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Molecular Cloning and Heterologous Expression of an Acid-Stable Endoxylanase Gene from *Penicillium oxalicum* in *Trichoderma reesei* S

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An endoxylanase gene (*PoxynA*) that belongs to the glycoside hydrolase (GH) family 11 was cloned from a xylanolytic strain, *Penicillium oxalicum* B3-11(2). *PoxynA* was overexpressed in *Trichoderma reesei* QM9414 by using a constitutive strong promoter of the encoding pyruvate decarboxylase (*pdc*). The high extracellular xylanase activities in the fermentation liquid of the transformants were maintained 29~35-fold higher compared with the wild strain. The recombinant POXYNA was purified to homogeneity, and its characters were analyzed. Its optimal temperature and pH value were 50°C and 5.0, respectively. The enzyme was stable at a pH range of 2.0 to 7.0. Using beechwood as the substrate, POXYNA had a high specific activity of 1,856 ± 53.5 IU/mg. In the presence of metal ions, such as Cu^{2+}, and Mg^{2+}, the activity of the enzyme increased. However, strong inhibition of the enzyme activity was observed in the presence of Mn^{2+} and Fe^{2+}. The recombinant POXYNA hydrolyzed birchwood xylan, beechwood xylan, and oat spelt xylan to produce short-chain xylooligosaccharides, xylopentaose, xylotriose, and xylobiose as the main products. This is the first report on the expression properties of a recombinant endoxylanase gene from *Penicillium oxalicum*. The properties of this endoxylanase make it promising for applications in the food and feed industries.

Key words: *Penicillium oxalicum*, xylanase, constitutive expression, pyruvate decarboxylase promoter, *Trichoderma reesei*

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Hemicellulose is widely present in plant cell walls. Xylan is the main component of hemicellulose and the second most abundant polysaccharide in nature. Among xylanolytic enzymes, endo-β-1,4-xylanase (E.C. 3.2.1.8) is the most foremost, which can catalyze the hydrolysis of long-chain xylan into xylose and short xylooligosaccharides of different sizes, which decreases the degree of polymerization of the substrate [2]. Xylanolytic enzymes are produced by fungi, bacteria, yeast, marine algae, protozoans, snails, crustaceans, insect, and seeds, but the principal commercial source is filamentous fungi [30]. According to sequence features and hydrophobic cluster analysis, xylanases are divided into different families. Most xylanases belong to the glycoside hydrolase families 10 and 11 [10]. These two families differ in enzyme action, molecular weight, and electric charge [11].

Xylanases have been applied in a variety of industrial processes [2]. In the food industry, xylanases are used as food additives to improve the dough handling and the quality of baked products [26]. In the feed industry, xylanases are used to enhance feed digestibility in the rumen of cattle, thereby reducing the FCR (feed conversion rate) [4]. Xylanases are also applied in textiles, bleaching of cellulose pulp, and ethanol and xylitol production [35].

Recently, numerous xylanases that have application in the above fields have been isolated and characterized from fungi and bacteria [15, 25, 42]. Thus, exploring new xylanases is still a current research trend [25]. This paper reports a xylanase from *Penicillium oxalicum* B3-11(2), a strain that was isolated from mangrove soil with significant xylanase activity. We describe the procedure of gene cloning, and its expression in *Trichoderma reesei* by using a strong constitutive promoter reported in our previous study [19], and the characterization of the purified xylanase POXYNA. At present, this is the first

report on the cloning and characterization of a xylanase gene from *P. oxalicum*. The recombinant xylanase has some superior properties, which makes POXYNA a good candidate for application in various industries.

Materials and Methods

Microorganism Isolation and Identification

The soil samples collected from the Mangrove Nature Reserve (located 114°03' E, 22°32' N, in Shenzhen, Guangdong Province, China) were diluted in sterile dilution solution (0.9% saline), aliquots were spread on modified Martin Agar Medium that contained 1% birchwood xylan as the sole carbon source, and the plates were incubated at 28°C for 48 h. Colonies harboring xylanase activity were determined using the xylan-Congo red clearance plate assay [39] based on their ability to produce a transparent zone on the selective plates. One strain, namely B3-11(2), with significant xylanase activity was selected for further study.

The difference in the metabolic rate of the strain in different carbon sources was analyzed by a microbial automatic analyzer (Biolog, USA) and the identification of the isolated strain was determined by using the Microlog system software, Release 6.0. For the sequence analysis, the ITS1-5.8 S-ITS2 rDNA region of the fungus was amplified by polymerase chain reaction (PCR) through the use of primer sets pITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and pITS4 (5'-TCCTCCGCTTATTGATATGC-3') [38]. The 593 bp amplicon thus obtained was cloned and sequenced.

Strains, Plasmids, and Cultivation Conditions

Escherichia coli (*E. coli*) Top10F' (Invitrogen, USA) was used for plasmid construction and maintenance. *T. reesei* QM9414 (ATCC 26921) was used as a parental strain throughout the study. The *E. coli* strain was cultivated in LB medium, in which ampicillin (100 μg/ml; Invitrogen) was supplemented when necessary.

The *T. reesei* and *P. oxalicum* strains were maintained on potato dextrose agar (PDA), and for liquid cultivation, grown in Mandel's medium that contained 2% glucose [24]. *P. oxalicum* produced xylanase with 30 g/l corncob powder as the inducer.

The recombinant *T. reesei* strains were placed on PDA agar supplemented with hygromycin B (100 μg/ml), and for recombinant xylanase production, the strains were cultivated in a modified Mandel's medium supplemented with 7% glucose, 5% soybean powder, and 1% peptone. *E. coli* was routinely cultured at 37°C, whereas *T. reesei* and *P. oxalicum* strains were cultured at 28°C. Plasmid pUC19 was used for the construction of *PoxynA* expression cassettes. Plasmid pAN7-1, which contained the hygromycin B resistant cassette, was used as an assisting plasmid for the transformation of *T. reesei* [31].

Chemicals and Reagents

PrimeSTAR HS DNA polymerase, TaKaRa LA Taq, plasmid pUC19, and all restriction enzymes used in this study were purchased from Takara Bio Inc. (Japan).

Substrates sodium carboxymethyl cellulose (CMC-Na), Avicel, and xylans from birchwood, beechwood, and oat spelts were purchased from Sigma (USA). Filter paper was purchased from Whatman (Germany). Corncob and wheat bran were purchased from Gao Tang Biotechnology Co., Ltd (Shandong, China). Mannan, locust bean gum, and konjac powder were purchased from Jiang Lai Biotechnology Co., Ltd (Shanghai, China). Xyloglucan oligosaccharide standards were purchased from Glycobio Co., Ltd. (Dalian, China). All other chemicals were of analytical grade.

DNA Isolation

Total cellular DNA from *P. oxalicum* B3-11(2) and *T. reesei* QM9414 was isolated by a Wizard Genomic DNA Purification kit (Promega, USA) by following the manufacturer's protocols.

Cloning of *PoxynA*

Degenerate primers (D1, D2) were designed for PCR amplification based on the conserved regions of the gene encoding family 11 xylanases of the *Penicillium* species. Then, thermal asymmetric interlaced polymerase chain reaction (TAIL PCR) [22] was performed to amplify the whole sequence of *PoxynA* by using 4 arbitrary degenerate primers of the Genome Walking Kit (TaKaRa, Dalian China) and 6 specific primers (3 UT-primers for upstream and 3 DT-primers for downstream amplifications). The complete DNA of *PoxynA* was amplified by using primers C1 and C2 based on the putative coding region. The primers used are listed in Table 1.

Construction of P*pdc*-*PoxynA*-T*pdc* Expression Cassette

The promoter (P*pdc*, 1,534 bp upstream fragment starting from the start codon of *pdc*) and the terminator of the *pdc* gene (T*pdc*, 1,030 bp downstream fragment starting from the stop codon of *pdc*) were amplified by using the primers P*pdc*-F, P*pdc*-R, and T*pdc*-F, T*pdc*-R, respectively (listed in Table 1), and using the genomic DNA of *T. reesei* QM9414 as the template. The signal peptide sequence of *cbh I* from *T. reesei* (S_{TrCbh1}) was used to construct the

Table 1. Primers used for cloning of *PoxynA* and the promoter and terminator of the PDC gene.

Primer name	Sequences[a]
D1	5'-TAYTCBTTYTGGACVRAYGG-3'
D2	5'-AARTGRTTNSCVGTRGTVAC-3'
UT1	5'-TTGACCTGCGTGTGCTTGTAGATA- 3'
UT2	5'-GTTGTAGCTTCCGAAGTTCTCCAGA-3'
UT3	5'-CGTTTGCCTTTGCTGCACTTACTGA -3'
DT1	5'-ACGGGCACCAACAACGGCTATTATTA-3'
DT2	5'-ACCTCGCATCAGGGACATCTCTTTCT-3'
DT3	5'-CTGACCAGTGACGGTTCGGACTATGA-3'
C1	5'-ATGATCTCCCTCTCCTCCGTGGCA-3'
C2	5'-CTACAGGCACTGGGAGTACCAT-3'
P*pdc*-F	AACTGCAGAGGACTTCCAGGGCTACTTGG
P*pdc*-R	TGACGGCCAACTTCCGATACATGATTGT GCTGTAGCTGCGCT
T*pdc*-F	CACCACCATCACCAC TAA CCCGGCATG-AAGTCTGAC
T*pdc*-R	GC TCTAGA TGGACGCCTCGATGTCTTCCTCT

[a]Restriction sites are underlined; overlapping regions in P*pdc*-F and T*pdc*-F are double underlined.

expression cassette. Exons 1, 2, and 3 of the *PoxynA* gene and 6× Histidine (His) Tag were fused by overlapping extension PCR. P*pdc*, S_{TrCbh1}, *PoxynA* gene with 6×His Tag, and T*pdc* were fused three times by overlapping extension PCR. The fusion product was amplified by PCR to regions upstream and downstream of the *Pst*I and *Xba*lI sites, respectively, which was cloned into a pUC19 vector and generated recombinant plasmid pUC19-P*pdc*-*PoxynA*.

Protoplast Preparation and Transformation of *T. reesei*

Protoplast preparation and transformation of *T. reesei* were performed by using the polyethylene glycol method as described in Penttilä *et al.* [28]. Lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich) were used in the *T. reesei* protoplast preparation. For the transformation, the expression cassettes were released from the plasmids through digestion with restriction enzyme *Xba*lI, and then purified and mixed with equal amounts of plasmid pAN7-1. The mixture was then used for the co-transformation of *T. reesei* protoplasts. Candidate transformants were streaked twice onto PDA plates that contained 100 μg/ml of hygromycin B, and then transferred to PDA plates to form conidia.

Purification of POXYNA

Transformants were spread onto PDA selection plates that contained 100 μg hygromycin B/mL and purified through a single spore culture for further analysis and cultured in shake flasks (200 rpm) in a modified Mandel's solution for 7 days at 28°C. After growth, culture supernatants were collected by filtration and used for enzymatic purification and analysis. Extracellular filtrate (1 L) from positive transformants of *T. reesei* QM9414 *PoxynA* was concentrated by using a Pellicon ultrafiltration device (Millipore Corporation, USA) fitted with a polyethersulfone membrane with a 10 kDa cut-off. Then, ammonium sulfate was added to the concentrated extract until the saturation was 75%. After 2 h at 4°C, the extract was centrifuged at 13,523 ×*g* for 10 min and the precipitation was collected. The concentrated material was freeze-dried and resuspended in 0.05 M Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer, 0.5 M sodium chloride (NaCl), at pH 8.0. The suspension was applied to a His-tag affinity chromatography column (chelating Sepharose fast flow prepacked columns XK-16, 1.6×60 cm; General Electric Company, USA) by a fast protein liquid chromatography (FPLC) system (General Electric Company, USA). The protein was eluted with a step gradient of 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 8.0) with 0.5 M imidazole at a flow rate of 1 ml/min. The collected fractions were eluted with a sodium acetate buffer (0.05 M, pH 4.8). A purified protein sample was centrifuged at 5,000 rpm for 20 min by a Pellicon ultrafiltration device (10,000 MW) for desalting and concentrating. Protein levels in the extracts were determined by using a Bradford reagent (Sangon Biotech, Shanghai, China).

Electrophoresis and Immunoblotting

Purified preparations of enzyme samples were analyzed for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel [18]. The protein concentration was determined by using the Bradford method [3] with bovine serum albumin as a standard. The bands were visualized by staining with Coomassie Brilliant Blue G250. Protein extract (60 μg) was loaded and separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Whatman, Germany). Western blotting and immunodetection of the *PoxynA* gene product were performed by a histidine tag antibody (mouse monoclonal antibody) and secondary antibody (alkaline phosphatase-conjugated Affinipure Goat Anti-Mouse IgG (H/L) (Protein Tech Group, Inc., Chicago, USA) by using the protocol specified by the manufacturer.

Enzyme Assays

For recombinant xylanase production, about 10^5 spores of the recombinant *T. reesei* strains were inoculated into 30 ml of Mandel's medium, and maintained at 28°C and 250 rpm for 48 h. Then, 1.5 ml of the above culture was transferred into 30 ml of a modified Mandel's medium and maintained at 28°C and 250 rpm for about 168 h. In the modified minimal medium, the glucose concentration was 7%, and a concentration of 1% peptone, and 5% soybean powder was added. Xylanase activity was assayed as described in Bailey *et al.* [1]. with 1% birchwood xylan as the substrate in 50 mM sodium pyrophosphate (Na_2HPO_4)-citric acid buffer, at pH 5.0, and incubated at 50°C for 10 min. Appropriate dilutions of the recombinant protein (culture supernatant) in 50 mM sodium citrate buffer (pH 5.0) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method described by Miller [27]. Xylanase activity was calculated from the calibration curve constructed by using D-xylose as the standard. One unit of enzyme activity was defined as the quantity of enzyme required to liberate 1 μmol of xylose equivalent per minute at 50°C, and specific activity was defined as units per milligram protein. Protein concentrations were measured with a method put forth by Sedmak and Grossberg [34].

Biochemical Characterization of the Purified Recombinant POXYNA

The optimal temperature for the xylanase enzyme was obtained by assaying the enzyme activity at temperatures that ranged from 30–80°C. The thermostability of POXYNA was determined under standard conditions (pH 5.0, 50°C, 10 min) after pre-incubation of the enzyme solution at temperatures 30°C, 40°C, and 50°C for various periods without substrate. Assays at different pH values were performed at an optimal temperature over a pH range of 1.0–8.0. Two different buffers (0.05 M) were used. A KCl-HCl buffer (0.05 M) was used for pH 1–2. A phosphate citrate buffer (0.05 M) was used for pH 2–8. The pH stability of recombinant POXYNA was determined under standard conditions after pre-incubation of the enzyme solution in the buffers mentioned above without substrate at 30°C for 1 h. The enzyme was respectively incubated with 10 mM solution of Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Na^{+}, K^{+}, and Li^{+}, and cysteine, ethylene diamine tetraacetic acid (EDTA), glycerin, and SDS at 30°C for 1 h. The system without any chemicals was treated as the control. Residual activity was measured under standard conditions. Each experiment was performed in triplicate.

Substrate Specificity Analysis

The substrate activity of purified recombinant POXYNA to different substrates was tested by measuring its enzyme activity against various substrates. For the substrate specificity study, each substrate had a concentration of 1.0% (w/v) and was placed in 50 mM Na_2HPO_4-citric acid buffer, at pH 5.0, and individually incubated at 50°C for 10 min. Xylanase activity was calculated per the method above.

Kinetic Parameters

The K_m and V_{max} values of POXYNA were determined by using 1–20 mg/ml of xylan from birchwood, beechwood, and oat spelts, respectively. The data were plotted according to the Lineweaver and Burk [20] method. Each experiment was repeated three times.

Analysis of Hydrolytic Products

The xylan-hydrolyzed products were analyzed by thin-layer chromatography (TLC) on plates of silica gel 60F 254 (E. Merck, Germany). Aliquots (100 ml) of the samples were collected at different times of the incubation period and 1 ml of the aliquot was spotted on the TLC plates. The plates were subsequently developed with two runs of acetonitrile–water [85:15 (v/v)] followed by heating for a few minutes at 130°C in an oven after spraying the plates with a methanol–sulfuric acid mixture [95:5 (v/v)] [13]. A xylooligosaccharide mixture that consisted of xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose was used as the standard.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the *Penicillium oxalicum* B3-11(2) rDNA, xylanase gene (*PoxynA*), and deduced amino acid sequence have been deposited in the GenBank database under the accession numbers JQ446378.1, HQ680966.1, and ADV31286.1 respectively.

RESULTS

Microorganism Identification

Strain B3-11(2) was isolated from soil samples collected from the Mangrove Nature Reserve, Shenzhen, Guangdong Province, China. The typical broom-like branch of the *Penicillium* species was observed by an optical microscope. The difference in the metabolic rate of the strain in different carbon sources was analyzed by a microbial automatic analyzer (Biolog, USA). The metabolic rates of the strain cultured at 48 h and 72 h were almost similar to those of *P. oxalicum*. The amplified internal transcribed spacer (ITS) rDNA gene sequence was compared with the ITS sequences in the GenBank database and the results indicated an identification of 100% with *P. oxalicum* isolate 413 (GenBank Accession No. GU724348.1), *P. oxalicum* strain ME28-1(GenBank Accession No. HM130515.1), and *P. oxalicum* strain RCEF4908 (GenBank Accession No. HM053477.1). Therefore, strain B3-11(2) is considered as a strain of *P. oxalicum*.

Cloning and Analysis of *PoxynA*

A full-length of DNA (1,073-bp) of *PoxynA* (GenBank Accession No. HQ680966.1), which encodes a protein of 310 amino acids, was cloned from *P. oxalicum* B3-11(2). On the basis of amino acid sequence similarities, this enzyme could be assigned to family 11 of the glycosyl hydrolase classification system. The deduced amino acid sequence of POXYNA showed high homologies with *Aspergillus fumigatus* Af293 (GenBank Accession No. XP_748367.1) (identity 77%), *Penicillium canescens* (GenBank Accession No. ACP27609.1) (identity 76%), and *Neosartorya fischeri* NRRL 181 (GenBank Accession No. XP_001258654.1) (identity 76%). The N-terminal region of POXYNA contained a signal peptide sequence of 19 amino acid residues, which indicates that it is an extracellular enzyme. The C-terminal region of POXYNA contained a common termination sequence "YSQCL" of glycosidic enzymes. The deduced protein POXYNA contained a GHF 11 catalytic domain and a carbohydrate-binding module (CBM) that belongs to family 1 (CBM1) which was connected by a Ser-Thr linker region.

Expression and Purification of the Recombinant Xylanases in *T. reesei*

We employed *T. reesei* QM 9414 as the host strain for the heterologous expression of the *xyn*A gene from *P. oxalicum*. The host strain was co-transformed with pAN7-1 and the linearized expression plasmid pUC19-P*pdc*-*PoxynA* that harbored the *xyn*A coding sequence with a signal peptide sequence of *cbh I* from *T. reesei* flanked by the *T. reesei pdc* promoter and terminator. Purified recombinant clones were cultivated in shake flasks. Protein secreted into the cultivation media was analyzed by SDS-PAGE (Fig. 1). The Western blot analysis showed only one band (37 kDa) in the locus for each transformant, which is slightly higher than that deduced from the protein sequence (32 kDa). The difference indicated overglycosylation for

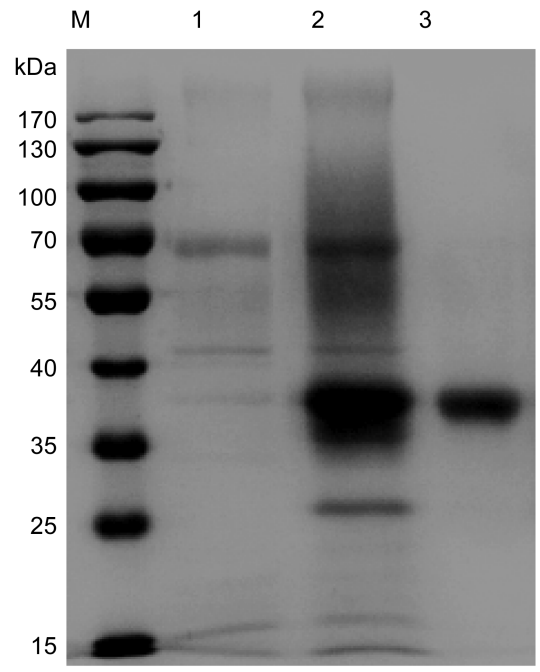

Fig. 1. SDS-PAGE analysis of POXYNA.
M: Marker; lane 1: Culture filtrate QM9414; lane 2: Culture filtrate from positive transformant of recombinant POXYNA; lane 3: purified recombinant POXYNA.

PoxynA expressed in *T. reesei*. The recombinant POXYNA was purified by immobilized metal ion affinity chromatography (IMAC) according to the 6×His Tag in the C terminal of the protein. POXYNA was purified to apparent homogeneity as described earlier.

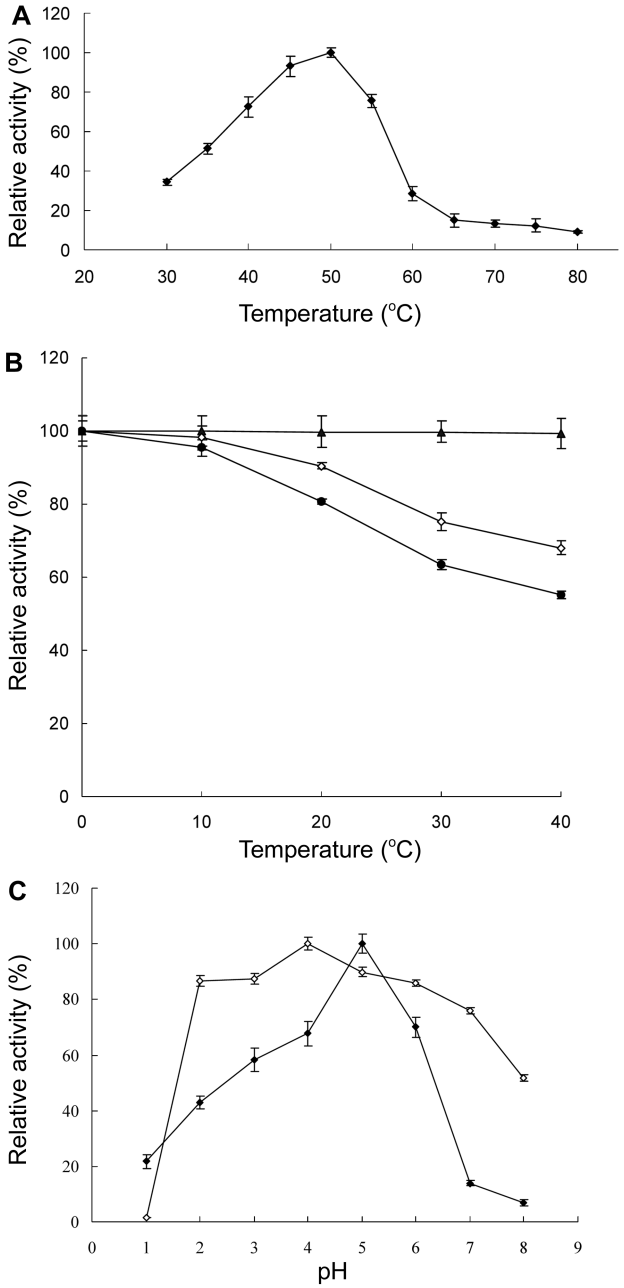

Fig. 2. Properties of the purified recombinant POXYNA.
(**A**) Effect of temperature on xylanase activity of POXYNA. The assay was performed at temperatures that ranged from 30°C to 80°C. (**B**) Temperature stability of POXYNA. The enzyme was pre-incubated at 30°C (▲), 40°C (◇), and 50°C (●) in 50 mM sodium pyrophosphate (Na_2HPO_4)-citric acid buffer (pH 5.0) without substrate. (**C**) Effect of pH on activity and pH stability of POXYNA. For pH effect, assays were performed at an optimal temperature over a pH range of 1.0–8.0 (◆). For pH stability, the enzyme was incubated at 30°C for 1 h in buffers of pH 1.0–8.0, then the activity was measured under standard conditions (◇). The following buffers (0.05 M) were used: KCI-HCI (pH 1–2), phosphate citrate (pH 2–8).

Optimization of Temperature and pH for POXYNA Activity and Stability

The optimum temperature of hydrolytic activity on birchwood xylan was 50°C (Fig. 2A). The enzyme showed relative activity of approximately 75% at 40°C for 30 min, while about 63% of the activity was retained at 50°C for 30 min (Fig. 2B). The pH activity profile of recombinant POXYNA on birchwood xylan showed that the optimum pH is 5.0 (Fig. 2C). The enzyme was stable at pH 2.0 ~ 7.0, retaining more than 80% of the initial activity after incubation at 30°C for 1 h.

Additional Effect of Chemicals on POXYNA Activity

The xylanase activity of purified POXYNA in the presence of 10 mM different metal ions or chemical reagents is shown in Table 2. The activity was almost completely inhibited by SDS and strongly inhibited by Mn^{2+} and Fe^{2+}. Partial inhibition was observed in the presence of some metals and reagents, such as Ca^{2+}, Na^{+}, K^{+}, Li^{+}, Zn^{2+}, and glycerol, whereas the presence of Mg^{2+}, Cu^{2+}, and EDTA was observed to obviously promote activity. Cys has no significant effect on the activity of POXYNA.

Substrate Specificity and Kinetic Parameters

The hydrolytic activities of purified recombinant POXYNA toward various substrates were assayed (Table 3). The recombinant protein showed high activity towards the three kinds of xylan substrates. POXYNA had the highest specific activity to beechwood xylan, up to 1,856 ± 53.5 IU/mg of protein. The recombinant protein showed some activity

Table 2. Effects of metal ions and chemical reagents on the activity of POXYNA.

Chemical (10 mM)	Relative activity (%)
Control	100.0 ± 4.6
Mg^{2+}	108.5 ± 3.4
Cu^{2+}	114.7 ± 2.8
Ca^{2+}	93.8 ± 4.0
Fe^{2+}	67.3 ± 4.6
Mn^{2+}	46.7 ± 2.8
Na^{+}	89.2 ± 3.2
K^{+}	82.8 ± 1.3
Li^{2+}	84.6 ± 4.5
Zn^{2+}	86.9 ± 2.1
Cys	101.7 ± 2.7
EDTA	106.5 ± 2.7
Glycerin	88.5 ± 2.9
SDS	7.1 ± 1.4

Table 3. Hydrolytic activity of recombinant POXYNA towards various glycan substrates.

Substrate	Specific activity (IU/mg of protein)
Xylan from birchwood	1,544 ± 59.2
Xylan from beechwood	1,856 ± 53.5
Xylan from oat spelts	1,522 ± 67.1
Corncob	52.0 ± 7.5
Wheat bran	36.0 ± 8.6
Mannan	ND
Locust bean gum	ND
Konjac powder	2.2 ± 0.1
CMC	ND
Avicel	1.6 ± 0.2
Filter paper	ND

Note: ND means xylanase activity was not detected after 120 h of incubation.

towards corncob powder and wheat bran, a trace of activity towards konjac powder and Avicel, and finally, no activity towards mannan, locust bean gum, CMC, and filter paper. POXYNA has a CBM1 in its C-terminal, which is probably the reason for the trace activities towards konjac powder and Avicel.

Kinetic parameters were determined for the three kinds of xylans. The calculated K_m and V_{max} were 5.0 mg/ml and 2,000 μmol/mg/min, respectively, for birchwood xylan, 4.6 mg/ml and 2,000 μmol/mg/min, respectively, for beechwood xylan, and 11.3 mg/ml and 2,500 μmol/mg/min, respectively, for oat spelt xylan (Table 4).

Analysis of Hydrolytic Products

The hydrolysis products of birchwood, oat spelt, and beechwood xylans from purified recombinant POXYNA were analyzed by TLC. The predominant end products of POXYNA were xylobiose, xylotriose, and xylopentaose, and short-chain xylooligosaccharides (Fig. 3). The products were produced after 1.5 h of reaction. As the reaction time increased, both the xylotriose and xylobiose concentrations increased. Xylotetraose was produced after 1.5 h of reaction, increased at 4.5 h, but decreased after 9 h. The reduced xylotetraose may be broken down into xylobiose. No xylose was found in the production, which indicated that the enzyme is unable to hydrolyze xylobiose. The results confirmed that POXYNA is an endoxylanase.

Discussion

Mangrove fungi play an important part in the decomposition of lingo-cellulose materials in tropical or subtropical coastal ecosystems where mangrove debris is abundant [41]. The activities of lingo-cellulolytic enzymes such as cellulases, laccases, and peroxidases in the mangrove fungi have been reported [29]. However, little attention has been given to xylanase. Yuan *et al*. [41] isolated mangrove fungi and evaluated their capability to produce xylanase

Table 4. Specific activity and kinetic parameters of the purified recombinant POXYNA.

Substrate	Specific activity (IU/mg)	K_m (mg/ml)	V_{max} (μmol/mg/min)
Birchwood xylan	1,544 ± 59.2	5.0	2,000
Beechwood xylan	1,856 ± 53.5	4.6	2,000
Oat spelt xylan	1,522 ± 67.1	11.3	2,500

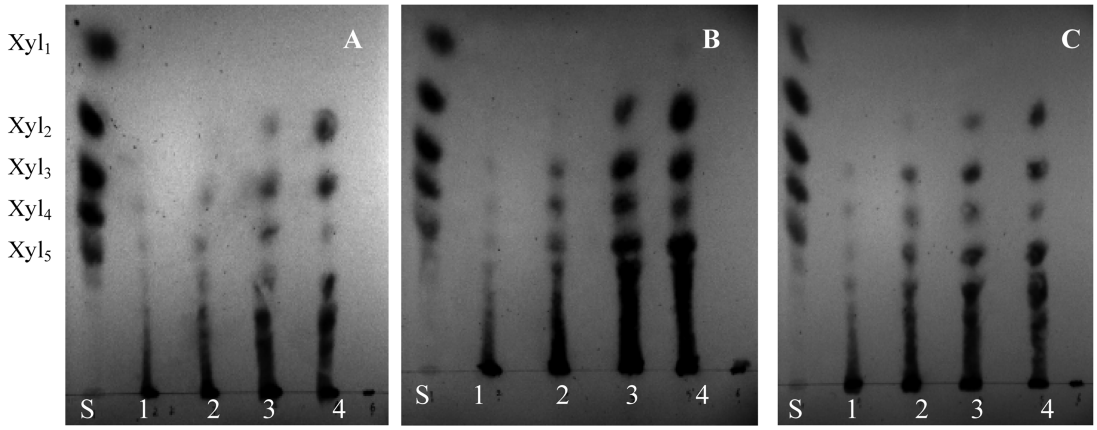

Fig. 3. Thin-layer chromatography of the hydrolysis products from different xylan substrates with purified POXYNA.
(**A**) Birchwood xylan; (**B**) Oat spelt xylan; (**C**) Beechwood xylan. S: Standards. Lanes 1, 2, 3, 4 illustrate POXYNA incubated with substrates for 0.5 h, 1.5 h, 4.5 h, and 9.0 h, respectively.

under different culture and assay conditions. However, few xylanase genes were cloned and expressed until now. In the present study, the strain *Penicillium oxalicum* B3-11(2), isolated from mangrove soil, has significant xylanase activity. This is the first report on the cloning and characterization of a xylanase gene from *P. oxalicum*.

Use of fungal expression systems for production of enzymes by fermentation has a long history in industry [14]. A large number of foreign genes have been expressed in *T. reesei* in recent years, especially genes from filamentous fungi [23, 32, 33, 44]. *T. reesei* is an important native producer of hydrolytic enzymes, including xylanases. However, the production of xylanase is often mixed with cellulase or other hydrolysis enzymes in the process of induced expression. Therefore, constitutive expression, which allows for the high-level production of a single xylanolytic enzyme, has advantages in the production of cellulase-free xylanase and separation of target protein. In the present study, *PoxynA* is the first heterologous gene overexpressed in *T. reesei* by using a constitutive promoter of the *pdc* reported in our previous study [19]. The xylanase activities in the transformant when cultured for 7 days without an inducer reach the maximum, while it required 5 days for xylanase activities in *P. oxalicum* when corncob powder was used as the inducer to reach the maximum. The extracellular xylanase activities in the fermentation liquid of the transformants were maintained 30.87-, 29.27-, and 34.92-folds higher when compared with the wild strain towards xylans from oat spelts, birchwood, and beechwood, respectively (Table 5). There was no cellulase activity in the culture supernatants. The recombinant protein accounted for approximately 61.9% of the total protein secreted by *T. reesei.* The production of recombinant POXYNA was 2.0 g/l, which is almost three times higher than that of xylanase from *Penicillium occitanis* Pol6 expressed in *Pichia pastoris* under the control of the glyceraldehyde 3-phosphate dehydrogenase (GAP) constitutive promoter [6]. These results indicate that *T. reesei* is a suitable host for the expression of xylanases from other filamentous fungi. At any rate, the high expression of POXYNA makes it more economical in xylanase production.

The optimum temperature and the optimum pH of POXYNA were similar to other family 11 endoxylanases from many mesophilic fungi [30]. POXYNA displayed superior properties on acid stability. The enzyme retained more than 80% of the initial activity at pH 2.0 ~ 7.0 after incubation at 30°C for 1 h. Furthermore, POXYNA was relatively stable at 50°C and below. Additionally, the recombinant protein could hydrolyze xylans from different sources, showed high activity to three kinds of substrates (beech wood xylan, birchwood xylan, and oat spelts xylan), a certain activity to wheat bran and corncob, and a trace of activity to konjac powder and Avicel. These enzyme properties suggest that POXYNA could be used as a feed additive working in the gastrointestinal tract under acidic conditions.

Some metal ions and reagents significantly affect xylanase activities. A common trend has been recognized in many cases for enzyme activity negatively affected by heavy metals like Fe^{2+}, Mn^{2+}, Cu^{2+}, and Pb^{2+} and reagents like EDTA as inhibitor, whereas $Ca^{2+}$and Mg^{2+} have been reported to be enzyme activators [25, 40]. In the present study, xylanase activity was assayed in the presence of metal ions and chemicals. Xylanase activities were also negatively affected by Mn^{2+}, Fe^{2+}, Zn^{2+}, K^{+}, Na^{+}, and Li^{+}. The xylanase activity was almost completely inhibited by SDS in this study, as was demonstrated in previous research [5, 9, 21]. Mg^{2+} was an activator to POXYNA in this study. It is predicted that Mg^{2+} ions help stabilize the enzyme–substrate complex, thereby elevating enzyme activity [43]. However, the observed increase in the xylanase activity in the presence of Cu^{2+} is similar to that reported by Fu *et al.* [7] and Driss *et al.* [6]. Cu^{2+} is known to catalyze autooxidation of cysteines, which leads to the formation of intramolecular and intermolecular disulfide bridges or formation of sulfenic acid [37]. It is not certain from these studies whether these ions binding to the enzyme was due to conformational changes resulting in increased enzyme activity. Previous publications reported that EDTA was an inhibitor to some xylanases [6, 21]; however, EDTA, as a chelating agent, enhanced the activity of POXYNA in this study. A similar result was detected in endoxylanase from *Aspergillus usamii* [43] and *Aspergillus niger* IBT-90 [12]. The specific reason is still unknown. The results suggest that xylanases from different sources may have different catalytic mechanisms.

The three-dimensional structures of acidophilic xylanase from *Aspergillus niger* [17], *T. reesei* [36], and *Aspergillus kawachii* [8] have been reported and the importance of the Asp residues for their acidophilic feature were highlighted.

Table 5. Extracellular xylanase activities of transformant and *Penicillium oxalicum*.

Substrates	Transformant (IU/ml) (7^{th} day)	*Penicillium oxalicum* B3-11-2 (IU/ml) (5^{th} day)	Improved multiples
Birchwood xylan	3,064 ± 170.8	87.8 ± 1.3	35
Beechwood xylan	3,033 ± 25.3	103.6 ± 2.6	29
Oat spelt xylan	3,075 ± 84.9	99.6 ± 3.3	31

ASP76, which is common in acidophilic xylanases, is also conserved in POXYNA (ASP78 in the amino acid sequence). Our sequence result also supports the importance of this Asp residue in pH profiles of fungal acid-stable xylanases, similar to acidophilic xylanase from *Penicillium* sp. 40 [16]. These residues are replaced by Asn in other fungal xylanases. However, two sites (ASN73, ASN125) were replaced by Asn in POXYNA compared with the responding sites (ASP59, ASP112) of acid xylanase (GenBank: AAC60542.1) of *Aspergillus kawachii*, which may be the reason that POXYNA is an acid-stable xylanase, but has weak acidophilic feature with optimal pH 5.0. This suggests that the acid stability or acidophilic feature of xylanases depends on the higher structure [16].

In summary, in this study we cloned and obtained the full-length *PoxynA* DNA from *P. oxalicum*. *PoxynA* was constitutively overexpressed in *T. reesei* QM9414. The recombinant POXYNA was purified and its characters were analyzed. POXYNA is an acid-stable xylanase with high specific activity and production. The hydrolyzate of POXYNA to three kinds of xylans were detected by TLC. The results confirm that POXYNA is an endoxylanase. The properties of this xylanase make it a potential economical candidate for use in feed and food industrial applications.

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