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**RESEARCH**

# Expression and Characterization of a Recombinant Endoglucanase From Western Corn Rootworm, in *Pichia pastoris*

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**ABSTRACT.** The endoglucanase cDNA, Dvv-ENGase I, from western corn rootworm, *Diabrotica virgifera virgifera* LeConte was expressed using the GS115 methylotrophic strain of *Pichia pastoris*. The *Dvv-ENGase I* gene was cloned into the integrative plasmid pPICZαA under the control of AOX1, which is a methanol-inducible promoter. Positive clones were selected for their ability to produce the recombinant endoglucanase upon continuous methanol induction. The secreted recombinant insect endoglucanase Dvv-ENGase I has an apparent molecular mass of 29 kDa. The recombinant endo-1,4-β-glucanase (ENGase) was able to digest the substrates: hydroxyethyl cellulose (HEC), carboxymethyl cellulose (CMC), and Whatman No. 1 filter paper. A higher accumulation of reducing sugar was evident when the *P. pastoris* expression medium contained HEC (1%) instead of CMC (1%). An enzymatic activity band was detected after performing electrophoretic separation under nondenaturing conditions. The biological activity of the recombinant Dvv-ENGase I was influenced by the presence of protease inhibitors in the culture medium.

**Key Words**: glycosyl hydrolase, cellulase, recombinant expression, *Pichia pastoris*

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the most economically important insect pest of corn plants, *Zea mays* (L.) in the United States (Levine and Oloumi-Sadeghi 1991). Recently, it was shown that not only endogenous endoglucanase activity is present in three gut regions of this insect pest but also Dvv-ENGase I, a β-1,4-endoglucanase (ENGase), is a monomeric protein with a molecular mass of 26 kDa that belongs to the glycoside hydrolase family 45 (GHF45), a large and growing hydrolase family (Valencia Jiménez et al. 2013). It has been shown that the presence of one or more possible posttranslational modifications may be necessary for secretion and biological activity of recombinant cellulases that have been cloned and expressed from other coleopteran insects (Wei et al. 2005, 2006). The Dvv-ENGase I identified from *D. v. virgifera* contains a possible posttranslational modification at the N-glycosylation site, 100–102 (N-S-T) in the amino acid sequence (Valencia Jiménez et al. 2013), which could affect the final biological activity of the recombinant enzyme, especially if a prokaryotic expression system is used.

For many years, *Escherichia coli*-based expression systems have been the most common method for the expression of recombinant proteins due to rapid cell growth, inexpensive cell culture media, and relative ease of genetic transformation (Sivashanmugam et al. 2009, Makino et al. 2011). However, bacterial-based systems can generate misfolded proteins as well as the formation of inclusion bodies and thus may yield nonfunctional proteins (Biely et al. 1985). In addition, some reports have described recombinant proteins that do not fold properly in bacterial systems but which can be expressed successfully in yeast systems (Cereghino and Cregg 2000, Wysocka-Kapcinska et al. 2010). In recent years, the methylotrophic yeast, *Pichia pastoris*, has become a successful and useful biotechnological tool for the expression of different heterologous proteins (Cregg et al. 1987, 1993). *P. pastoris* provides a eukaryotic expression system that is not only well known as an efficient system for expression and secretion of heterologous proteins (Cereghino and Cregg 2000) but also as an alternative to *E. coli* for expression of eukaryotic proteins that may require posttranslational modification (Wysocka-Kapcinska et al. 2010). *P. pastoris* has

been successfully used to express proteins for both basic research and industrial uses (Higgins and Cregg 1998), using low-cost and well-established culture methods to produce large amounts of a recombinant product based on the presence of a highly expressed alcohol oxidase promoter (Cregg et al. 1987, 1993; Canales et al. 1997; Stratton et al. 1998). A number of different recombinant proteins from a variety of eukaryotic sources have been successfully produced in this eukaryotic expression system (Cereghino and Cregg 2000).

*P. pastoris* provides an important expression system or a variety of reasons including: 1) it is easily genetically manipulated, 2) it is capable of producing recombinant proteins in higher yields, and 3) it is capable of structural and posttranslational protein modification including glycosylation, disulfide-bond formation, and proteolytic processing and protein folding (Cregg et al. 2000). As a consequence, some proteins that cannot be expressed efficiently in other expression systems may be successfully produced as functional recombinant proteins in *P. pastoris* (Cereghino et al. 2002). *P. pastoris* is generally considered to be a recombinant culture system that is capable of producing proteins in higher yields than mammalian or insect cell expression systems (Higgins and Cregg 1998). Here we present the expression and biological characterization of an active form of Dvv-ENGase I using the eukaryotic expression system of *P. pastoris* GS115 and demonstrate the utility of *P. pastoris* as an appropriate system for expression of insect heterologous proteins.

## Materials and Methods

**Strains and Reagents.** pEXP5-CT/TOPO-DvvENGI, a plasmid containing the gene for expression of an endogenous ENGase (Dvv-ENGase I), was obtained from an *E. coli* colony that was previously transformed in our laboratory and kept at −80°C. *P. pastoris* GS115, the *Pichia* EasyComp Kit, and pPICZαA vector were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes were purchased from Fermentas (Hanover, MD). Carboxymethyl cellulose (CMC) and ostazin brilliant red-hydroxyethyl cellulose (OBR-HEC) were purchased from Sigma Chemical (St. Louis, MO).

**Construction and Cloning of the Recombinant Expression Plasmid.** The coding region of *Dvv-ENGase I* gene was amplified by polymerase chain reaction (PCR) from plasmid pEXP5-CT/TOPO-DvvENGI using the following two primers: pEXP5 forward [5-GAATTCATGAAGATCGCTATCCTT-3] (EcoRI) and pEXP5 reverse [5-GCGGCCGCTTAAAGACTGCAACCA-3] (NotI) (restriction sites are underlined and the reverse stop codon TTA is bold). The PCR product containing the *Dvv-ENGase I* was submitted to double digestion with EcoRI and NotI restriction enzymes and ligated into pPICZαA vector which utilizes the AOX1 promoter of *P. pastoris* and the alpha-factor leader sequence from *Saccharomyces cerevisiae* for final product secretion. Finally, the plasmid pPICZαA-DvvENGI (20 µg) was linearized using SacI, and the DNA product was used to transform *P. pastoris* GS115 competent cells.

**Preparation and Transformation of Competent Pichia Cells.** The preparation and transformation of competent *Pichia* cells was done using the *Pichia* EasyCompKit (Invitrogen Carlsbad, CA). In general, transformation was performed by using 50 µl of competent cells with 3 µg of linearized plasmid DNA. Transformation efficiencies may vary not only based on the strain that is used but also on the efficiency of plasmid integration into the *Pichia* genome. The integration of the linearized pPICZαA-DvvENGI plasmid into the *Pichia* genome was confirmed by cloning PCR according to the manufacturer's instructions using the 5′ *AOX1* primer paired with the 3′ *AOX1* primer included in the kit. Recombinant DNA manipulations were carried out in *E. coli* TOP10 chemical competent cells (Invitrogen). *E. coli* cells were cultured in LB medium (0.5% yeast extract, 1% glucose, and 0.5% NaCl) at 37°C with antibiotics at the following final concentrations: 100 µg/ml ampicillin and 25 µg/ml Zeocin for plasmid selection. Plasmid DNA was purified from *E. coli* cell cultures using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA). DNA digested with restriction enzymes was resolved on TBE (Tris-Borate-EDTA buffer) agarose gels and purified using the QIAquick Gel extraction kit (Qiagen, Chatsworth, CA). A *P. pastoris* colony that was transformed with the empty pPICZαA vector was used as a control for documenting expression of a functional protein.

**Small Scale Expression.** Transformants were selected on YPD plates (1% yeast extract, 2% peptone, and 2% glucose) containing 100 µg/ml Zeocin as the selective marker. Positive *P. pastoris* transformants were grown in BMGY medium (buffered glycerol-complex medium, containing 1% glycerol, 2% peptone, and 1% yeast extract) at 30°C and 250 rpm until an OD600 of approximately 2–6 was reached (∼16–18 h). The *Pichia* cells were harvested by centrifugation at $2,500 \times g$ for 5 min at room temperature (RT), and the supernatant was decanted and the cell pellet resuspended to an OD600 of 1.0 in BMGY medium to induce expression (approximately 100–200 ml). Induction was initiated by adding 100% methanol to a final concentration of 0.5% methanol every 24 h. At 24-h intervals after initiating induction, 1 ml aliquots of the expression culture samples were collected in a 1.5-ml microcentrifuge tube and centrifuged at $15,000 \times g$ at RT for 2–3 min. Supernatants were immediately frozen and stored at −80°C and then used to analyze expression levels and determine the optimal time for post-induction harvest. A similar induction system containing protease inhibitors was tested in order to evaluate the effect of endogenous proteolytic activity on the enzymatic activity from the recombinant Dvv-ENGase I expressed using *P. pastoris* GS115.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting.** SDS–polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970). All protein samples were diluted in $2 \times$ SDS-PAGE sample buffer and separated on 10% SDS–PAGE. Proteins in the SDS-gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 90 mA overnight at 4°C. After blocking overnight at 4°C with 5% (w/v) nonfat dry milk prepared in TTBS (Tris-buffered saline, containing 150 mM NaCl, 20 mM Tris, 0.05% Tween-20, pH 7.5), the membranes were incubated for 1 h at RT with an anti-DvvENGase I polyclonal antiserum (1:10,000 v/v), generated using a 19 amino acid synthetic peptide (T P L E Q S P E I K F I E K G I S G E), which was designed based on the protein target sequence and using PeptideSelect Online (Invitrogen, Carlsbad, CA; Valencia Jiménez et al. 2013). After five washes with TTBS, the nitrocellulose membrane was incubated for 1 h at RT with anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:10,000 v/v, Sigma-Aldrich Life Science). Finally, the membrane was washed three times and the specific protein bands were visualized by using Sigma Fast 5-bromo-4-chloro-3'-indolyphosphate/Nitro-blue tetrazolium (BCIP/NBT; Sigma-Aldrich, St. Louis, MO). Precision Plus protein dual color standards (broad range, 10–250 kDa) were used as a control for protein transfer and estimation of molecular mass.

**OBR-HEC Hydrolyzing Activity.** ENGase was assayed in 50 mM sodium acetate (pH 5.0) with OBR-HEC (Sigma, St. Louis, MO) as substrate (Biely et al. 1985). The enzyme fraction (10 µl) was incubated with 50 µl of OBR-HEC solution (5 mg/ml) in a final volume of 250 µl for 15 min at 45°C. After 30 min, the reaction was stopped by adding 900 µl of ethanol. The reaction mixture was then centrifuged at $10,000 \times g$ and the final absorbance was read at 550 nm. The enzyme activity was expressed as the ΔA550/min. One unit of OBR-HECase activity was defined as the amount of enzyme changing the absorbance (0.001/h).

**Monitoring of Reducing Sugar Accumulation.** The recombinant ENGase activity was also determined by measuring reducing sugar concentration using the dinitrosalicylic acid (DNS) method (Miller 1959). After 5 d of *P. pastoris* GS115 culturing in a cell culture medium containing the substrates CMC and HEC (1%), 100 µl of supernatant was collected from each treatment, centrifuged at $10,000 \times g$ for 15 min at RT and combined with 100 µl of 1% DNS (DNS containing 30% potassium tartrate and 0.4 M sodium hydroxide) and then boiled for 10 min at 100°C. Finally, the absorbance was determined at 550 nm. The recombinant ENGase activity was also determined using a modification of the method described in Mandels et al. (1976). Briefly, two filter paper discs (Whatman No. 1, 42.5 mm, GE Healthcare, Buckinghamshire, UK) were incubated with 10 ml of the 6-d *P. pastoris* cell culture supernatant in 40 ml of 50 mM citrate buffer pH 5.0 and incubated at 45°C for six consecutive days. Aliquots (100 µl) of the final incubation mix were collected daily, centrifuged at $10,000 \times g$ for 15 min at RT and combined with 100 µl of 1% DNS. The final absorbance was determined at λ550 nm after boiling for 5 min at 100°C. The final ENGase activity was expressed as relative reducing sugar accumulation (%). Each experiment was carried out in triplicate.

**ENGase Activity in Native Polyacrylamide Gels.** Zymograms for ENGase activity were conducted under semidenaturing conditions on 7.5% SDS-PAGE gels containing 0.25% of copolymerized HEC (Fluka, Milwaukee, WI). After electrophoretic separation, the SDS gel was washed in 1% Triton X-100 solution for 30 min at 4°C followed by incubation in 50 mM sodium acetate buffer solution (pH 5.0) at 45°C to allow the enzyme to digest HEC. Finally, the gel was stained with 0.1% Congo red for 1 h at RT and destained in 1 M NaCl. The reaction was stopped by immersing the gel in a 10% (w/v) acetic acid solution and then photographed. A clear zone on a dark blue background indicated the hydrolysis of HEC as a result of ENGase activity.

**Radial Diffusion Assay to Detect ENGase Activity.** HEC-agar plates were prepared by dissolving agarose (1%), Congo red (0.02%), and HEC (0.5%) in 50 mM sodium acetate buffer (pH 5.0). After solidification into Petri plates to a depth of 10 mm, circular wells were punched into the agar with a 5-mm diameter cork borer. To detect the recombinant ENGase activity, 50 µl of *P. pastoris* cell culture supernatant was added to wells and incubated at 45°C overnight. After 24 h, the residual Congo red dye was removed by rinsing the plate with distilled water and then fixed by flooding the plate with dilute acetic acid (10%) for 1 h at RT. ENGase activity zones appeared as white haloes on a dark blue background.
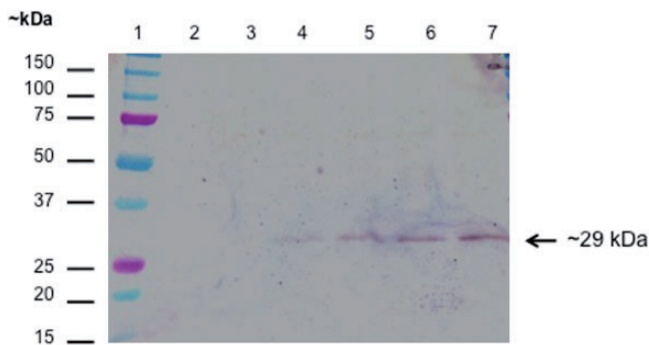
**Fig. 1.** Western-blot analysis of proteins from the supernatant of a *P. pastoris* cell-culture. *P. pastoris* GS115 was transformed with the pPICZαA vector containing the endoglucanase gene (DvvENG1) from *D. v. virgifera*. Lane 1, molecular mass markers; Lanes 2–7 represent fractions of cell culture fractions of 6 d of continuous cell-culture. Every 24 h the concentration of methanol was adjusted to 0.5% to maintain expression of the recombinant protein. A polyclonal antibody against an endoglucanase from *D. v. virgifera* was used as the primary antibody. The protein samples were run using a 10% SDS gel.

**Protein Determination.** The protein concentration was determined by the method of Bradford (1976) using bovine gamma globulin as protein standard. Protein samples were incubated for 15 min at RT and then read at 595 nm.

**pH Optimum.** The optimal pH was determined by preincubating 10 μl of the recombinant ENGase for 15 min at 45°C in appropriate buffers: 50 mM sodium phosphate (pH 2 and 7), 50 mM sodium citrate (pH 3, 4 and 6), 50 mM sodium acetate (pH 5), and 50 mM Tris–HCl (pH 8 and 9) and then starting the enzymatic reactions by adding 50 μl of OBR-HEC (5 mg/ml) as substrate. After 30 min, the reaction was stopped adding 900 μl of ethanol and then centrifuged at $10,000 \times g$ at RT, and the final absorbance was read at 550 nm. Each pH determination was replicated three times.

## Results

**Expression of Dvv-ENGase I From *P. pastoris*.** *P. pastoris* GS115 cells were transformed with the linearized pPICZαA-DvvENG I plasmid, which contained the coding sequence for *DvvENG I* gene from *D. v. virgifera* and then plated on YPD plates containing zeocin as a selective marker for positive colony selection. The expression of the recombinant protein was induced by adding methanol to the cell-culture every 24 h for 6 d and then the supernatant was collected daily for SDS-PAGE and western blot analyses. Western blotting analysis indicates that the secreted recombinant DvvENG I protein has an apparent molecular mass of 29 kDa and is clearly visible in the growth medium after 3 d of induced expression (Fig. 1). In addition, the accumulation of the recombinant protein during cell culture was also confirmed by enzymatic assays in which OBR-HEC was used as a substrate (Fig. 2). The accumulation of recombinant protein increased each day and reached a maximum by 6 d after induction.

**Characterization and Biological Activity of the Recombinant Protein.** The specific biological activity of the recombinant ENGase was evaluated by testing its capacity to digest the substrates including HEC, CMC, and Whatman No. 1 filter paper. Reducing sugar accumulation (%) was identified by incubating filter paper discs with the 6-d *P. pastoris* cell culture supernatant that contained the secreted recombinant enzyme (Fig. 3). The reducing sugar accumulation (%) was significantly increased when the cell culture medium was originally supplemented with CMC (1%) or with HEC (1%), although a higher accumulation of reducing sugar was evident when the expression medium contained HEC compared with CMC. No reducing sugar accumulation was observed for a nontransformed *P. pastoris* colony or a
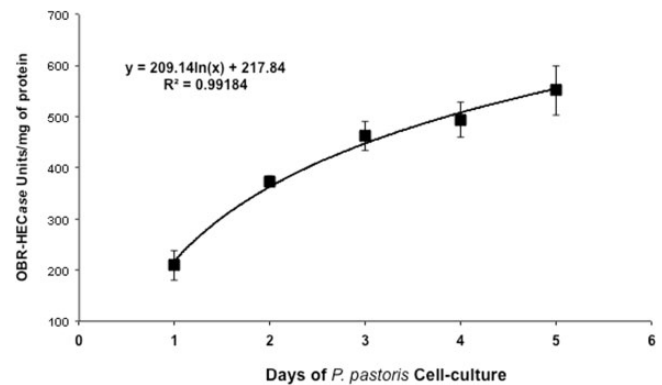


**Fig. 2.** Enzymatic activity from Dvv-ENG I recombinant protein expressed using *P. pastoris* GS115 after 5 d of Cell-culture. The enzymatic reaction was initiated by adding 50 μl of OBR-HEC (5 mg/ml) as substrate. One unit of OBR-HECase activity was defined as the amount of enzyme changing the absorbance (0.001/h).
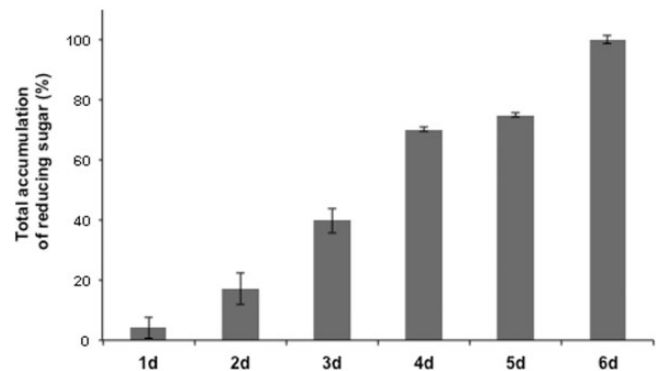


**Fig. 3.** Relative reducing sugar accumulation (%) during the degradation of filter paper (Whatman No. 1) by the enzymatic action of a recombinant endoglucanase gene from *D. v. virgifera* expressed in *P. pastoris* GS115. Two filter paper discs (Whatman No. 1; 42.5 mm) were incubated with 10 ml of 6-d *P. pastoris* supernatant and 40 ml of 50 mM citrate buffer pH 5.0 and then incubated at 45°C for six consecutive days. The final absorbance was determined at λ550 nm after adding 500 μl of distilled water. Each experiment was carried out in triplicate.

transformed *P. pastoris* colony carrying the empty vector grown in a medium supplemented with HEC (1%) (Fig. 4). The recombinant ENGase exhibited optimal enzymatic activity at pH 5.0 using OBR-HC as a substrate and appropriate buffer solutions (Fig. 5).

**ENGase Activity on HEC-Based Gels.** A single enzymatic activity band was identified (Fig. 6A) after electrophoretic separation under semidenaturing conditions with SDS-PAGE gels containing 1% HEC (Fig. 6A; lanes T1–T4). This band was absent in the supernatant from the *P. pastoris* colony that was transformed with the empty pPICZαA vector (lanes E1–E4). The expressed protein also showed enzymatic activity based on the zone of clearing in the radial diffusion assays (Fig. 6B), and the activity was clearly enhanced by the presence of protease inhibitors based on the increased size of the clear zone (Fig. 6B1).

## Discussion

We have previously reported the cloning and functional characterization of the *Dvv-ENG I* gene, which codes for an ENGase belonging to GHF45 from *D. v. virgifera* (Valencia Jiménez et al. 2013). It is important to note that high-level expression of recombinant ENGase's from different insect sources has been efficiently achieved using either *E. coli* or the aforementioned *P. pastoris* expression system
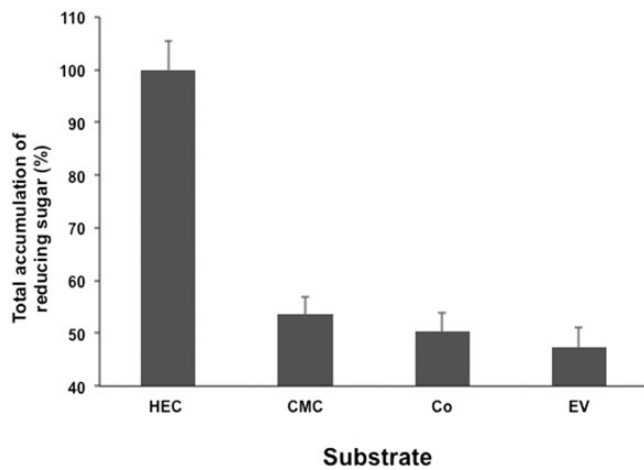
**Fig. 4.** Reducing sugar accumulation (%) during the culture of *P. pastoris* GS115 that was transformed with the Dvv-ENG I gene from *D. v. virgifera*. After 5 d of culturing, 100 µl of supernatant were collected from each treatment and combined with 100 µl of 1% DNS and then boiled for 5 min at 100°C. The final absorbance was determined at λ550 nm. HEC, *P. pastoris* cell culture containing 1% of HEC. CMC, *P. pastoris* cell culture containing 1% of CMC. EV, *P. pastoris* GS115 strain transformed with the empty vector as control. Each experiment was carried out in triplicate.
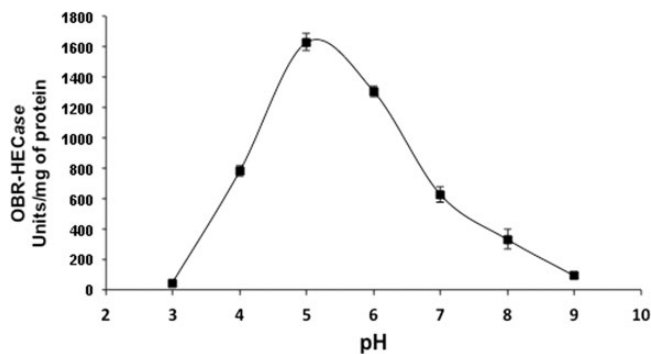


**Fig. 5.** Effect of the pH on the enzymatic activity of recombinant endoglucanase from *D. v. virgifera* that was expressed in *P. pastoris* GS115 cell-culture after 6 d of cell-culture. The enzymatic reaction was initiated by adding 50 µl of OBR-HEC (5 mg/ml) as substrate. Each point is the average of three measurements.

(Girard and Jouanin 1999, Lee et al. 2004, Inoue et al. 2005, Wei et al. 2005). The *P. pastoris* expression system is a widely reported method for the efficient production and secretion of recombinant proteins (Cregg et al. 2000) that can be expressed either extracellularly or intracellularly with high yields using well-established culture conditions. This recombinant expression system is also known for being a superior alternative to *E. coli*-based expression systems because of its natural capacity to express recombinant proteins that include posttranslational modifications not possible with *E. coli*-based expression (Wysocka-Kapcinska et al. 2010). The production of a functionally active form of the recombinant ENGase Dvv-ENGase I using the *P. pastoris* GS115 strain for expression constitute the successful expression of this protein in a eukaryotic system.

The recombinant Dvv-ENG I protein was detected by SDS-PAGE and western blotting and indicates that the apparent molecular mass of the recombinant ENGase from *P. pastoris* (29 kDa) was greater than the predicted molecular mass of 26-kDa from the translated cDNA sequence (Valencia Jiménez et al. 2013). This difference in the molecular mass may be due to the posttranslational modifications, such as
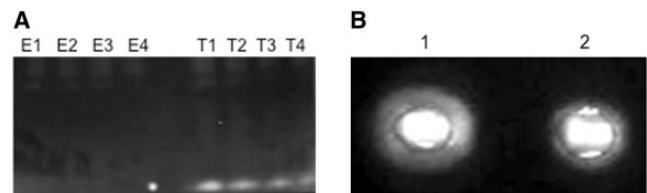


**Fig. 6.** (A) Endoglucanase activity assay on 7.5% semidenaturing SDS-PAGE gels containing 0.25 % (w/v) HEC of two *P. pastoris* cell cultures. Lanes E1–E4 represent four different protein concentrations from the supernatant of a *P. pastoris* GS115 strain that was transformed with the pPICZαA empty vector. Lanes T1–T4 represent four different protein concentrations from the supernatant of a *P. pastoris* GS115 strain that was transformed with the pPICZαA vector containing the endoglucanase gene (DvvENG1) from *D. v. virgifera*. The clear zones at the bottom of the gel indicate the hydrolysis of HEC as a result of endoglucanase activity. (B) Radial diffusion assay using agarose medium containing 0.5 % (w/v) HEC and 0.1% of Congo red. Samples were incubated at 45°C overnight; 1) 50 µl of total extract from *P. pastoris* cell culture containing protease inhibitors; 2) 50 µl of total extract from *P. pastoris* cell culture without protease inhibitors.

glycosylation, which can increase the apparent molecular mass in SDS-PAGE gels. It is well known that *P. pastoris* is able to add both *O*-linked and *N*-linked carbohydrate moieties to secreted proteins (Sivashanmugam et al. 2009).

The enzymatic activity of the recombinant ENGase Dvv-ENG I from *P. pastoris* was higher than the biological activity of the recombinant ENGase that was initially expressed using the *E. coli* cell-free expression system (data not shown). This increased enzymatic activity from the recombinant ENGase expressed in *P. pastoris* may be related to a potential N-glycosylation site at the Dvv-ENGase protein structure. It has been shown that the enzymatic activity of recombinant insect cellulases, with the presence of one or more possible N-glycosylation sites, is essential not only for enzymatic activity but also for stability of the recombinant protein (Howard et al. 1991, Flesher et al. 1995, Wei et al. 2005, 2006).

The recombinant ENGase Dvv-ENG I was successfully expressed under the control of the alcohol oxidase (AOX1) promoter, which is strongly repressed in cells grown on glucose, but is highly expressed when cells are grown in a cell culture medium containing methanol as a main carbon source (Cereghino et al. 2002). Monitoring of reducing sugar accumulation was possible by adding supplemental HEC and CMC to the original *P. pastoris* cell culture medium. The reducing sugar accumulation (%) in the medium supplemented with HEC was significantly greater when compared with the *P. pastoris* cell culture medium that was prepared with CMC as an additional ingredient. This phenomenon can be explained by either a higher recombinant ENGase activity or an easier digestion of HEC, which is a nonionic substituted cellulose that has been recommended to determine ENGase activity (Wood and Bhat 1988). It is important to point out that no significant accumulation of reducing sugar was observed for transformed *P. pastoris* expressing the empty vector (data not shown).

By using appropriate buffer solutions and reaction conditions, it was found that the ENGase activity of the recombinant DvvENG I protein expressed in *P. pastoris* GS115 cells has an optimum pH around 5.0. This result is similar to the previous report of enzymatic properties on optimum pH of the Ag-EGase I from *Apriona germari* (Lee et al. 2004) and from the mollusk *Ampullaria crossean* (Guo et al. 2008).

The higher enzymatic activity that was detected in the supernatant from the *P. pastoris* cell medium supplemented with protease inhibitors could be explained by susceptibility of the secreted recombinant ENGase to endogenous neutral proteolysis. Because part of the expressed protein is degraded during its expression, an unbuffered

medium may then be important for future recombinant protein expression experiments. As *Pichia* expression progresses in an unbuffered medium, the pH becomes more acidic, inactivating many neutral pH proteases without affecting the cell growth (Brierley et al. 1994).

In conclusion, we have established an expression system using the methylotrophic yeast, *P. pastoris* GS115 strain, which will facilitate the production and expression of large amounts of a soluble and functionally active form of DvvENG I that will allow further biochemical and biophysical studies. Expression of insect ENGases and other cellulase genes should help develop an understanding about the role of insect cellulolytic enzymes during the cellulose hydrolysis that takes place in the insect gut.

## Acknowledgments

## References Cited

Biely, P., D. Mislovicová, and R. Toman. 1985. Soluble chromogenic substrates for the assay of endo-1,4-beta-xylanases and endo-1,4-beta-glucanases. Anal. Biochem. 144: 142–146.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brierley, R. A., G. R. Davis, and G. C. Holtz. 1994. Production of insulin-like growth factor-1 in *methylotrophic yeast cells*. U.S. patent 5,324,639.

Canales, M., A. Enríquez, E. Ramos, D. Cabrera, H. Dandie, A. Soto, V. Falcón, M. Rodríguez, and J. de la Fuente. 1997. Large-scale production in *Pichia pastoris* of the recombinant vaccine Gavac TM against cattle tick. Vaccine 15: 414–422.

Cereghino, J. L., and J. M. Cregg. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24: 45–66.

Cereghino, L. G. P., J. L. Cereghino, C. Ilgen, and J. M. Cregg. 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Curr. Opin. Biotechnol. 13: 329–332.

Cregg, J. M., J. F. Tschopp, C. Stillman, R. Siegel, M. Akong, W. S. Craig, R. G. Buckholz, K. R. Madden, P. A. Kellaris, G. R. Davis, et al. 1987. High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. Biotechnol. 5: 479–485.

Cregg J. M., T. S. Vedvick, and W. C. Raschke. 1993. Recent advances in the expression of foreign genes in *Pichia pastoris*. Biotechnol. 11: 905–910.

Cregg J. M., L. Cereghino, J. Shi, and D. R. Higgins. 2000. Recombinant protein expression in *Pichia pastoris*. Mol. Biotechnol. 16: 23–52.

Flesher A. R., J. Marzowski, W. C. Wang, and H. V. Raff. 1995. Fluorophore-labeled carbohydrate analysis of immunoglobulin fusion proteins: correlation of oligosaccharide content with in vivo clearance profile. Biotechnol. Bioeng. 46: 399–407.

Girard C., and L. Jouanin. 1999. Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae*. Insect Biochem. Mol. Biol. 29: 1129–1142.

Guo R., M. Ding, S. Zhang, G. Xu, and F. Zhao. 2008. Expression and characterization of two secreted His6-tagged endo-β-1,4-glucanases from the mollusc *Ampullaria crossean* in Pichia pastoris. Acta Biochim. Biophys. Sinica 40: 419–425.

Higgins D. R., and J. M. Cregg. 1998. Introduction to *Pichia pastoris*, pp. 1–15. *In* D. R. Higgins and J. M. Cregg (eds.), Methods in molecular biology. *Pichia* protocols. Humana Press, Totawa, NJ.

Howard S. C., A. J. Wittwer, and J. K. Welply. 1991. Oligosaccharides at each glycosylation site make structure-dependent contributions to biological properties of human tissue plasminogen activator. Glycobiology 1: 411–418.

Inoue T., S. Moriya, M. Ohkuma, and T. Kudo. 2005. Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite, *Coptotermes formosanus*. Gene 349: 67–75.

Laemmli U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lee S. J., S. R. Kim, H. J. Yoon, I. Kim, K. S. Lee, Y. H. Je, S. M. Lee, S. J. Seo, H. D. Sohn, and B. R. Jin. 2004. cDNA cloning, expression, and enzyme activity of a cellulase from the mulberry longicorn beetle, *Apriona germari*. Comp. Biochem. Physiol. B 139: 107–116.

Levine E., and H. Oloumi-Sadeghi. 1991. Management of diabroticite rootworms in corn. Ann. Rev. Entomol. 36: 229–255.

Makino T., G. Skretas, and G. Georgiou. 2011. Strain engineering for improved expression of recombinant proteins in bacteria. Microb. Cell Fact. 10: 1–10.

Mandels M., R. Andreotti, and C. Roche. 1976. Measurement of saccharifying cellulase. Biotechnol. Bioeng. Symp. 6: 21–33.

Miller G. L. 1959. Use of the dinitrosalicylic acid reagent for the determination of reducing sugar. Anal. Chem. 31: 426–428.

Sivashanmugam A., V. Murray, C. Cui, Y. Zhang, J. Wang, and Q. Li. 2009. Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*. Protein Sci. 18: 936–948.

Stratton J., V. Chiruvolu, M. Meagher. 1998. High cell-density fermentation, pp. 107–120. *In* D. R. Higgins and J. M. Cregg (eds.), Methods in molecular biology. *Pichia* protocols. Humana Press, Totawa, NJ.

Valencia Jiménez A., A. P. Alves, and B. D. Siegfried. 2013. Molecular cloning and cell-free expression of an endogenous endoglucanase belonging to GHF45 from the western corn rootworm, *Diabrotica virgifera virgifera*. Gene 513: 260–267.

Wei Y. D., S. J. Lee, K. S. Lee, Z. Z. Gui, H. J. Yoon, I. Kim, Y. H. Je, X. Guo, H. D. Sohn, and B. R. Jin. 2005. N-glycosylation is necessary for enzymatic activity of a beetle (*Apriona germari*) cellulase. Biochem. Biophys. Res. Commun. 329: 331–336.

Wei Y. D., K. S. Lee, Z. Z. Gui, H. J. Yoon, I. Kim, Y. H. Je, S. M. Lee, G. Z. Zhang, X. Guo, H. D. Sohn, et al. 2006. N-linked glycosylation of a beetle (*Apriona germari*) cellulase Ag-EGase II is necessary for enzymatic activity. Insect Biochem. Mol. Biol. 36: 435–441.

Wood T. M., and K. M. Bhat. 1988. Methods for measuring cellulase activities. Methods Enzymol. 160: 87–117.

Wysocka-Kapcinska M., J. A. Campos-Sandoval, A. Pal, and J. B. C. Findlay. 2010. Expression and characterization of recombinant human retinol-binding protein in *Pichia pastoris*. Protein Expr. Purif. 71: 28–32.