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Characterization of three novel thermophilic xylanases from *Humicola insolens* Y1 with application potentials in the brewing industry

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HIGHLIGHTS

- One xylanase and three GH10 xylanase genes were identified in *Humicola insolens* Y1.
- The genes had identities of <83% to known fungal xylanases and ⩽38% to each other.
- The natural xylanase was identical to one of the deduced proteins in sequences.
- The natural and recombinant xylanases had similar enzyme properties.
- Recombinant xylanase combination showed better mashing performance than Ultraflo.

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ABSTRACT

Three xylanase genes (*xynA*, *xynB*, *xynC*) of glycosyl hydrolase family 10 were identified in *Humicola insolens* Y1. The deduced protein sequences showed the highest identity of ⩽83% to known fungal xylanases and of ⩽38% with each other. Recombinant XynA–C produced in *Pichia pastoris* showed optimal activities at pH 6.0–7.0 and at high temperature (70–80 °C), and exhibited good stability over a broad pH range and temperatures at 60 °C. The gene *xynC* produced by *H. insolens* Y1 (named XynW) was similar in enzyme properties with XynC expressed by *Pichia*. XynA exhibited better alkaline adaptation and thermostability, and had higher catalytic efficiency and wider substrate specificity. Under simulated mashing conditions, addition of XynA–C showed better performance on filtration acceleration (37.4%) and viscosity reduction (13.5%) than Ultraflo from Novozyme. Thus the three xylanases represent good candidates for application in the brewing industry.

1. Introduction

Plant cell wall consists mainly of cellulose, hemicellulose, lignin and pectin (Polizeli et al., 2005; Prade, 1996). Xylan as the major component of hemicellulose is composed of a backbone of β-1,4-linked D-xylopyranosyl residues and side chains of different substituents. The complete breakdown of xylan requires a variety of hydrolytic enzymes, including two backbone-hydrolyzing enzymes endo-β-1,4-xylanase and β-D-xylosidase, and five debranching enzymes α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase, and feruloyl or coumaroyl esterase (Chávez et al., 2006). Among them, endo-β-1,4-xylanase is the crucial enzyme in xylan deg- radation.

Enzymatic hydrolysis of xylan has become attractive due to its biotechnological applications in the food, animal feed, waste treatment, ethanol production, textile, and pulp and paper industries (Collins et al., 2005). For commercial purposes, many xylanases have been highly expressed in heterologous systems, such as *Escherichia coli*, *Bacillus* spp. and *Pichia pastoris* (Prade, 1996; Jhamb and Sahoo, 2012). The most widely used xylanases are from the fungal genera of *Trichoderma*, *Aspergillus* and *Penicillium*, and these enzymes are generally highly active over a temperature range of 40–60 °C (Ahmed et al., 2009). At these temperatures, complete saccharification of biomass polysaccharides requires a long reaction time with high contamination risks (Berka et al., 2011). Thus high-temperature active xylanases are necessary to enhance the mass transfer and reduce the substrate viscosity (Margaritis and Merchant, 1986).

Thermophilic *Humicola* spp. are well-known microbial sources for their capacity to produce xylanases (Anand and Vithayathil,

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1996; Luo et al., 2012). In this study, direct purification of one xylanse and heterologous expression of three xylanases of *Humicola insolens* Y1 were reported. The xylanases showed optimal activities at high temperature and better stability under alkaline conditions. Moreover, the enzyme combination showed better performance than commercial enzyme under simulated mashing conditions.

2. Methods

2.1. Strains, media, vectors and chemicals

The *H. insolens* Y1 donor strain was isolated from forest soil sample (Luo et al., 2012). *E. coli* Trans1-T1 (TransGen, Beijing, China) was cultivated in Luria-Bertani (LB) medium at 37 °C for gene cloning and sequencing. *P. pastoris* GS115 (Invitrogen, Carlsbad, CA) cultivated in yeast peptone dextrose (YPD) medium at 30 °C was used for gene expression. The plasmids pGEM-T Easy (Promega, Madison, WI) and pPIC9 (Invitrogen) were used as cloning and expression vectors, respectively.

Birchwood xylan, beechwood xylan, barley β-glucan, carboxymethyl cellulose sodium (CMC-Na) and Avicel were purchased from Sigma–Aldrich (St. Louis, MO). Soluble and insoluble wheat arabinoxylan were obtained from Megazyme (Wicklow, Ireland). The DNA purification kit, LA *Taq* DNA polymerase and restriction endonucleases were purchased from TaKaRa (Otsu, Japan). T4 DNA ligase was from New England Biolabs (Hitchin, UK). All chemicals were of analytical grade and commercially available.

2.2. Induction, purification and identification of one natural xylanase (XynW)

To induce xylanase production, *H. insolens* Y1 was grown in the wheat bran medium containing 5 g/L NaCl, 5 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L $CaCl_2$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 10 g/L agar and 24 g/L wheat bran at 45 °C for 6 days. The induced culture was harvested by centrifugation at 12,000*g*, 4 °C for 10 min to remove cell debris. The cell-free supernatant was concentrated by ultrafiltration with Vivaflow 200 ultrafiltration membrane of 5-kDa molecular weight cut-off (Vivascience, Hannova, Germany). The crude enzyme was then dialyzed against buffer A (20 mM Tris–HCl, pH 7.0) and loaded onto a HiTrap Q Sepharose XL FPLC column (GE Healthcare, Uppsala, Sweden) which was previously equilibrated with buffer A. Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer at a flow rate of 4 mL/min. Fractions exhibiting xylanase activity were combined, concentrated and loaded onto a Superdex 75 10/300 GL column (GE Healthcare) that was equilibrated with buffer B (50 mM McIlvaine buffer, pH 6.0). Proteins were eluted at a flow rate of 0.5 mL/min. The fractions with enzyme activity were collected, concentrated and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The protein concentration was determined by Bradford assay, using bovine serum albumin as the standard.

Purified XynW was excised from the SDS–PAGE gel and digested with trypsin. The peptide-fragments were subject to Nano liquid chromatography–electrospray ionization-collision induced dissociation–tandem mass spectrometry (LC–ESI–CID–MS/MS) analysis using a LTQ Orbitrap Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) at Jisijiayang Science & Technology (Beijing, China).

2.3. Cloning of the full-length genomic sequences of xylanase-encoding genes

Genomic DNA of *H. insolens* Y1 was extracted with Fungal DNA Isolation Kit (Omega Bio-tek, Norcross, GA). The core region of the xylanase-encoding genes were amplified by the degenerate primers XCP1 and XCP2 (Table S1) specific for glycosyl hydrolase (GH) family 10 xylanases of filamentous fungi with the genomic DNA of strain Y1 as the template. The PCR products were ligated into the pGEM-T Easy vector for sequencing and BLAST analysis. The 5′ and 3′ flanking regions of the core regions were obtained using thermal asymmetric interlaced (TAIL)–PCR technique (Liu and Whittier, 1995). The PCR products with appropriate size were sequenced and assembled with the core regions to obtain the full-length genes.

2.4. Cloning of the cDNAs of xylanase-encoding genes

Mycelia of strain Y1 were collected after 3 days' growth on wheat bran agar plate as described above and ground to a fine powder in liquid nitrogen. The total RNA was extracted and purified using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNAs were synthesized *in vitro* using the ReverTra Ace-α-™ kit (TOYOBO, Osaka, Japan) with the total RNA as the template. The full-length cDNAs of xylanase-encoding genes were amplified using the specific primers (Table S1), which were designed based on the genomic DNA sequences. The specific PCR products were ligated into the pGEM-T Easy vector for sequencing.

2.5. Sequence analysis

The nucleotide and protein sequences were aligned using the BLASTn and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. The nucleotide sequence was analyzed using the NCBI ORF Finder tool (http://www.ncbi.him.nih.gov/gorf/gorf/gorf.html). The sequence assembly was performed using the Vector NTI Advance 10.0 software (Invitrogen). Genes, introns, exons and transcription initiation sites were predicted using the online software FGENESH (http://linux1.softberry.com/berry.phtml). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The potential *N*-glycolyzation sites were predicted online (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequence alignments were performed with ClustalW (Thompson et al., 1994). Homology modeling and calculations of protein ionization and electrostatic potential were performed using the Accelrys Discovery Studio software (DS 2.5, http://www.accelrys.com).

2.6. Heterologous expression in P. pastoris

The full-length cDNAs of xylanase-encoding genes without the signal peptide-coding sequences were amplified with expression specific primers (Table S1) and digested with *Eco*RI and *Not*I, then cloned into the pPIC9 vector in-frame fusion of the α-factor signal peptide to construct the recombinant plasmids. The recombinant plasmids were linearized using *Bgl*II and transformed into *P. pastoris* GS115 competent cells by electroporation, respectively. The positive transformants were screened based on their enzymatic activities in shake tubes, and transformants exhibiting the highest activities were selected for fermentation in a 1-L Erlenmayer flask as described by Luo et al. (2012).

2.7. Purification of recombinant xylanases

The cell-free culture supernatants were collected as described above and loaded onto the HiTrap Q Sepharose XL FPLC column equilibrated with buffer A (20 mM Tris–HCl, pH 8.0). Proteins were eluted with NaCl gradients (0–1.0 M) in the same buffer at a flow rate of 3 mL/min. Fractions exhibiting xylanase activity were