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Heterologous Expression of Endo-1,4-beta-xylanaseA from *Phanerochaete chrysosporium* in *Pichia pastoris*

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The cDNA of endo-1,4-β-xylanaseA, isolated from *Phaenerocheate chrysosporium* was expressed in *Pichia pastoris*. Using either the intrinsic leader peptide of XynA or the α-factor signal peptide of *Saccharomyces cerevisiae*, xylanaseA is efficiently secreted into the medium at maximum concentrations of 1,946 U/L and 2,496 U/L, respectively.

KEYWORDS : Hemicellulose, *Phanerochaete chsysosporium*, *Pichia pastoris*, Xylan, Xylanase

Hemicellulose is a heterogeneous group of branched and linear polysaccharides that are bound, via hydrogen bonds, to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming a highly complex structure together with cellulose. Based on the current stage of knowledge, hemicelluloses can be divided into four general classes of structurally different cell-wall polysaccharide types: xylans, mannans, mixed linkage β-glucans, and xyloglucans [1]. The major component of hemicellulose, xylan, is composed of D-xylopyranosyl units linked by β-1,4-glycosidic bonds. Xylan is a major structural polysaccharide in plant cells and is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on Earth [2-4]. Degradation of xylan requires endo-xylanase (Ec. 3.2.1.8), which hydrolyses the β-1,4 bond in the xylan backbone, yielding short xylooligomers. Most of the known xylanases belong to GH families 10 and 11 (over 300 gene sequences are known), and about 20 more xylanase genes are distributed between families 5, 8 and 43. Xylanases are naturally secreted by various micro-organisms, including bacteria, yeasts, and filamentous fungi [5].

The white rot basidiomycete, *Phanerochaete chrysosporium*, can degrade all complex woody materials through the release of extracellular enzymes such as lignin peroxidases, manganase peroxidase, glyoxal oxidase, cellobiose dehydrogenase, and xylanase [6-8]. Although the lignin and hemicellulose hydrolyzing capabilities of fungi have been described previously, the purification and characterization of individual enzymes can be complicated by the presence of several enzymes with similar activities [4]. Therefore, it is advantageous to clone and express genes coding for individual hydrolytic enzymes in a heterologous host.

The heterologous expression system of *Pichia pastoris* provides many advantages for the production of eukaryotic recombinant proteins and is a powerful tool for industrial-scale fermentation [9-12]. In this study, we report the cloning of *XynA*, which encodes an endo-1,4-β-xylanaseA (XynA) from *P. chrysosporium*, in *P. pastoris*. Expression of the *XynA* gene in *P. pastoris* was obtained under the control of the methanol inducible alcohol oxidase I (*AOXI*) promoter.

P. chrysosporium BKM-F-1767 was obtained from the Korean Collection for Type Culture and maintained in the medium described by Tien and Kirk [13]. *Escherichia coli* Top10 was maintained in an LB agar medium containing 50 μg/mL ampicillin, IPTG and X-Gal for PCR products selection, and in a low-salt LB medium, containing 50 μg/mL Zeocin, for xylanase gene constructs with expression vectors. The host strain used for heterologous expression was *Pichia pastoris* GS115 (*his4*). It was grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar).

The vectors pPICZA and pPICZαA (Invitrogen, Carlsbad, CA, USA) were used for expression in *P. pastoris*. Expression of inserts in both vectors is controlled by the methanol inducible AOX1 promoter. The pPICZαA possesses the α-secretion factor from *Saccharomyces cerevisiae,* while pPICZA does not contain a secretion signal.

P. chrysosporium was cultured under nitrogen limitation in stationary flasks at 30°C for 5 days to induce the

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expression of Xylanase genes. Total mRNA was extracted using the Oligotex mRNA mini kit (Qiagen, Hilden, Germany) and total cDNA was synthesized using the SMARTer PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA).

The XynA cDNA of *P. chrysosporium* BKM-F-1767 including signal peptide was amplified using PCR with the forward primer 5′-GCGAATTCATGAAGCTCTCTGCCTCCTTCGCG-3′ and the reverse primer 5′-CGGGGCCCATTGCCGAAGCCAATAGCGATA-3′. For PCR amplification, the following conditions were employed: 94°C 1 min, 94°C 30 sec, 51°C 30 sec, and 72°C 2 min (30 cycles). The purified PCR products were cloned into the pGEM-T vector, excised using *Eco*RI and *Apa*I, purified from agarose gel, and inserted into pPICZA, resulting in the pPICZA/ss-*XynA* construct. For cloning into pPICZαA, PCR amplification of the XynA cDNA in the pGEM-T vector was performed using the forward primer 5′-CGAATTCCAGTCGCCTGTTTGGGGCC-3′ and the reverse primer 5′-CGCCGCGGAATTGCCGAAGCCAATAG-3′. The forward primer does not contain a *P. chrysosporium* XynA secretion signal with the amplified region because the *S. cerevisiae* α-secretion signal is present in the pPICZαA. PCR conditions employed were the same as described for XynA cDNA. The resulting PCR product, *XynA*, was cloned into the pGEM-T vector, excised using *Eco*RI and *Sac*II, purified from agarose gel, and inserted into pPICZαA, resulting in the pPICZαA/*XynA* construct. After transformation into *E. coli* and isolation of plasmid DNA, the presence of the inserts was determined by PCR and by restriction enzyme digestion followed by agarose-gel electrophoresis. Sequence analysis was performed on pPICZA/ss-*XynA* and pPICZαA/*XynA*.

For transformation of the yeast strain, 10 μg of plasmid was linearized using *Pme*I and transformed into *P. pastoris* by electroporation methods, as recommended by the manufacture (Bio-Rad, Hercules, CA, USA). Transformed cells were selected on YPD agar plates containing 100 μg/mL Zeocin, at 30°C until a colony could be formed (2~3 days). The recombinant *P. pastoris* was confirmed by PCR using AOX1 primers.

Ten *P. pastoris* transformants were cultured in 50 mL YP medium containing 1% methanol to determine extracellular enzyme activities by enzyme assay. To measure the expression of XynA, a single colony was grown in 5 mL YPD medium overnight at 30°C and 200 rpm. After overnight incubation, 5 mL cultures were transferred into 100 mL of fresh YPD medium in a shaking incubator at 30°C and 150 rpm for 2 days. The cell pellets were harvested by centrifugation at 2,000 rpm for 5 min and the cell pellet was resuspended in 10 mL YP medium (1% yeast extract, 2% peptone). The suspensions were slowly added to 90 mL of fresh YP medium until the optical density (OD) reached 1. Fresh YP medium was added to make a final volume of 100 mL. For the induction of xylanase, 1 mL of 100% methanol was added every 24 hr to a final concentration of 1% for 7 days at 25°C. One mL of cultivation fluid was collected every 24 hr, centrifuged for 5 min at 15,000 rpm, and measured for enzyme activity. The clone that released xylanase at the highest level was selected and stored at −80°C.

To purify XynA, on the second day of cultivation, cell-free supernatants were collected by centrifugation at 3,000 rpm for 5 min and filtered through 0.45 μm filters. One hundred mL of filtered supernatant was mixed with 10 mL 10× binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4). The mixture was applied to the Ni^{2+} his-tag column (Histrap; GE Healthcare, München, Germany) using the ΔKTA FPLC purification system. Protein was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4) and collected in a 15 mL conical tube. All fractions containing purified enzyme were dialyzed in distilled water to remove salt and imidazole. Protein concentration was measured by the Bradford method using a Thermo Scientific protein assay kit (Rockford, IL, USA) with serum albumin as the standard protein. SDS-PAGE was performed as described in Sambrook and Russell [14].

Xylanase activity was assayed by measuring the production of reducing-sugar ends from birchwood xylan (Sigma, St. Louis, MO, USA) with 3,5-dinitrosalicylic acid (DNSA), as described by Decelle *et al*. [4], with minor modifications. Culture supernatant (100 μL) or purified xylanase (1 μg) were mixed with double distilled water to make a 200 μL volume. The mixture was incubated with 200 μL of 1% (w/v) birchwood xylan in a sodium citrate buffer (100 mM, pH 4.5) at 70°C for 10 min. Then, 200 μL of DNSA reagent was added and the mixture was boiled for 10 min. The amount of released reducing sugar was determined at 540 nm, as described in Sengupta *et al.* [15]. A standard curve was built using D-xylose as a substrate and the absorbance was converted into moles of reducing sugars released. One unit of xylanase activity was defined as the release of 1 μmol/min of xylose. The optimum temperature of xylanase activity was measured by performing the activity assay, as described above, at a range of temperatures (30 to 80°C). Sodium citrate and sodium phosphate buffers were used to generate a pH range (3.0 to 8.0) to determine the optimum pH at the optimal temperature.

The full-length cDNA fragment that encodes XynA in *P. pastoris* was 99% identical to the nucleotide sequence of the cDNA encoding for *XynA* from *P. chrysosporium* strain ME446 (GeneBank accession No. AF301903.1). However, the predicted amino acid sequence was 100% identical. Thus, the selected cDNA was expressed in *P. pastoris*. To study the expression of XynA in *P. pastoris*, two distinct expression vectors were used. The construct

pPICZA/ss-*XynA* is composed of the inducible promoter AOX1, the native intrinsic signal peptide of *XynA*, the open reading frame for mature *XynA*, and a termination transcription signal. The second construct vector pPICZαA/*XynA* differs from the first in that it contains the *S. cerevisiae* α-factor secretion signal upstream of the sequence of *XynA* cDNA. In addition, to facilitate purification of the recombinant XynA, both recombinant *XynA* genes were fused to the his-tag site on the vectors. For each construct, approximately 50~100 transformants were obtained after selection of recombinants on a zeocin containing plate. For each construct, six colonies were checked by PCR using AOX primers. They exhibited unique bands of the correct size on agarose gel and produced enzyme activity in the culture medium one day after inoculation. Enzyme assays showed that all positive colonies secreted extracellular XynA into the culture medium.

P. pastoris recombinants were induced to express XynA at OD 1 for 7 days. Every day, 1 mL methanol was added for induction and an enzyme assay was performed using 1 mL of the culture medium (Fig. 1). The results indicated that both native and α-factor secretion signal peptides were efficient in mediating xylanase secretion. Enzyme activity secreted using α-factor peptides is higher than when using intrinsic native signal peptide. Enzyme activities reached 1,536 U/L and 1,089 U/L one day after induction, for α-factor and native signal peptide, respectively. The maximal enzyme activities occurred two days after induction for both secretion signal peptides (2,496 U/L and 1,946 U/L, respectively). These results show that the enzyme activity in this study is four times higher than previously reported XynA expression in *Aspergillus niger* [4].

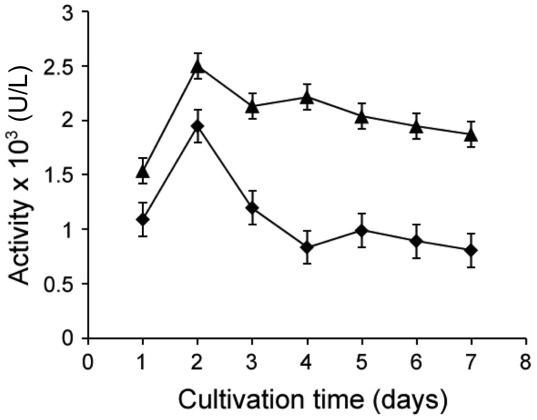

Fig. 1. Time course changes of XynA expression using the intrinsic secretion signal (pPICZA/ss-*XnA*) and the α-factor secretion signal (pPICZαA/*XynA*) in *Pichia pastoris*. Data shown are a representative profile based on two separate experiments, each with three samples for each data point. The closed triangles represent XynA secreted by the α-factor secretion signal (pPICZαA/*XynA*) and the closed diamonds represent XynA secreted by the intrinsic secretion signal (pPICZA/ss-*XynA*).

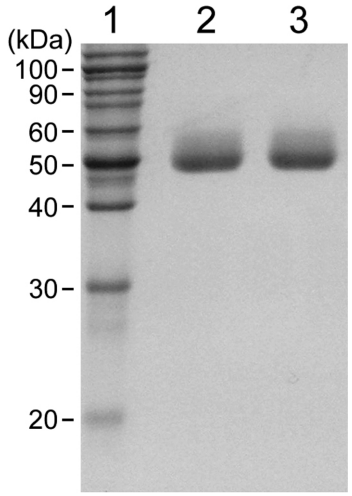

Fig. 2. SDS-PAGE analysis of XynA purified from *Pichia pastoris*. Lane 1, molecular weight markers; lane 2, pPICZαA/*XynA*; lane 3, pPICZA/ss-*XynA*.

To purify the enzyme product, culture media were directly applied on his-tag column Histrap (GE Healthcare) and eluted, as per manufacturor's recommendations (Fig. 2). However, the molecular mass of purified XynA on polyacrylamide gel was approximately 50 kDa, which was larger than the predicted molecular mass of 43 kDa for pPICZA and pPICZαA. The discrepancies between the predicted and recombinant enzymes seem to be due to post-translational modifications, such as glycosylation [16-18]. To confirm that the purified proteins were XynA, we performed matrix-assisted laser desorption/ionization mass spectrometry. The purified XynAs were subjected to SDS-PAGE and digested by trypsin. Peptide mapping of the fragments performed by mass spectrometry confirmed the identity of the purified proteins (data not shown).

The optimum temperature of recombinant XynA was determined by assay within a temperature range of 30 to 80°C (Fig. 3A). The results showed that recombinant XynA had maximum activity at 70°C, which is similar to XynA from *P. chrysosporium* expressed in *A. niger* [4]. Enzymatic assays suggested an optimal activity of XynA at approximately pH 5.0 (Fig. 3B). Enzyme activity decreased rapidly at a pH of below 4.0 or above 6.0. The optimum pH is comparable with XynA from *P. chrysosporium* [4], although the result was slightly different (5.0 compared to 4.5). The differences in protein folding form may be an explanation for this.

Although *P. chrysosporium* hydrolyzes lignin and polysaccharide, the purification and characterization of individual enzymes can be complicated because of the presence of several enzymes with similar activities. As a result, it is

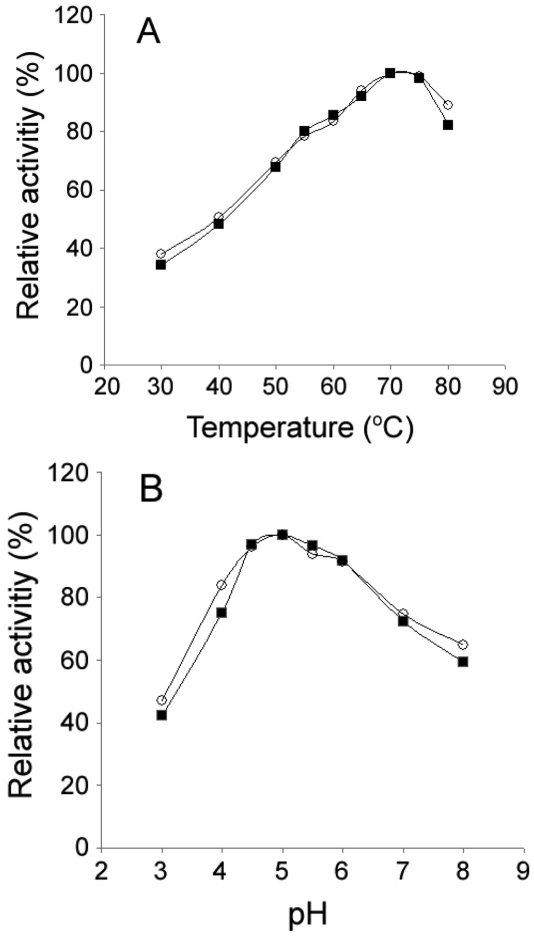

Fig. 3. Effect of temperature (A) and pH (B) on enzyme activity. The closed squares represent XynA secreted by the intrinsic secretion signal (pPICZA/ss-*XynA*) and the open circles represent XynA secreted by the α-factor secretion signal (pPICZαA/*XynA*).

often advantageous to clone and express genes of individual hydrolytic enzymes in a heterologous host. The present study has reported the cloning of the gene that encodes XynA from *P. chrysosporium* and its expression in *P. pastoris*, which can utilize methanol as its sole carbon and energy source. Heterologous expression of XynA in *P. pastoris* makes four times more XynA than was previously reported in *A. niger* [4]. It is possible to over-produce XynA without any contamination by other xylolytic enzymes. The enzyme produced in this study offers a candidate for use in biobleaching or enzymatic pretreatment of biomass prior to utilization.

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