

## Regular Paper

# Xylanase from Marine Filamentous Fungus *Pestalotiopsis* sp. AN-7 Was Activated with Diluted Salt Solution Like Brackish Water

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**Abstract:** The genus *Pestalotiopsis* are endophytic fungi that have recently been identified as cellulolytic system producers. We herein cloned a gene coding for a xylanase belonging to glycoside hydrolase (GH) family 10 (*PesXyn10A*) from *Pestalotiopsis* sp. AN-7, which was isolated from the soil of a mangrove forest. This protein was heterologously expressed by *Pichia pastoris* as a host, and its enzymatic properties were characterized. *PesXyn10A* was produced as a glycosylated protein and coincident to theoretical molecular weight (35.3 kDa) after deglycosylation by peptide-*N*-glycosidase F. Purified recombinant *PesXyn10A* exhibited maximal activity at pH 6.0 and 50 °C, and activity was maintained at 90 % at pH 5.0 and temperatures lower than 30 °C for 24 h. The substrate specificity of *PesXyn10A* was limited and it hydrolyzed glucuronoxylan and arabinoxylan, but not  $\beta$ -glucan. The final hydrolysis products from birchwood xylan were xylose, xylobiose, and 1,2<sup>3</sup>- $\alpha$ -D-(4-*O*-methyl-glucuronyl)-1,4- $\beta$ -D-xylotriose. The addition of metallic salts (NaCl, KCl, MgCl<sub>2</sub>, and CaCl<sub>2</sub>) activated *PesXyn10A* for xylan degradation, and maximal activation by these divalent cations was approximately 160 % at a concentration of 5 mM. The thermostability of *PesXyn10A* significantly increased in the presence of 50 mM NaCl or 5 mM MgCl<sub>2</sub>. The present results suggest that the presence of metallic salts at a low concentration, similar to brackish water, exerts positive effects on the enzyme activity and thermal stability of *PesXyn10A*.

**Key words:** GH10 xylanase, *Pestalotiopsis*, marine fungus, MeGlcA<sup>3</sup>Xyl<sub>3</sub>, thermal stability, metallic salt

## INTRODUCTION

The plant cell wall is attractive feedstock for biorefineries because it contains abundant amounts of polysaccharides, such as cellulose and hemicellulose, and phenolic compounds, including lignin. Cellulose is a major component of the plant cell wall and a polymer in which the glucose unit is polymerized by the  $\beta$ -1,4-glucosidic linkage. Although the degrees of crystallinity and polymerization vary depending on the plant origin and part of the cell wall, the primary structure of cellulose is simple regardless of the plant species. In contrast, the structure of hemicellulose

strongly depends on the plant species and is more complex in composition and linkage type of constituent sugars. Xylan is the most abundant polysaccharide in hardwood hemicellulose, and forms the  $\beta$ -1,4-linked D-xylopyranosyl main chain. In glucuronoxylan or arabinoglucuronoxylan, D-glucuronic acid (GlcA) or 4-*O*-methyl-D-glucuronic acid (MeGlcA) residues modify to the backbone by  $\alpha$ -1,2-glycosidic linkages. Furthermore, acetyl groups are ester-linked at position C2, C3 or C2/C3 of xylopyranose.<sup>1)</sup> These modifications considered to be prevented the decomposition of the xylan chain by xylanase.

Endo- $\beta$ -1,4-xylanase (EC 3.2.1.8) is an enzyme that randomly cleaves the  $\beta$ -1,4-xylosidic linkage of xylan and releases  $\beta$ -anomer products. Many xylanases have been reported from a wide range of organisms, such as archaea, bacteria, and eukaryotes, and are categorized into glycoside hydrolase (GH) families 5, 8, 10, 11, 30, and 43 in the Carbohydrate-Active enZYmes (CAZy) database (www.CA-Zy.org).<sup>2)</sup> GH10 and GH11 xylanases are major xylanolytic enzymes in most microorganisms. GH10 xylanase has a molecular weight of more than 30,000 and its core struc-

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Abbreviations: GlcA, D-glucuronic acid; MeGlcA, 4-*O*-methyl-D-glucuronic acid; GH, glycoside hydrolase; CAZy, carbohydrate-active enzymes; ORF, open reading frame; PNGaseF, peptide-*N*-glycosidase F; Xyl, xylose; Xyl<sub>2</sub>, xylobiose; Xyl<sub>3</sub>, xylotriose; MeGlcA<sup>3</sup>Xyl<sub>3</sub>, 1,2<sup>3</sup>- $\alpha$ -D-(4-*O*-methyl-glucuronyl)-1,4- $\beta$ -D-xylotriose.

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ture motif is an  $(\alpha/\beta)_8$  barrel, whereas GH11 xylanase is a smaller protein and its core structure motif is a  $\beta$ -jelly roll. Although GH10 and GH11 xylanases are typical enzymes that act on xylan, their sites of action on xylan with side chains differ.<sup>3)</sup> In contrast to GH10 and GH11 xylanases, GH30 xylanase specifically recognizes the 4-*O*-methyl glucuronyl residue as a side chain branching from the xylan main chain.<sup>4)5)6)</sup> Thus, xylanases exhibit diverse substrate recognition and degradation modes to accommodate the degradation of xylan with different structures depending on the plant source.

Due to the usefulness of xylanase in various industrial fields, such as pulp and paper, extremophilic xylanases, including thermophilic, alkaliphilic, and acidophilic enzymes, have been attracting increasing attention. The properties of these xylanases have already been summarized in detail.<sup>7)</sup> Mangroves are salt-tolerant forest ecosystems that are situated between terrestrial and marine environments of tropical and subtropical regions. These ecosystems are regarded as unique and dynamic environments because their geochemical characteristics, such as salinity, soil humidity, and nutrient concentration, are cyclically modified by periodic tidal flooding. Mangrove-isolated fungus, which are adapted to dynamic environments, might be a rich source for novel enzyme discovery. In this study, a filamentous fungus *Pestalotiopsis* sp. AN-7 was isolated from the soil of a mangrove forest of Iriomote Island in Japan. Furthermore, a cDNA coding for an endo- $\beta$ -1,4-xylanase was cloned from it and heterologously expressed in yeast *Pichia pastoris*. Properties of the recombinant *PesXyn10A* on the effect of metallic salts have been characterized in this report.

## MATERIALS AND METHODS

**Organisms and cultivation.** *Pestalotiopsis* sp. AN-7 was subcultured in potato dextrose agar plate medium. *Escherichia coli* DH5 $\alpha$ , purchased from Takara Bio Inc. (Shiga, Japan), was used for gene manipulation and grown at 37 °C in Luria-Bertani medium. *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was used for the expression of the recombinant enzyme as a host. Yeast extract (BD Biosciences, Miami, USA), peptone (BD Biosciences), yeast nitrogen base (BD Biosciences) were used for the medium.

**Isolation and identification of fungus.** A mangrove soil sample was collected from the tidal flats of Nakamagawa river mouth in Iriomote Island (Okinawa, Japan). Diluted soil suspension was spread onto CZapek-Dox agar medium (Sigma-Aldrich, St. Louis, MO, USA) containing 0.5 % lignin as a carbon source, and it was incubated at 27 °C for one week. The isolated fungus was named AN-7, and its morphological characteristics were microscopically observed. Identification of AN-7 was carried out by sequencing 18S rDNA as described elsewhere.<sup>8)</sup>

**Isolation of cDNA coding for  $\beta$ -1,4-xylanase.** *Pestalotiopsis* sp. AN-7 was grown in culture medium containing 1.0 % yeast extract, 3.0 % NaCl, 0.07 % KCl, 1.0 % MgCl<sub>2</sub>, 0.53 % MgSO<sub>4</sub>, 0.1 % CaCl<sub>2</sub>, 0.1 % NH<sub>4</sub>NO<sub>3</sub>, and 0.1 % Na<sub>2</sub>HPO<sub>4</sub> at 25 °C with orbital shaking (140 rpm) for 7 days. Three types of 1 % carbon sources (Avicel,

alkaline-treated *Erianthus*, and hydrothermal-treated *Erianthus*) were added to the culture medium. Total mRNA was prepared using the ISOPLANT II kit (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions for filamentous fungi. Isolated total RNA was sent to the National Institute of Technology and Evaluation (NITE, Tokyo, Japan) for RNA-seq. Full-length cDNA encompassing the whole GH10 xylanase open reading frame (ORF) was amplified from the above first-strand cDNA with KOD-plus DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) using two primers; *PesGH10-01 F* (5'-ATGAAGACCGCACTTATTCTCC-3') and *PesGH10-01 R* (5'-TTACTGCAGTGCCTTGACAAC-3'). These two primers were designed based on sequence data obtained from the RNA-seq analysis. The amplified fragment was subcloned into a pCR2.1 vector (Thermo Fisher Scientific, MA, USA) after the addition of adenine to the 3'-end of the PCR product by LA taq DNA polymerase (Takara Bio Inc., Shiga, Japan).

**Transformation and expression in *P. pastoris*.** Regarding expression using *P. pastoris*, the insertion fragments of the mature region *xyn10a* were amplified by two expression primer pairs containing the restriction enzyme site, *PesGH10-Pic-F1* (5'-GAATTC-CAGTCGACCACCTCGATCGTC-3' with the EcoRI site shown with underlines) and *PesGH10-Pic-R1* (5'-GCGGCCGCTTACTGCAGTGCCTT-3' with the NotI site shown with underlines) and cloned into the restriction sites of EcoRI and NotI in the expression vector pPIC9K by digestion and ligation. Approximately 10  $\mu$ g of pPIC9K expression plasmid DNA was linearized with SacI (Takara Bio Inc.) and transformed into *P. pastoris* GS115-competent cells by electroporation. Selection transformants and the production of the recombinant protein were performed according to the instruction manual of the EasySelect *Pichia* expression kit (Invitrogen, Carlsbad, CA, USA).

**Purification of *PesXyn10A*.** The *P. pastoris* strain expressing *PesXyn10A* was grown in 200 mL of BMGY medium containing 1 % glycerol, 1 % yeast extract, 2 % peptone, 1.34 % yeast nitrogen base, and 0.1 M potassium phosphate buffer (pH 6.0) at 30 °C and 180 rpm for 2 days. Cells were collected by centrifugation at 3,000  $\times$  G for 5 min, resuspended in 400 mL of BMMY medium containing 0.5 % methanol, 1 % yeast extract, 2 % peptone, 1.34 % yeast nitrogen base, and 0.1 M potassium phosphate buffer (pH 6.0) to induce expression, and then incubated further at 30 °C. Every 24 h, methanol was added to the culture medium to maintain the concentration at 0.5 %. After cultivating for 5 days, the cell-free supernatant was harvested by centrifugation at 10,000  $\times$  G for 20 min. The recombinant protein in the supernatant was precipitated by the addition of ammonium sulfate at a concentration of 90 % saturation. The precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl (pH 7.5) containing 30 % saturated ammonium sulfate. Precipitated recombinant enzyme solution was applied to Toyopearl Butyl 650M column chromatography using the BioLogic DuoFlow system (Bio-Rad, CA, USA). Non-adsorbed proteins were washed by 50 mM Tris-HCl (pH 7.5) containing 30 % saturated ammonium sulfate, and adsorbed proteins were eluted by decreasing

the concentration of ammonium sulfate. The eluted fraction was applied to SDS-PAGE. The amount of protein was measured by the Protein assay kit (Bio-Rad, CA, USA) and bovine serum albumin was used as the standard. In order to examine the glycosylation of the recombinant protein, we treated purified *PesXyn10A* with peptide-*N*-glycosidase F (PNGase F, Roche Diagnostics K.K., Basel, Switzerland) according to the instruction procedure.

**Measurements of enzyme activity.** Enzymatic activity was measured as follows. Birchwood xylan, beechwood xylan, oat spelt xylan, and carboxymethyl cellulose were purchased from Sigma-Aldrich and glucomannan (Shimizu Kagaku, Hiroshima, Japan) were used as substrates. Each substrate was prepared to 0.5 wt%, and 25  $\mu$ L of the substrate solution was mixed with 50  $\mu$ L of 50 mM sodium acetate buffer (pH 5.0). The enzymatic reaction was started by the addition of 25  $\mu$ L of the enzyme solution (1.2 U/mL), and was then stopped by the addition of Somogyi reagent. The amount of reducing sugars produced was detected by the Somogyi-Nelson method using xylose as a standard saccharide. One unit of enzyme activity was defined as the amount of enzyme that produces reducing sugar corresponding to 1  $\mu$ mol of xylose. Kinetic parameters for *PesXyn10A* were assessed in 50 mM sodium acetate buffer (pH 5.0) containing 0.1-5.0 mg/mL birchwood xylan and 0.036  $\mu$ g of the enzyme solution at 40 °C.  $K_m$  and  $k_{cat}$  values were calculated by the non-linear least optimization of the Michaelis-Menten equation.

**Analysis of reaction products from birchwood xylan.** In the product analysis, 5 mL of substrate solution (0.5 wt%) was mixed with 10 mL of 50 mM sodium acetate buffer (pH 5.0) and the reaction was started by the addition of 25  $\mu$ L of the enzyme solution (96 U/mL). To confirm the final reaction product, an additional 25  $\mu$ L of enzyme solution was added to reaction mixture at 24 h, and the reaction mixture was incubated for an additional 24 h. After 0.5-48 h, 1 mL of the reaction mixture was removed, and the reaction was stopped by heating to a boiling temperature for 10 min. Each reaction mixture was applied to the HPLC system for the sugar analysis as follows. The LC-2000 (JASCO, Co. Ltd., Tokyo, Japan) system was used to connect with Shodex Asahipak NH<sub>2</sub>P 50-4E (4.6  $\times$  250 mm). The mobile phase was used as the gradient concentration of the mixture with acetonitrile : 85 % H<sub>3</sub>PO<sub>4</sub> (98.5 : 1.5) and distilled water : 85 % H<sub>3</sub>PO<sub>4</sub> (99 : 1), and the flow rate was 1 mL/min. Products were detected using a fluorescence detector GL-7453A (GL Science, Tokyo, Japan) as post labeled method modified with 85 % H<sub>3</sub>PO<sub>4</sub> : acetic acid : phenyl hydrazine (220 : 180 : 6) at 0.4 mL/min (flow rate). The standard sugars used were xylose (Xyl), xylobiose (Xyl<sub>2</sub>), xylotriose (Xyl<sub>3</sub>), and 1,2<sup>3</sup>- $\alpha$ -D-(4-*O*-methyl-glucuronyl)-1,4- $\beta$ -D-xylotriose (MeGlcA<sup>3</sup>Xyl<sub>3</sub>) (Megazyme, Wicklow, Ireland).

**Properties of pH, temperature, and salts.** The properties of *PesXyn10A* against pH and temperature were investigated by measuring the release of the reducing sugar from birchwood xylan under various reaction conditions. In the optimum pH measurement, the enzymatic reaction was conducted at 40 °C for 30 min in Britton-Robinson's buffer (pH

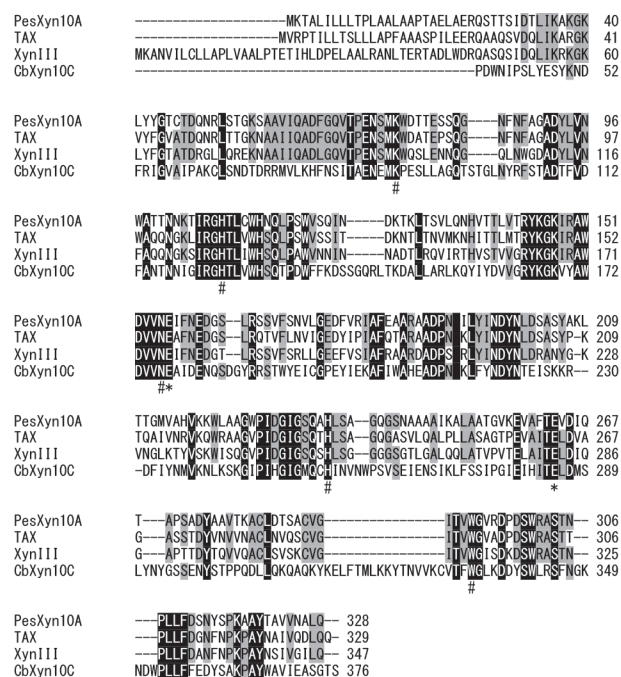
2 to 12). In the pH stability measurement, *PesXyn10A* was mixed with the same buffer adjusted to each pH, incubated at 4 °C for 24 h, and then reacted at 40 °C and pH 6.0 for 30 min. In the optimum temperature measurement, the enzymatic reaction was performed at each temperature (30 to 70 °C) at pH 5.0 for 30 min. To assess the influence of salt, 5 mM sodium acetate buffer with various concentrations of salt (10 mM-2.0 M) was prepared, and 25  $\mu$ L of birchwood xylan (0.5 wt%), 50  $\mu$ L of the buffer solution, and 25  $\mu$ L of the enzyme solution (1.2 U/mL) was incubated at 40 °C for 30 min. The reducing sugar produced from this substrate was examined using the method described above. The effects of salt on enzyme stability were also analyzed as follows. Five hundred microliters of 5 mM sodium acetate buffer with NaCl (100 mM), MgCl<sub>2</sub> (10 mM) was mixed with 500  $\mu$ L of the enzyme solution (1.2 U/mL) and then incubated at 20-50 °C for 30 min-2 days. After incubations for several time periods, 75  $\mu$ L of the mixture was removed and cooled on ice for 10 min, and the reaction was then started at 40 °C for 30 min by the addition of 25  $\mu$ L of birchwood xylan solution (0.5 wt%).

## RESULTS and DISCUSSION

### Primary structural analysis of *PesXyn10A*.

Morphological characteristics of the strain AN-7 isolated from the soil of a mangrove forest in this study were greatly coincident with those of the genus *Pestalotiopsis* (Ascomycota, Xylariales, Amphispheariaceae). Although the 18S rDNA sequence of the strain AN-7 also showed high similarity to those of other *Pestalotiopsis* species, the species could not be identified. Here, the strain AN-7 was designated as *Pestalotiopsis* sp. AN-7.

The ORF of *xyn10a* has 987 bp with the ATG start codon and TAA stop codon. It encodes a protein of 328 amino acid residues, and the theoretical molecular weight and isoelectric point are 35.3 kDa and 6.52, respectively (named *PesXyn10A*). The nucleotide sequence of the cDNA coding the full length of *xyn10a* was deposited in the DDBJ/EMBL/GenBank database under accession no. LC584173. A Signal P analysis (Signal P server 4.1, <http://www.cbs.dtu.dk/services/SignalP-4.1/>)<sup>9</sup> predicted the presence of a signal peptide at 16 residues of the N-terminal sequence (Met1 to Ala16). A protein BLAST analysis of *PesXyn10A* showed that characterized xylanases from Ascomycota such as *Trichoderma reesei* (*TrXyn* III, accession number; BAA89465.2)<sup>10</sup> shared high homology with 60-70 % identities. A hypothetical GH10 xylanase (accession number; XP\_007828407.1) in five homolog genes was found in *P. fici* W106-1,<sup>11</sup> and it showed the highest homology (95 % identity) with *PesXyn10A*. The primary amino acid sequences of *PesXyn10A* with three structurally elucidated xylanases were aligned (Fig. 1). Two highly conserved residues, Glu156 and Glu263, which are regarded as catalytic residues of family 10 glycosyl hydrolases,<sup>12</sup> were identified in the two conserved regions. Five amino acid residues (Lys75, His108, Asn155, His235, and Trp293), which are considered to be involved in xylan-binding subsites -2, -1, and +1,<sup>13</sup> were also conserved. According to



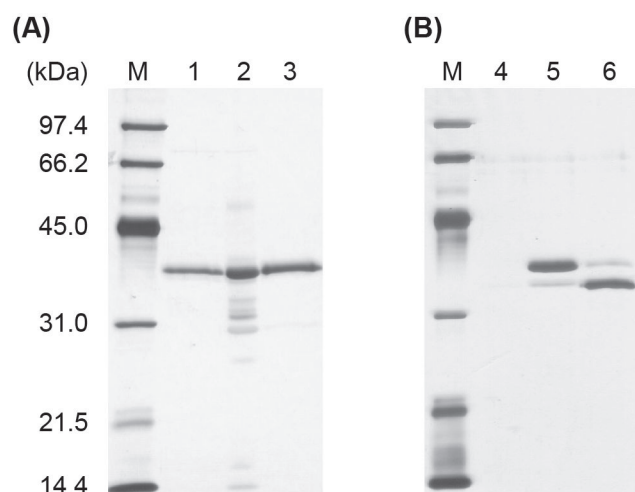
**Fig. 1.** Multiple amino acid sequence alignment of *PesXyn10A* with characterized GH10 xylanases.

The asterisks (\*) indicate the putative catalytic residues. The sharps (#) indicate the conserved residues which involved in xylan-binding subsites -2, -1, and +1.<sup>13</sup> Identical residues are shared in black and conserved residues are shared in gray. *PesXyn10A*, xylanase in this study; *TAX*, a thermostable xylanase from *Thermoascus aurantiacus* (AAF24127.1); *TrXyn111*, fungal xylanase from *T. reesei* (BAA89465.2); *CbXyn10C*, bacterial xylanase from *Caldicellulosiruptor bescii* (ACM60945).

the sequence homology analysis, *PesXyn10A* was predicted to be an extracellular GH10 xylanase involved in plant cell wall degradation.

**Expression and purification of *PesXyn10A*.** The mature region of *PesXyn10A* was successfully expressed by *P. pastoris* as an active enzyme. The enzyme was purified from culture medium by hydrophobic column chromatography. Purified *PesXyn10A* was finally obtained at a recovery rate of 59.1 % from 1 L of culture medium. The purified protein was detected as a single band at approximately 35 kDa, which was slightly higher than the theoretical molecular weight calculated from mature *PesXyn10A* (approximately 33.4 kDa) (Fig. 2A). After the treatment with PNGaseF under denatured conditions, the band of *PesXyn10A* shifted to the position corresponding to the theoretical molecular weight (Fig. 2B). Since *PesXyn10A* has two potential sites for *N*-glycosylation at Asn102 and Asn313, *PesXyn10A* expressed by *P. pastoris* may be glycosylated. In the present study, the experiment was performed using the purified enzyme obtained without degrading the sugar chain.

**Properties of *PesXyn10A* for pH and temperature.** To confirm the properties for pH and temperature, the enzymatic activity of *PesXyn10A* was measured at various conditions using birchwood xylan as a substrate. *PesXyn10A* optimally hydrolyzed birchwood xylan at pH 6.0, and the pH treatment at 4 °C for 24 h maintained the activity of the untreated enzyme at more than 80 % between pH 3 and



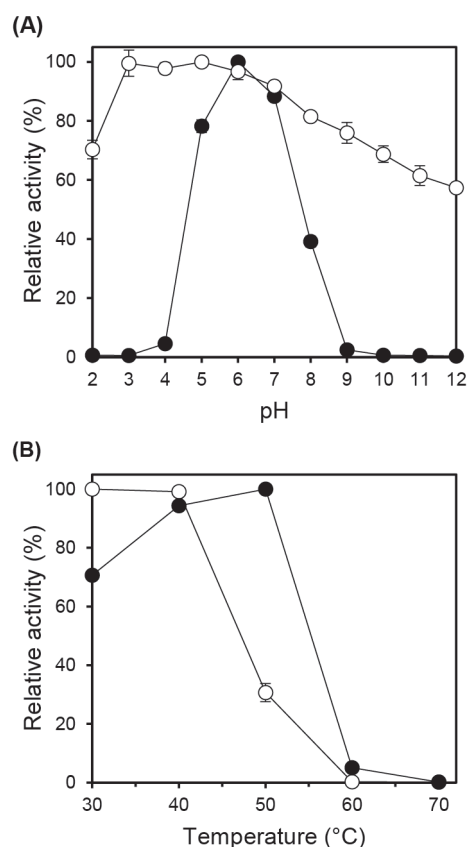
**Fig. 2.** SDS-PAGE of purified *PesXyn10A*.

(A) Purification steps of expressed *PesXyn10A*, (B) Deglycosylation of *PesXyn10A*. Lane M, molecular weight marker; lane 1, culture medium; lane 2, after precipitation with  $(\text{NH}_4)_2\text{SO}_4$ ; lane 3, after Butyl TOYOPEARL 650 M; lane 4, PNGaseF; lane 5, purified *PesXyn10A*; lane 6, *PesXyn10A* treated with PNGaseF.

8 (Fig. 3A). Under optimal pH conditions, *PesXyn10A* exhibited the strongest activity at 50 °C and relative activity markedly decreased at temperatures higher than 50 °C. After the thermal treatment at each temperature for 30 min, the residual activity of *PesXyn10A* decreased at temperatures higher than 40 °C (Fig. 3B). Thus, despite having high primary structural homology (91 %) with a xylanase (*TAX*) from *Thermoascus aurantiacus*,<sup>14</sup> *PesXyn10A* is not hyperthermia. The optimum temperature of xylanase from *T. aurantiacus* was 80 °C, and the half-live at 70 °C was approximately 204 h.<sup>15</sup>

**Substrate specificity of *PesXyn10A*.** The substrate specificity of *PesXyn10A* was summarized in Table 1. *PesXyn10A* showed similar specific activities for birchwood xylan and beechwood xylan with 33.1 and 33.0 U/mg, respectively. The specific activity of 10.2 U/mg for oat spelt xylan was lower than those for birchwood and beechwood xylan. There was no activity for carboxymethyl cellulose or glucomannan. The  $K_m$  and  $k_{cat}$  values of *PesXyn10A* for birchwood xylan were  $4.7 \pm 1.3$  mg/mL and  $31.4 \pm 1.3$  s<sup>-1</sup>, respectively (Fig. 1S; see J. Appl. Glycosci. Web site). These values were similar to those reported for other xylanases, such as XynA from the marine bacterium *Glaciecola mesophila* KMM 241.<sup>16</sup> Although some types of xylanases, including *AICMC*ase (WP\_111373332.1) from the Arctic marine bacterium *Arcticibacterium luteifluviistationis*, act on  $\beta$ -1,4-glucan as the substrate,<sup>17</sup> the present results demonstrated that *PesXyn10A* is a strict endo- $\beta$ -1,4-xylanase.

The final products from birchwood xylan after the *PesXyn10A* reaction were confirmed by HPLC (Fig. 4). Endo-type xylanases, such as GH10 xylanase, hydrolyze xylan in a random manner and release xylooligosaccharides. However, they are sterically hindered by acetyl- and 4-*O*-methylglucuronic acid substituents. Therefore, in addition to xylooligosaccharides, xylooligosaccharides substituted with the 4-*O*-methyl glucuronide residue were also liberated. In the first period, xylooligosaccharides, such as xylotri-ose



**Fig. 3.** Properties of *PesXyn10A* for pH and temperature. (A) Optimum pH and stability for pH were shown by closed and open circles, respectively. (B) Optimum temperature and stability for temperature were shown by closed and open circles, respectively.

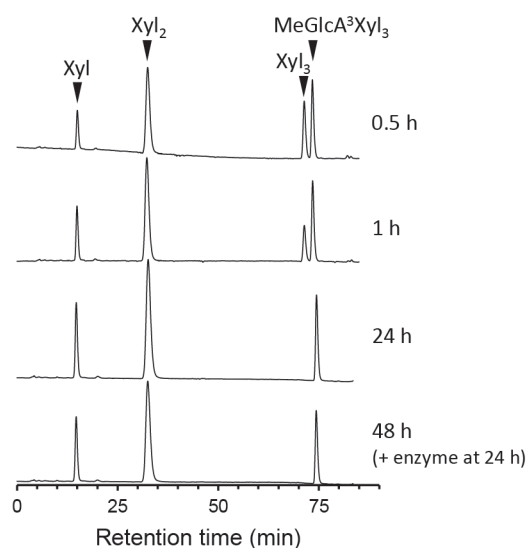
**Table 1.** Substrate specificity of *PesXyn10A*.

Substrates	Specific activity (U/mg)	Relative activity (%)
Birchwood xylan	33.1±0.5	100.0
Beechwood xylan	33.0±1.9	99.7
Oat spelt xylan	10.2±0.6	30.8
Carboxymethyl cellulose	N.D. <sup>a</sup>	—
Glucomanan	N.D. <sup>a</sup>	—

<sup>a</sup> N.D., not detected.

and xylotetraose, were detected as major products. Xyl, Xyl<sub>2</sub>, and MeGlcA<sup>3</sup>Xyl<sub>3</sub> were detected as some of the final products. MeGlcA<sup>3</sup>Xyl<sub>3</sub> is a unit composed of xylotriase branched with 4-*O*-methyl glucuronate by an  $\alpha$ -1,2-glycosidic linkage at the non-reducing end of xylotriase. This result was consistent with previous findings showing that the end product from the degradation of glucuronoxylan with xylanase was MeGlcA<sup>3</sup>Xyl<sub>3</sub>.<sup>3</sup> Therefore, *PesXyn10A* was a typical GH10  $\beta$ -1,4-xylanase, similar to other fungal xylanases, because GH11 xylanase produced longer acidic xylooligosaccharides from glucuronoxylan.

**Properties of *PesXyn10A* for various inorganic salts.** The genus *Pestalotiopsis* are halo-tolerant fungi and have a high number of carbohydrate-active enzyme (CAZymes)-coding genes for the utilization of carbohydrates from plants.<sup>11</sup> Arfi et al. isolated *Pestalotiopsis* sp. NCi6 from a mangrove forest, and examined the effects of salinity on secretomes



**Fig. 4.** HPLC analysis of the hydrolysate from birchwood xylan treated by *PesXyn10A*.

Xyl, xylose; Xyl<sub>2</sub>, xylobiose; Xyl<sub>3</sub>, xylotriase; MeGlcA<sup>3</sup>Xyl<sub>3</sub>, 1,2<sup>3</sup>- $\alpha$ -D-(4-*O*-methyl-glucuronyl)-1,4- $\beta$ -D-xylotriase.

of CAZymes.<sup>18</sup> In *Pestalotiopsis* sp. NCi6, the presence of salt (saline) increased the secretion of xylanases and cellulases and decreased the production of oxidases.<sup>18</sup> The adaptation of enzymes to salt environments is of great interest for the industrial processing of marine products and food with a high salt content.<sup>19)20)21</sup> Although the secretion of lignocellulosic enzymes from *Pestalotiopsis* sp. is known to be affected by sea salt, limited information is currently available on the properties of each enzyme. In the present study, the effects of the addition of inorganic salts (NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, and NH<sub>4</sub>Cl) on *PesXyn10A* activity were assessed (Table 2). Enzymatic activity was weaker when NH<sub>4</sub>Cl was added to the reaction solution than in its absence. On the other hand, when other metallic salts (NaCl, MgCl<sub>2</sub>, KCl, and CaCl<sub>2</sub>) were added, enzymatic activity increased. In the case of 5 mM MgCl<sub>2</sub> and CaCl<sub>2</sub>, enzymatic activity increased to approximately 166 and 161 %, respectively, but gradually decreased in the presence of concentrations higher than 5 mM. In addition, enzymatic activities also increased to 149 and 148 % in the presence of 50 mM NaCl and KCl, respectively, and gradually decreased at higher concentrations. These results revealed that metallic salts influence the activation of *PesXyn10A* activity. The activation of xylanase activity by metallic salts has been observed in xylanases isolated from marine bacteria, such as *Bacillus* sp. SN5 (134 % at 0.5 M NaCl),<sup>22</sup> *Thermoanaerobacterium saccharolyticum* NT0U1 (190 % at 0.4 M NaCl),<sup>23</sup> and *G. mesophila* KMM 241 (120 % at 0.5 M NaCl)<sup>16</sup>. It has been also reported that xylanase from *Planococcus* sp. SL4 isolated from the sediment soda lake was activated 134 % at 5 mM CaCl<sub>2</sub> and 117 % at 5 mM MgCl<sub>2</sub>.<sup>24</sup> Furthermore, there is a report investigating the effects of salt addition on kinetic parameters. In marine bacterial xylanase from *Zunongwangia profunda*, addition of 3 M NaCl caused a 0.4-fold decrease of  $K_m$  value, a 1.7-fold increase of  $k_{cat}$  value, and a 4.4-fold increase of  $k_{cat}/K_m$  value compared to no added NaCl.<sup>25</sup> This suggests that metallic

**Table 2.** Effect of salts on the xylanase activity of *PesXyn10A*..

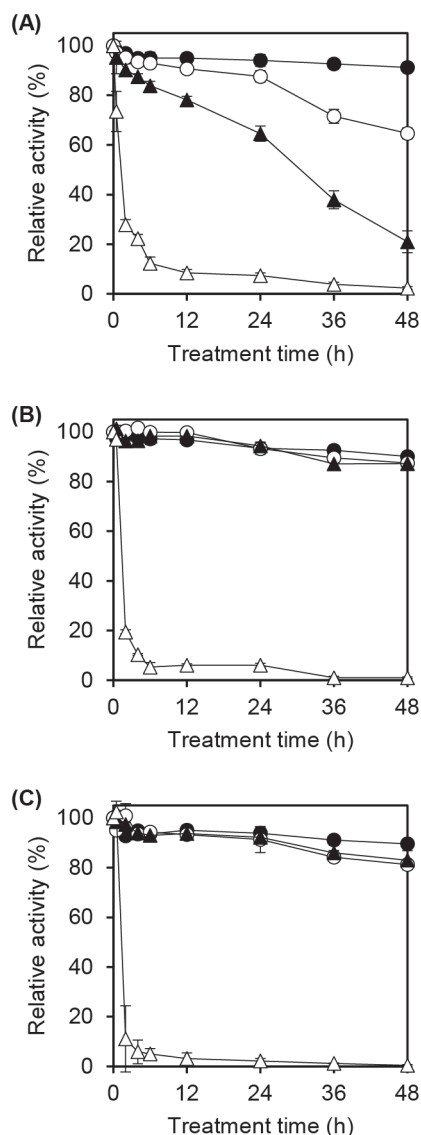
Salts	Relative activity (%) <sup>a</sup>	
	5 mM	50 mM
Control	100	100
NaCl	107.4±1.3	149.2±2.7
KCl	101.7±4.1	148.1±4.2
NH <sub>4</sub> Cl	71.3±3.2	38.5±1.9
MgCl <sub>2</sub>	165.6±6.2	126.8±4.2
CaCl <sub>2</sub>	161.4±5.3	116.0±4.4

<sup>a</sup> The activity of *PesXyn10A* without any salt was taken as control (100%). The data represent the mean ± SD of three independent experiments.

salts are involved in the catalytic mechanism of xylanase. Although marine bacterial xylanases described above were activated at NaCl concentrations of between 0.5-3.0 M, corresponding to average sea salinity of 3.5 % (w/v) (the final concentration of NaCl was approximately 0.6 M), the activation of *PesXyn10A* was caused at approximately 10 times lower NaCl concentration. (Fig. 2S; see J. Appl. Glycosci. Web site). *Pestalotiopsis* sp. AN-7 was isolated from a mangrove forest in a brackish water area at which seawater and freshwater mix. Therefore, the favorable salt concentration for enzyme activity may differ and depends on the habitat of each microorganism.

To investigate the thermal stability of *PesXyn10A* in the presence of salt, the influence of NaCl on thermal stability at 20 to 50 °C was evaluated and compared to without salts. Although optimum pH was observed at pH 6, residual activity was the highest at pH 5 and markedly decreased at pH > 6 (Fig. 3S; see J. Appl. Glycosci. Web site). Therefore, the thermal stability of *PesXyn10A* without salts was measured at pH 5 for a heat treatment time of 48 h (Fig. 5A). When the temperature was 30 °C or less, stability was maintained 90 % or higher for 24 h, but was decreased to approximately 65 % by the treatment at 30 °C for 48 h. *PesXyn10A* was rapidly inactivated at temperatures above 40 °C. By the addition of 50 mM NaCl, the thermal stability of *PesXyn10A* at temperatures less than 40 °C was significantly improved in contrast to without salts (Fig. 5B). Liu *et al.* previously reported that the thermostability of xylanase from *Z. profunda* increased in the presence of NaCl,<sup>25</sup> and these ions may affect the conformation of the protein structure. In addition to NaCl, 5 mM MgCl<sub>2</sub> exerted positive effects on xylanase stability, even at low concentrations (Fig. 5C). After a 48-hour incubation with these salts, enzyme stability was maintained at 90 % at temperatures less than 40 °C. The present results indicate that NaCl and MgCl<sub>2</sub> contribute not only to the activation of enzyme activity, but also to the stabilization of the enzyme structure.

To the best of our knowledge, there are few reports about terrestrial filamentous fungal xylanases activated by the addition of metallic salts. The activity of an xylanase from Ascomycota *Aspergillus terreus* S9 was not significantly influenced by the presence of Mg<sup>2+</sup>, Ca<sup>2+</sup>, and K<sup>+</sup> (96, 112, and 101 %, respectively) at 10 mM.<sup>26</sup> In two xylanases from Basidiomycota *Irpex lacteus*, the presence of Mg<sup>2+</sup>,



**Fig. 5.** Stability of *PesXyn10A* at different temperatures without salts (A) and with 50 mM NaCl (B) and 5 mM MgCl<sub>2</sub> (C). ●, 20 °C; ○, 30 °C; ▲, 40 °C; △, 50 °C.

Ca<sup>2+</sup>, Na<sup>+</sup>, and K<sup>+</sup> at 2 mM showed little positive effect on enzymatic activities (90-105 %).<sup>27</sup> Thus, the positive effect by metallic salts seems to be a feature of xylanases produced by marine organisms. However, the mechanisms responsible for both activation and stabilization in the presence of metallic salts have not yet been elucidated in detail. Therefore, research is underway to elucidate the molecular mechanisms by which metallic salts activate enzyme activity and increase thermal stability in *PesXyn10A*. Understanding the molecular basis of activation and stabilization by metallic salt in *PesXyn10A* may contribute to develop a robust xylanase for industrial applications.

## CONCLUSION

We herein isolated a cDNA coding  $\beta$ -1,4-endoxylanase (*PesXyn10A*) from the marine fungus *Pestalotiopsis* sp. AN-7 that was successfully expressed by *P. pastoris* as a functional enzyme. Based on substrate specificities and primary structure similarities, *PesXyn10A* belongs to a typical

GH10 family xylanase. The presence of diluted inorganic salt induced both the activation and stabilization of *Pes-Xyn10A*. In addition, divalent cations exert stronger effects than monovalent cations, even at 10 times lower concentrations. Divalent cations, such as  $Mg^{2+}$ , exerted positive effects on enzyme structures, resulting in both activation and stabilization, even at low concentrations. These properties may be attributed to the habitat in the forest in a brackish water area. This is the first study to isolate and characterize a xylanase from *Pestalotiopsis* species.

### CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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