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NOTE

Heterologous expression of endo-1,4-beta-xylanaseC from *Phanerochaete chrysosporium* in *Pichia pastoris*

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The cDNA of endo-1,4-β-xylanaseC, isolated from *Phanerochaete chrysosporium*, was expressed in *Pichia pastoris*, under the control of the alcohol oxidase I promoter. Using either the intrinsic leader peptide of xylanaseC or the α-factor signal peptide of *Saccharomyces cerevisiae*, xylanaseC is efficiently secreted into the medium, at a maximum concentration of 2500 $U \cdot l^{-1}$.

[**Key words:** *Phanerochaete chrysosporium*; *Pichia pastoris*; Xylan; Xylanase; Glycoside hydrolase]

Endo-β-1,4-xylanases (EC.3.2.1.8) are key enzymes for the degradation of xylan, a major component of hemicelluloses, which constitutes up to 20% of the total dry weight of plant primary cell walls. Endo-β-1,4-xylanases hydrolyse internal linkages in xylan, through a random attack mechanism, yielding a mixture of xylo-oligosaccharides from the polymer (1). Endo-β-1,4-xylanases belong to two main groups of glycoside hydrolase families, known as 10 and 11 based on their physicochemical properties. The xylanase members of family 10 are characterized by being highly active on short xylo-oligosaccharides, having high molecular masses, and low pIs. Contrarily, xylanase members of family 11 are most active on long chain xylo-oligosaccharides, have high pIs, and low molecular weights (2,3).

Xylanases have important industrial applications due to their enormous potential to modify and transform lignocelluloses and cell wall materials, which are abundant components of the vegetative biomass that is used in a wide variety of industrial processes (1). Currently, xylanases, together with cellulases and pectinases, account for 20% of the global, industrial enzyme market (3). In the pulp and paper industry, hemicellulases, especially endo-β-xylanases, that decrease chemical consumption, reduce environmental loading, or increase the final brightness of pulp have been reported, which present economic and environmental advantages over the non-enzymatic process. There are other biotechnological applications of xylanases in industry, such as preparation of animal feed, bread and biscuit-making in the food industry, and other applications within the juice and wine industries, and the textile industry (2,3).

Xylanases are naturally secreted by various micro-organisms, including bacteria, yeasts, and filamentous fungi (4). The white rot basidiomycete, *Phanerochaete chrysosporium*, is known to be able to degrade all complex woody materials, through the release of extracellular enzymes, such as lignin peroxidases, manganase peroxidase, glyoxal oxidase, cellobiose dehydrogenase, and xylanase (5–7). Although the fungus' lignin and hemicellulose hydrolyzing capabilities have been described previously, the purification and characterization of individual enzymes can be complicated by the presence of other enzymes that have similar activities (8). 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, as well as a most powerful tool for industrial-scale fermentation (9–12). In this study, we report the cloning of *XynC*, which encodes an endo-1,4-β-xylanaseC (XynC) from *P. chrysosporium* and its expression in *P. pastoris* under the control of the methanol inducible alcohol oxidase I (*AOXI*) promoter, which was followed by either the intrinsic leader peptide of XynC or the α-factor signal peptide of *Saccharomyces cerevisiae*. The intrinsic leader peptide of XynC efficiently directed the secretion of xylanase, by *P. pastoris*, to the culture medium as much as α-factor signal peptide of *S. cerevisiae*.

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

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

P. chrysosporium was cultured under nitrogen limited conditions in stationary flasks, at 30°C, for 5 days to induce the expression of Xylanase gene. Total mRNA was extracted using the Oligotex mRNA Mini Kit (Qiagen); and total cDNA was synthesized using the SMARTer PCR cDNA synthesis Kit (Clontech).

The XynC cDNA of *P. chrysosporium* BKM-F-1767 including the signal peptide was amplified using PCR with a forward primer of 5′-GCGAATTCATGTTCAAGTTCTCCGCGTCCCT-3′ and the reverse primer of 5′- GCTCTAGACATGCGCTGAAGCCAGCGGC-3′. For PCR amplification, the following conditions were employed: 94°C for 1 min, 94°C for 30 s, 51°C for 30 s, and 72°C for 2 min (for 30 cycles). The purified PCR products were cloned into the pGEM-T vector and, subsequently, excised using *Eco*RI and *Xba*I; this was followed by purification from the agarose gel and insertion into pPICZB, which resulted in the construct pPICZB/ss-*XynC*. For cloning into pPICZαA, PCR amplification of the XynC cDNA in the pGEM-T vector was performed, using the forward primer 5′-GCGAATTCCAGTCACCAGAGTGGGGCCAA-3′ and the reverse primer 5′- GCTCTAGACATGCGCTGAAGCCAGCGGC-3′. The forward primer did not contain a *P. chrysosporium* XynC secretion signal within the amplified region, as the *S. cerevisiae* α-secretion signal is present in the pPICZαA. PCR conditions employed were the same as previously described for XynC cDNA. The resulting PCR product, *XynC,* was cloned into the pGEM-T vector and, subsequently, excised using *Eco*RI and *Xba*I, which was followed by purification from the agarose gel and insertion into pPICZαA, which resulted in the construct pPICZαA/*XynC*. After transformation into *E. coli* and isolation of plasmid DNA, the presence of the inserts was determined both by PCR and by restriction enzyme digestion followed by agarose-gel electrophoresis. Sequence analysis was performed on pPICZB/ss-*XynC* and pPICZαA/*XynC*.

For transformation of the yeast strain, 10 μg of plasmid was linearized using *Pme*I, and transformed into *P. pastoris* by an electroporation method, as recommended by the manufacturer (Bio-Rad). Transformed cells were selected, on YPD agar plates containing 100 μg/ml zeocin, at 30°C, until a colony was formed (2–3 days). The recombinant *P. pastoris* was confirmed by PCR using AOX1 primers.

Ten transformants of *P. pastoris* were cultured in 50 ml YP medium, containing 1% methanol, in order to determine extracellular enzyme activities by enzyme assay. To measure the expression of XynC, 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 onto 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 2000 rpm for 5 min, and the cell pellet was resuspended in 10 ml YP medium (1% yeast extract, 2% peptone). Then, the suspensions were added, slowly, to 90 ml of fresh YP medium until the OD reached 1; finally, fresh YP medium was added to make up a final volume of 100 ml. For the induction of xylanase, 1 ml 100% methanol was added every 24 hours, to a final concentration of 1%, for seven day cultivation at 25°C. One milliliter of cultivation fluid was collected every 24 hours and centrifuged for 5 min at 15,000 rpm, after which, enzyme activity was measured. The clone that released xylanase at the highest level was selected and stored at −80°C.

To purify XynC, on the second day of cultivation, the cell-free supernatants were collected, by centrifugation at 3000 rpm for 5 min, and filtered through 0.45 μm filters. A total of 100 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) using the ÄKTA 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 into 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 (USA) protein assay kit with serum albumin as the standard protein. SDS–PAGE was performed as described by Sambrook and Russell (14).

Xylanase activity was assayed by measuring the production of reducing-sugar ends, from birchwood xylan (Sigma), with 3,5-dinitrosalicylic acid (DNSA), as described by Decelle et al. (8). The amount of released reducing sugar was determined at 540 nm, as carried out by 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 full-length cDNA nucleotide sequence of XynC isolated from *P. chrysosporium* BKM-F-1767 has deposited in GenBank (accession no. AQ993045) and shows 99% identical to the nucleotide sequence of the cDNA encoding for XynC from *P. chrysosporium* strain ME446 (GeneBank accession no. EU302794.1); however, the predicted amino acid sequence was 100% identical. Thus, the selected cDNA was expressed in *P. pastoris*.

To express of XynC in *P. pastoris*, two distinct expression vectors were used. The construct pPICZB/ss-*XynC* was composed of the inducible promoter AOX1, the native intrinsic signal peptide of *XynC,* the open reading frame for mature *XynC*, and a termination transcription signal. The second construct vector pPICZαA/*XynC* differs from the first in that it contained the *S. cerevisiae* α-factor secretion signal in the upstream of the sequence of *XynC* cDNA. In addition, to facilitate purification of the recombinant XynC, both recombinant *XynC* 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, ten colonies were checked by PCR using AOX primers; these colonies exhibited a unique band at the correct size on agarose gel. Enzyme assay showed that all positive colonies secreted extracellular XynC into the culture medium.

P. pastoris recombinants were induced to express XynC at the first OD 1, for 7 days. Every day 1 ml methanol was added for induction, and an enzyme assay was performed on 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. Maximal enzyme activities were reached 2 days after induction for

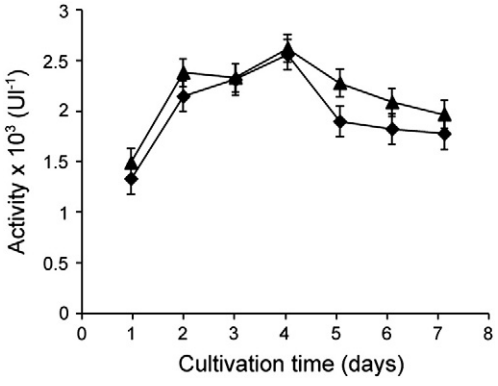

FIG. 1. Time course changes of XynC expression, using the intrinsic secretion signal (pPICZB/ss-*XynC*) and the α-factor secretion signal (pPICZαA/*XynC*), in *P. pastoris*. Data are shown as a representative profile based on two separate experiments, each with three samples for each data point. The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

both secretion signal peptides, 2369 $U \cdot l^{-1}$ and 2547 $U \cdot l^{-1}$, respectively. The activity of XynC recorded in this study was four times higher than the previously reported in *Aspergillus niger* (8). These results indicate that the intrinsic leader peptide of XynC efficiently directed the secretion of xylanase, in *P. pastoris*, to the culture medium as much as the α-factor signal peptide of *S. cerevisiae* which is most commonly used in *P. pastoris*. The signal peptide is responsible for translocation of the pro-protein into the ER and is subsequently cleaved by signal peptidase during the translocation process, and then the pro-protein is transported to the Golgi where the pro-region is cleaved by Kex2 protease to release the mature protein. The α-factor signal peptide comprises a pre-sequence signal peptide of nineteen amino acids (MRFPSIFTAVLFAASSALA) and a 60 hydrophilic amino acid pro-region. However the intrinsic leader peptide of XynC comprises only a signal peptide of twenty amino acids (MFKFSASLAALAALVPFVAA) without the hydrophilic pro-region and Kex2 cleavage site. No sequence similarity is also found between the pre-sequence of α-factor signal peptide and the intrinsic leader peptide of XynC. It is known that yeasts *S. cerevisiae* and *P. pastoris* have low specificity requirement for signal sequence recognition (9,10). There is, therefore, no definitive means to pre-determine whether a native signal will result in secretion as the success varies significantly.

To purify the enzyme product, culture media were directly applied on his-tag column Histrap (GE Healthcare) and eluted out, as per manufacturer's recommendations. Most of the enzyme fusion could be eluted well (Fig. 2). However, the molecular mass of purified XynC on polyacrylamide gel was approximately 55 kDa, larger than the predicted molecular mass of 43 kDa (403 amino acids, including 379 amino acids of mature XynC and 24 amino acids of extension fragment) for pPICZB and pPICZαA. The discrepancies between the predicted and recombinant enzymes seem to be due to post-translational modifications, such as glycosylation (16–18). Using the program support for detection of O-beta-GlcNAc attachment sites in eukaryotic protein sequences (http://www.cbs.dtu.dk/services/YinOYang), one sequence on XynC: Ser-Gly-Thr-Ser-Ser-Ala-Gly-Gly-Ser-Thr-Pro-Ser-Ser was considered as an O-glycosylation site, while there is no *N*-glycosylation site which can be determined on XynC sequence. To confirm that the purified proteins were indeed

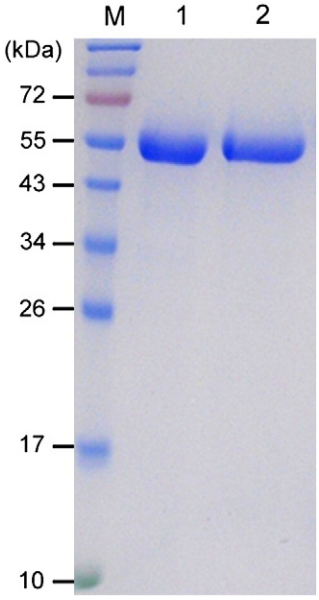

FIG. 2. SDS–PAGE analysis of XynC purified from *P. pastoris*. M, Molecular weight markers; Lane 1, pPICZαA/*XynC*; Lane 2, pPICZB/ss-*XynC*.

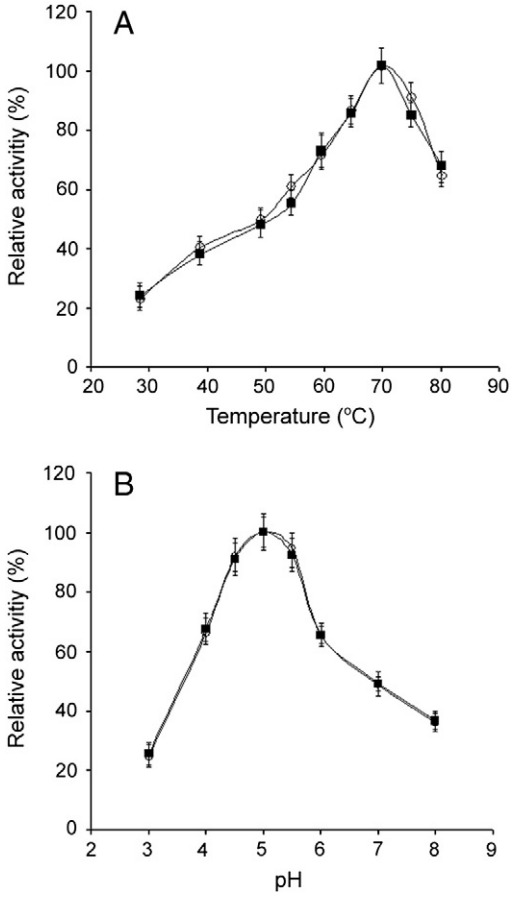

FIG. 3. Effect of temperature (A) and pH (B) on enzyme activity. The closed squares represent XynC secreted by the intrinsic secretion signal (pPICZB/ss-*XynC*), and the open circles represent XynC secreted by the α-factor secretion signal (pPICZαA/*XynC*).

XynC, we performed matrix-assisted laser desorption/ionization mass spectrometry. The purified XynCs were subjected to SDS–PAGE and digested by trypsin, and peptide mapping of the fragments performed by mass spectrometry confirmed the identity of the purified proteins (data not shown).

The optimum temperature of recombinant XynC was determined, by assay, within a temperature range of 30–80°C (Fig. 3A). The results showed that recombinant XynC exhibited maximum activity at 70°C, which is similar to XynC from *P. chrysosporium* expressed in *A. niger* (8). Concerning the effect of the pH on XynC, a pH range from 3.0 to 8.0 was generated; enzymatic assay suggested optimal activity at approximately pH 5.0 (Fig. 3B). The optimum pH is comparable with XynC from *P. chrysosporium* from a previous report (8). Specific activity of XynC at the optimum condition was 302 $U \cdot mg^{-1}$, while the Km and Vmax values of XynC were 15.9 and 12.92, respectively.

The present study has reported the cloning of the gene that encodes XynC from *P. chrysosporium*, and its first expression in *P. pastoris*, which can utilize methanol as its sole carbon and energy source. Heterologous expression of XynC, present in this study, makes it possible to over-produce XynC without any contamination by other xylolytic enzymes. The enzyme produced in this study offers a candidate for use in biobleaching or the enzymatic pretreatment of biomass prior to utilization.

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