus *Miscanthus* cellulose on cellulase production and secreted protein

concentrations of 0, 0.4, 1.2, 2.5 and 3.0%, the total protein produced was 0.82 mg mL^{-1}, 0.80 mg mL^{-1}, 0.50 mg mL^{-1}, 0.25 mg mL^{-1}, and 0.23 mg mL^{-1}, respectively (Fig. 3). However, four bands were an exception (Fig. 4, the arrows denote I, II, III, IV at about 42, 26, 25 and 16 kDa,

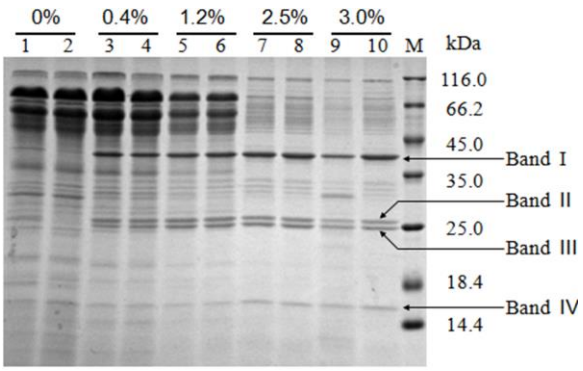

Fig. 4: SDS-PAGE analysis of secreted extracellular protein from *T. orientalis* EU7-22 on different glucose concentrations mixed with 2% cellulose. (Arrows I–IV indicates bands that needed to identify. lanes 1–2, 0 % glucose as control; lanes 3–4, 0.4 % glucose; lanes 5–6, 1.2 % glucose; lanes 7–8, 2.5 % glucose; lanes 9–10, 3.0 % glucose)

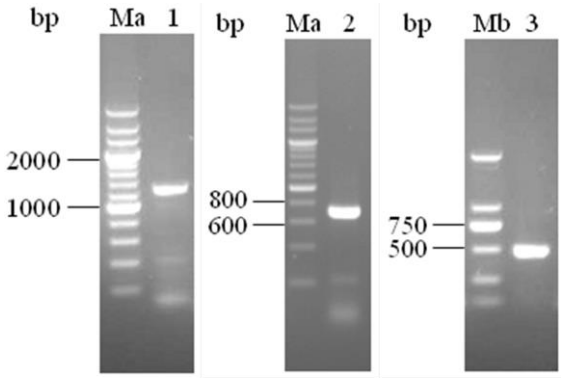

Fig. 5: Gel electrophoresis of gene fragment amplified by PCR. (Ma: 200 bp DNA Marker: Mb: DL2000 DNA Marker: 1: gene fragment for Band I:2: gene fragment for Band III: 3: gene fragment for Band IV)

respectively). Their color did not decrease but remained stable indicating that the proteins were immune to glucose repression. These proteins were considered suitable candidates for analysis, especially the protein from band I, because the amount of this protein secreted was much greater than that for proteins represented by bands II, III and IV.

Identification of proteins by MALDI-TOF mass spectrometry

The aforementioned four bands of the gel (I, II, III, and IV) were excised and digested with trypsin. The resulting peptides were extracted, analyzed by MALDI-TOF, and identified by a MASCOT search. The results are shown in Table 1 and supplementary S1. Bands I, III, and IV were identified, with no result for band II.

Band I was identified as an aspartic protease, which showed 25% coverage from *Hypocrea jecorina*. The protein contained 407 amino acids (GenBank accession No. CAJ44684.1) and the corresponding gene, *proA* (GenBank accession No. AM168137.1), had 1302 bp (from NCBInr, gi|83318620). Sample III was identified as a predicted protein, which showed only 3% coverage from *T. reesei* QM6a. The putative protein from *T. reesei* QM6a contained 236 amino acids (GenBank accession No. EGR49452.1) and its gene (GenBank accession No. GL985062) was 711 bp (from NCBInr, gi|340519213). Sample IV was also identified only as a predicted protein. It showed 47% coverage from *T. reesei* QM6a. The putative protein from *T. reesei* QM6a contained 138 amino acids (GenBank accession No. EGR44058) and its gene (GenBank accession No. GL985109.1) consisted of 417 bp (from NCBInr, gi|340513772).

Gene cloning of identified protein

The gene for the identified band I protein, band III protein and band IV protein were cloned by PCR with primers sample I-F&R, sample III-F&R and sample IV-F&R, respectively. Genomic DNA from *T. orientalis* EU7-22 was used as the template. The length of the gene fragment was fit for analysis (Fig. 5).

The sequencing results and NCBI Blast analysis showed that the cloned gene for sample I had 1280 bp, contained one intron, and encoded a 407 amino acid protein. ProtParam analysis showed that it had a molecular weight (MW) of 42.6 kDa and an isoelectric point (pI) of 4.86. The deduced amino acid sequences showed 96% amino acid homology to the aspartic protease from *H. jecorina* (GenBank No. CAJ44684.1) that belongs to the pepsin-retropepsin-like superfamily. The gene sequence was deposited in the GenBank database with accession number JQ728540.

The sequencing results and NCBI Blast analysis showed that the cloned gene for sample III had 711 bp without intron, and encoded a 236 amino acid protein. ProtParam analysis showed that it had a MW of 24.4 kDa and a pI of 4.38. The deduced amino acid sequences showed 95% amino acid homology to the predicted protein from *T. reesei* QM6a(GenBank No. EGR49452.1) that belongs to the peptidase-A4 superfamily. The gene sequence was deposited in the GenBank database with accession number JQ728541.

The sequencing results and NCBI Blast analysis showed that the cloned gene for sample IV had 483 bp, contained one intron, and encoded a 138 amino acid protein. ProtParam analysis showed that it had a MW of 14.3 kDa and a pI of 5.75. The deduced amino acid sequences showed 99% amino acid homology to the predicted protein from *T. reesei* QM6a (GenBank No. EGR44058) that belongs to the cerato-platanin superfamily. The gene sequence was deposited in the GenBank database with accession number JQ728542.

Discussion

Filamentous fungi as an industrial strain could be manufactured bioproducts with cellulosic biomass (Silva *et al*. 2015). However, the metabolite glucose from cellulosic

Table 1: List of identified proteins from *T. orientalis* EU7-22

Sample No.	Protein Name MASCOT	Organism	Accession No.	MASCOT Score	Peptides matches	Cov. (%)	Exp. Mr (Da)	Exp. pI
Band I	Aspartic protease	*Hypocrea jecorina*	gi|83318620	481	6	25%	42776	4.78
Band III	Predicted protein	*Trichoderma reesei* QM6a	gi|340519213	70	1	3%	24431	4.32
Band IV	Predicted protein	*Trichoderma reesei* QM6a	gi|340513772	254	4	47%	14491	5.75

Cov: coverage; Exp. Mr: expected molecular weight; Exp. pI: expected isoelectric point

biomass as a repressor could affect the yield of bioproducts. It is meaningful that find certain anti-glucose proteins, because the constitutive promoters from anti-glucose proteins are active in a glucose-rich medium.

We first compared the effects of three cellulose inducers (pretreated *Miscanthus*, Avicel and CMC-Na) on cellulase production. Compared to the wheat bran control, they all promoted cellulase production (Fig. 1), but the pretreated *Miscanthus* cellulose and Avicel were the best inducers for FPA activity amongst the four substrates. This proved that insoluble cellulose substrate could be regarded as an inducer. One hypothesis is that oligosaccharides (cellobiose and sophorose) released from cellulose trigger a high level of cellulase expression. However, the mechanism of this triggering of cellulase production is not currently understood (Schmoll and Kubicek 2003). Reports have shown that wheat bran can increase cellulase production by filamentous fungi (Palmarola-Adrados *et al*. 2005; Sun *et al*. 2008). However, in our cultures without cellulose, wheat bran alone did not provide sufficient cellulose for maximum cellulase production (Fig. 1). A similar finding was reported in the fungus *Penicillium decumbens* (Sun *et al*. 2008).

Glucose was found to be a repressor inhibiting cellulase gene expression and protein synthesis (Ilmen *et al*. 1996; Vautard *et al*. 1999). In this study, we found similar results from *T. orientalis* EU7-22 (Fig. 3). Cellulase synthesis was completely stopped when the glucose concentration was very high.

We further performed SDS-PAGE analysis of secreted extracellular proteins from *T. orientalis* EU7-22 grown on various cellulose substrates (Fig. 2). This showed that pretreated *Miscanthus* cellulose and Avicel were good inducers of cellulase. And the secreted extracellular protein from the pretreated *Miscanthus* cellulose and Avicel fermentation samples were 0.78 mg mL^{-1} and 0.71 mg mL^{-1}, respectively. SDS-PAGE showed the protein bands for the pretreated *Miscanthus* cellulose were much stronger than those for Avicel. Herpoël-Gimbert *et al*. (2008) analyzed two *T. reesei* hypersecretory strains (RUT-C30 and CL847) by comparing secretomes and reported the cellulase protein weights of cellobiohydrolase I (57 and 63 kDa), cellobiohydrolase II (38, 42, 55, 58 and 59 kDa), endoglucanase I (55 kDa), endoglucanase II (43 and 48 kDa) and β-glucosidase (81 kDa). This suggests that the protein bands distributed from 25–116 kDa were almost completely represented by cellulase and hemicellulase molecules.

When the soluble sugar like glucose was added to medium containing cellulose, they all repressed cellulase production. The secreted extracellular proteins were analyzed by SDS-PAGE (Fig. 4). Secreted cellulase protein (Herpoël-Gimbert *et al*. 2008) mostly declined as the sugar concentration increased. However, it seemed that certain proteins were not repressed, as shown in Fig. 4 (indicated by arrows). The molecular weights of these proteins was 32, 25, 26 and 16 kDa in glucose. They were stably secreted under both induction and repression conditions. Data showed that the four protein bands were immune to glucose repression. The bands I, III and IV were successfully identified by MALDI-TOF.

Sample I was identified as an aspartic protease. It encoded a 407 amino acid, and showed that it had 42.6 kDa and an pI of 4.86. It was different from aspartyl protease from *T. harzianum* CECT 2413 (Delgado-Jarana *et al*. 2002). The *T. harzianum* CECT 2413 *papA* gene has an intronless ORF which consists of 404 amino acids and a pI of 4.35. It also differed from aspartic protease from *T. harzianum* T88 (Liu and Yang 2007). The *T. harzianum* T88 gene sequence, *SA76*, is 1593 bp long, encoding a polypeptide of 530 amino acids with a predicted molecular mass of 55 kDa and pI of 4.5. The function of this protein was related to biocontrol processes (Kredics *et al*. 2005), and aspartic proteases have been associated with problems in protein production due to proteolytic degradation (Delgado-Jarana *et al*. 2000; Kredics *et al*. 2005). Therefore, it was important in biocontrol and biotechnology industries involving detergent, leather, dairy, baking and pharmaceuticals (Rao *et al*. 1998).

Sample III was identified only as a predicted protein. The function of this protein had little description. Sample IV was again identified only as a predicted protein. This hypothetical protein bore a cerato-platanin domain, and was potentially involved in induction of systemic resistance in plants (Pazzagli *et al*. 2006; Fontana *et al*. 2008).

Further studies of these proteins are needed because we could potentially make use of their corresponding gene promoters to construct high-expression cellulolytic enzyme vectors with applications for the fuel industry.

Conclusion

This study focused on a method to screen anti-glucose repression proteins in *T. orientalis* EU7-22. Enzyme activity and concentration assay data indicate that the pretreated *Miscanthus* is a powerful inducer for cellulase production and secretion of extracellular protein. Supplementation with 3% glucose with pretreated *Miscanthus* cellulose reduced the FPA activity by 96.9% and the total protein content was decreased by 71.95%. Surprisingly, there was a significant quantity of anti-glucose repression protein successfully identified by MALDI-TOF (one was aspartic protease, two

were predicted proteins) and their corresponding genes were cloned by PCR. The deduced protein sequences from cloned gene completely accorded with the proteins identified by MALDI-TOF.

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