

Characterization of Hexenuronosyl Xylan-degrading Enzymes Produced by *Paenibacillus* sp. 07

Krisna Septiningrum,^{a,b,c} Hiroshi Ohi,^a Akiko Nakagawa-izumi,^a and Akihiko Kosugi^{a,b,*}

The enzyme involved in hexenuronic acid (HexA) removal from kraft pulp was identified in *Paenibacillus* sp. strain 07. Extracellular and intracellular enzymes of *Paenibacillus* sp. were assessed for their hexenuronosyl-xylotriase ($\Delta X3$) degradation activity. First, $\Delta X3$ was obtained from hardwood kraft pulp by enzymatic hydrolysis using three commercial enzymes. Crude extracellular and intracellular enzyme fractions were obtained from *Paenibacillus* cultures cultivated in 0.5% (w/v) birch wood xylan as the sole carbon source. The $\Delta X3$ -degrading activities of the enzyme fractions were measured by hydrolysis assays in sodium acetate buffer containing $\Delta X3$ substrate (pH 6) at 50 °C. The reaction products were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection. The enzyme fractions displayed different chromatogram patterns. After treatment with the intracellular enzyme fraction, the chromatograms displayed xylose and hexenuronosyl xylobiose ($\Delta X2$) peaks. The chromatogram patterns of the extracellular fraction assays indicated xylose, xylotriase, and $\Delta X2$ production. Thus, the intracellular enzymes of *Paenibacillus* can hydrolyze the xylosidic linkages at the reducing ends of $\Delta X3$, whereas a specific extracellular enzyme can hydrolyze HexA. This enzyme is potentially applicable to HexA removal during bio-bleaching.

Keywords: Enzyme activity; Hexenuronic acid; Hexenuronosyl xylotriase; Pulp bleaching; *Paenibacillus* sp.

Contact information: a: Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan; b: Biological Resources and Post-harvest Division, Japan International Research Center for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan; c: Center for Pulp and Paper, Agency for Research and Development of Industry, Ministry of Industry, Jl. Raya Dayeuhkolot 132, Bandung 40258, Indonesia; *Corresponding author: akosugi@affrc.go.jp

INTRODUCTION

Hemicellulose is an important wood component that influences the yield, strength, and bleaching ability of pulp (Lyytikäinen *et al.* 2011). During the heat treatment step in kraft pulping of wood chips, approximately 50% of the 4-O-methylglucuronic acid (MeGlcA) group residues in xylan are converted to hexenuronic acid (HexA) groups by β -elimination reactions (Gellerstedt and Li 1996; Teleman *et al.* 1996). The HexA groups inflate the measured kappa number (KN) of the pulp. The KN, which ideally indicates the lignin content of the pulp, is useful for estimating the quantity of bleaching agents in the pulp treatment. HexA inflates the KN of hardwood and softwood pulps by approximately 3–6 KN units and 1–3 KN units, respectively (Gellerstedt and Li 1996; Teleman *et al.* 1996; Jiang *et al.* 2000; Takahashi *et al.* 2011). Thus, the presence of HexA in unbleached pulp adversely affects the pulp bleaching operations, as it increases the requisite amounts of bleaching chemicals and decreases the brightness stability of the pulp (Sevastyanova *et al.* 2006; Kuwabara *et al.* 2012).

Previous efforts have focused on the removal of HexA from kraft pulp (Kuwabara *et al.* 2011; Tavast *et al.* 2011). Kraft pulp is often oxygen-bleached by chlorine dioxide, which does not efficiently degrade HexA. Moreover, the chlorine (Cl₂) formed during this process may chlorinate HexA and other lignin and carbohydrate structures. Chlorinated HexA and organic compounds may have negative environmental effects (Tavast *et al.* 2011). To alleviate the problems associated with HexA removal, a hot chlorine dioxide bleaching stage or a hot acid treatment stage is introduced. However, these treatments reduce the cellulose viscosity (Tavast *et al.* 2011). Thus, a HexA-removal method that is highly specific under mild treatment conditions is required.

In a recently proposed procedure for enzymatic degradation of HexA, the HexA is indirectly removed in a mixture of xylanase and laccase from sisal and kraft pulps (Aracri and Vidal 2011; Thakur *et al.* 2012). Enzyme-based methods have several advantages over chemical-based methods.

Because enzymes have high specificities, they are unlikely to yield unwanted by-products; hence they improve the quality of the pulp. In addition, as enzymatic reactions do not require high reaction temperatures, they conserve energy and are ecofriendly (Thakur *et al.* 2012). Thus, enzyme-based methods are potentially employable in clean bleaching processes and the development of value-added products.

The pulp quality can be improved by adding HexA-degrading enzymes, which selectively remove the HexA. Winyasuk *et al.* (2012) showed that a soil bacterium, *Paenibacillus* sp. strain 07-G-dH (*Paenibacillus* sp. strain 07), can utilize HexA-substituted xylotriase (hexenuronosyl xylotriase, ΔX3), a model compound of hexenuronoxylan. In order to clear reaction of HexA-degrading enzymes, ΔX3 appears to be suitable as alternative substrate of hexenuronoxylan. Whereas Winyasuk *et al.* (2012) also examined the HexA-hydrolysing ability of the enzymes such as xylanase and β-xylosidase secreted by the *Paenibacillus* strain in ΔX3, the ability was indirect degradation. It is still unclear how these enzymes cooperate to degrade ΔX3. In particular, there are no reports how they can directly remove HexA from ΔX3. We here prepare extracellular and intracellular enzymes, and characterize the HexA degradation entities in the *Paenibacillus* strain. We reveal a novel enzymatic ability in the extracellular fraction that directly releases HexA from ΔX3.

EXPERIMENTAL

Bacterial Strain and Medium

The strain was *Paenibacillus* sp. strain 07-G-dH (hereafter referred to as *Paenibacillus* sp. strain 07), provided by Dr. Shigeki Yoshida (University of Tsukuba, Ibaraki, Japan). The strain was grown on a production medium (pH 7.0) described by Winyasuk *et al.* (2012). Briefly, the medium contained 1 g of yeast extract, 1 g of polypeptone, 1 g of yeast nitrogen base, 1 g of KH₂PO₄, and 1 g of MgSO₄·7H₂O, and was supplemented with 5 g of birch wood xylan (Sigma–Aldrich, St. Louis, MO, USA) per liter of distilled water (pH 7.0).

Chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nihon Seiyaku Kogyo Co., Ltd. (Nagoya, Japan), and Difco BD Laboratories (Franklin Lakes, NJ, USA).

Preparation of $\Delta X3$ by Enzymatic Hydrolysis of Kraft Pulp Xylan

Eucalyptus oxygen-bleached kraft pulp (obtained from Hokuetsu Kishu Paper Co., Ltd., Niigata Mill, Japan) was soaked in 15% (w/v) NaOH for 24 h, then filtered through a cotton cloth. The filtrate was neutralized by adjusting the pH to 7.0 with sulfuric acid (H₂SO₄). The supernatant was separated from the solid phase by centrifugation at 8,500 × g for 30 min. The pellet containing the modified xylan was suspended in distilled water and dried under vacuum at room temperature. The modified xylan was hydrolyzed by incubation with xylanase (Shearzyme; Novozymes A/S, Bagsvørd, Denmark) (544 U/g) and “Onozuka” R-10 from *Trichoderma viride* (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) (74 U/g) at pH 4.5 (50 mM acetic acid buffer) at 45 °C for 72 h. To stop the reaction, the mixture was heated at 100 °C for 10 min and then centrifuged. The supernatant was applied to a chromatography column packed with activated carbon (Wako Pure Chemical Industries) at a flow rate of 60 mL/h. Next, the column was washed with 12 L of distilled water to remove the monomers. Once all monomers were removed from the column, the oligosaccharides were eluted by applying aqueous 40% ethanol solution. The eluate was concentrated in a rotary vacuum evaporator. To eliminate contamination with xylooligosaccharides (XOs), the eluate was treated with β -xylosidase (10 U/mL) obtained from *Bacillus pumilus* (Megazyme, Bray, Ireland). The enzyme treatment was performed at pH 7.0 (100 mM phosphate buffer) at 35 °C for 3 h. The purity and concentration of $\Delta X3$ were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD).

Preparation of Extracellular and Intracellular Enzymes from *Paenibacillus* sp. Strain 07

Paenibacillus cells were grown for 1 day at 37 °C on production medium supplemented with 0.5% (w/v) birch wood xylan (Sigma–Aldrich) as the sole carbon source. The culture supernatant was separated from the bacterial cells by centrifugation (7,800 g, 15 min, 4 °C) and precipitated in 80% ammonium sulfate for 24 h at 4 °C. The precipitated pellet was harvested by centrifugation (7,800 × g, 45 min, 4 °C), suspended in Milli-Q water (EMD Millipore, Billerica, MA, USA), and dialyzed against the same solution at 4 °C in an Econo-Pac® 10 DG Desalting column (Bio-Rad, Hercules, CA, USA). This suspension constituted the extracellular fraction.

The cell pellets were frozen at –80 °C for 24 h. The frozen pellets were thawed, washed with phosphate buffer saline (pH 7), and resuspended in 30 mL of the same buffer. A cell-free extract was prepared by sonication for 30 min. The sonicated cells were separated into cell debris and cell lysate fractions by centrifugation. The lysate constituted the intracellular fraction.

Hydrolytic Activity of the Enzyme Fraction against $\Delta X3$ and Enzymatic Product Analysis

The extracellular and intracellular fractions were subjected to a $\Delta X3$ hydrolysis assay in 50 mM sodium acetate buffer (pH 6) at 50 °C for 6 h. The reaction was stopped by heating the reaction mixture at 100 °C for 10 min. The enzyme fractions (30–90 μ g/mL) were incubated with the prepared $\Delta X3$ (3.5 mM), and the extracellular and intracellular hydrolysis products were analyzed by an HPAEC-PAD DX-500 series chromatograph with a PAD II pulsed amperometric detector (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA100 analytical column (250 mm × 4 mm). The column was equilibrated with 100 mM NaOH at 30 °C at a flow rate of 1 mL/min. A gradient elution was performed

using 100 mM NaOH and 100 mM NaOH/1 M CH₃COONa. The standard was authentic ΔX3, provided by Dr. Shigeki Yoshida (University of Tsukuba, Ibaraki, Japan).

Enzyme Activities

To measure the α-glucuronidase activities in the extracellular and intracellular fractions of *Paenibacillus* sp. strain 07, the amount of glucuronic acid liberated from aldouronic acid (Megazyme) was measured by colorimetric assay (Milner and Avigad 1967). The incubation mixture for the α-glucuronidase assay (total volume 0.2 mL) contained 0.16 mL of substrate (2 mg in 100 mM sodium acetate buffer, pH 6.0) and 0.04 mL of the target enzyme solution. The reaction was started by adding the enzyme. After 30 min of incubation at 40 °C, the reaction was stopped by boiling the samples for 4 min. Next, 0.6 mL of copper reagent, prepared as described by Milner and Avigad (1967), was added to each tube, and the samples were boiled for 10 min and cooled on ice. Subsequently, 0.4 mL of arsenomolybdate reagent was added (Nelson 1944). The samples were gently mixed, 0.8 mL of H₂O was added, and the absorbance at 620 nm was measured against a H₂O blank. Controls were prepared by boiling a complete assay mixture at time 0, and incubating the mixture at 40 °C. As a substrate control, the enzyme solution was replaced with water. A standard curve was prepared from D-glucuronic acid (Sigma–Aldrich). One α-glucuronidase unit was defined as the amount of enzyme liberating 1 μmol/min of glucuronic acid under standard assay conditions. The activities of the extracellular and intracellular fractions toward xylan were measured by colorimetric assay (Milner and Avigad 1967; Nelson 1944). The activities toward 4-nitrophenyl β-D-xylopyranoside and 4-nitrophenyl β-D-glucopyranoside (Sigma–Aldrich) were determined from the absorbance of 4-nitrophenol at 410 nm.

RESULTS AND DISCUSSION

Preparation of ΔX3 as a Model HexA Compound

ΔX3 was prepared from *Eucalyptus* kraft pulp; fractionation was conducted as described by Winyasuk *et al.* (2012). ΔX3 was produced by alkaline extraction followed by enzymatic hydrolysis. The alkaline extraction process yielded 87 g of xylan from 2 kg of kraft pulp. The xylan was derived from the alkali-extracted precipitate formed by adding acidic solution during the neutralization process.

The enzymatic hydrolysis employed two commercial enzymes (Shearzyme and Onozuka R10). Xylan is a heteropolymer with a homopolymeric backbone composed of β-1,4-linked xylose units and various branching units. To completely hydrolyze this complex structure, we require the synergistic action of different enzymes. In this study, xylan was first hydrolyzed by the xylanase *Shearzyme*, whose main ingredient is endo-xylanase. Endo-β-1, 4-xylanases (primarily from GH10) attack the β-1,4-bonds between the xylose units of xylan, degrading the xylan to XOs (Rantanen *et al.* 2007). GH10 xylanases liberate shorter XO products than other xylanases (such as GH11). The GH11 enzyme activity is abrogated by additional groups, which restrict access to the β-1,4-linkages in the xylan backbone (Biely *et al.* 1997; Rantanen *et al.* 2007). In addition, GH10 xylanases target the sites near the substituted xylose residue. Consequently, the XO degradation products carry the substituent at the non-reducing terminal xylopyranose residue. ΔX3 is the shortest acidic oligosaccharide liberated by GH10 treatment. The hydrolysate from this step also contains xylose, XOs, and various acidic oligosaccharides. This mixture can be further

hydrolyzed by the cellulase Onozuka R10, which contains β -xylosidase and α -glucuronidase (Teleman *et al.* 1996; Park *et al.* 2001; Winyasuk *et al.* 2012). β -Xylosidase converts XOs with lower degrees of polymerization (DPs) into monomeric xylose, whereas α -glucuronidase is a debranching enzyme that cleaves the xylan side groups (MeGlcA).

The hydrolysate from the previous step was further purified by separating the XOs from undesirable compounds such as monosaccharides and disaccharides. This was achieved by charcoal column chromatography, followed by elution with ethanol. Charcoal chromatography is the preferred method for sugar purification because of its high loading capacity (Sun *et al.* 2002). The XOs remaining in the column were then eluted by applying 40% ethanol in water, which fractionated the XOs by their molecular weight. The resulting hydrolysate contained 5.8 g Δ X3. The structure of Δ X3 was characterized in a previous study (Teleman *et al.* 1996; Winyasuk *et al.* 2012).

After treatment with commercial xylanases and cellulases, HPAEC–PAD revealed the presence of xylotriose in the Δ X3 fraction (Fig. 1). Remnant Δ X3 was expected because XOs are not easily separated from high-DP XOs and acidic oligosaccharides (XOs with uronic acid substituents). Next, the contaminated Δ X3 was further purified by β -xylosidase. Analysis of the hydrolyzed Δ X3 confirmed that the xylotrioses were successfully removed from the substrate without loss of Δ X3. Similar data were reported by Tenkanen *et al.* (1996) and Biely *et al.* (1997). The liberated glucuronoxylan was hydrolyzed by xylanase GH10 and was resistant to β -xylosidase. As β -xylosidases successively remove the terminal xylose unit from the non-reducing end of XOs (Tenkanen *et al.* 1996), these findings indicate substitution of the xylopyranosyl residue at the reducing end of the liberated glucuronoxylan with MeGlcA. Xylotriose constitutes the main product of the target enzyme, which cleaves the 1,2-linkage of Δ X3. Thus, the xylotriose must be removed from the Δ X3 fraction. Chromatograms of the β -xylosidase-treated Δ X3 are shown in Fig. 1. The Δ X3 yield is 5.1 g.

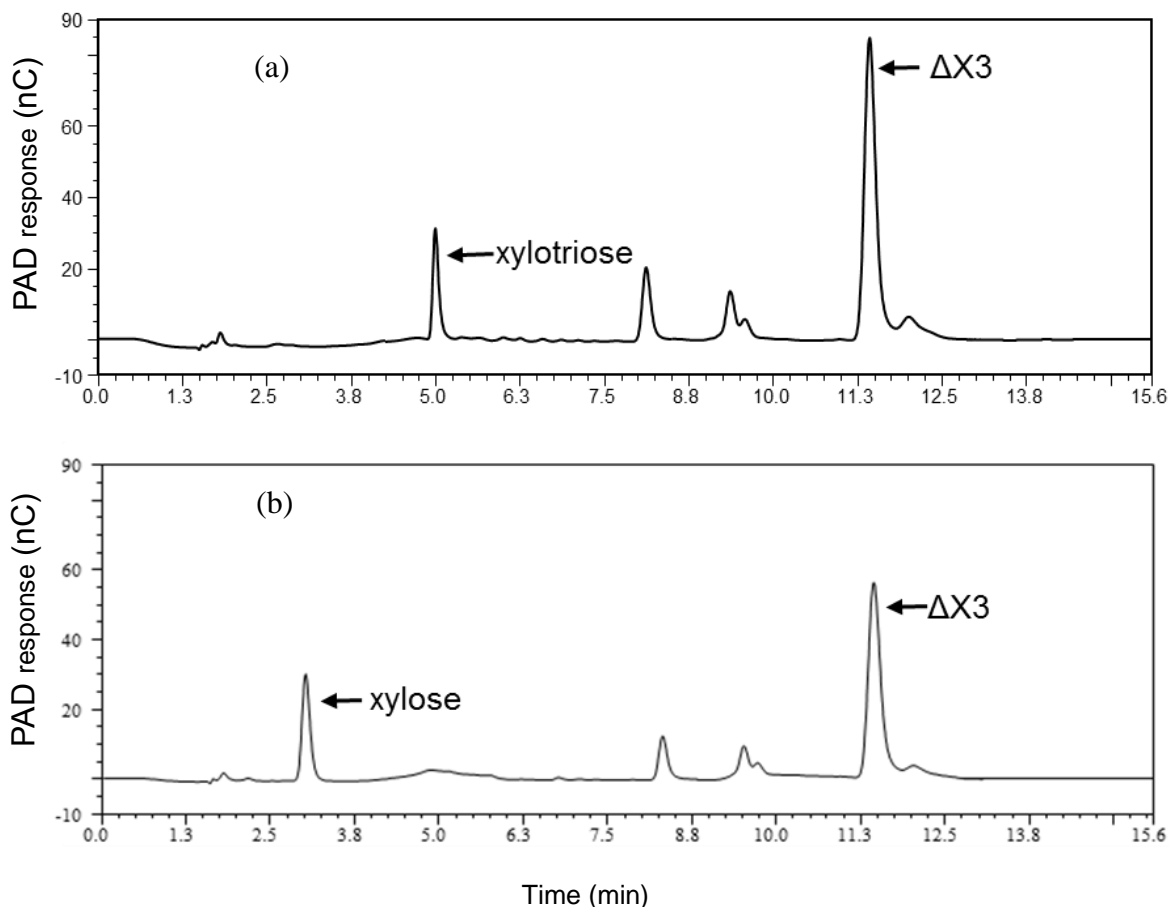


Fig. 1. HPAEC chromatograms of hexenuronosyl xylotriase ($\Delta X3$) before (a) and after (b) β -xylosidase treatment. The x- and y-axes represent the retention time (min) and PAD response (nC), respectively. The standard is authentic $\Delta X3$.

Characterization of Intracellular and Extracellular Enzymes from *Paenibacillus* sp. Strain 07

This section examines the activities of the extracellular and intracellular enzymes of *Paenibacillus* sp. strain 07 on $\Delta X3$ substrate. Both fractions were prepared from cells and culture supernatant of *Paenibacillus* cultivated on birch wood xylan as the sole carbon source, and analyzed for their $\Delta X3$ hydrolyzing patterns.

The intracellular fraction of *Paenibacillus* sp. strain 07 rapidly degraded the substrate and afforded two major products, xylose and $\Delta X2$, after 30 min of hydrolysis (Fig. 2). The $\Delta X3$ degradation rate was higher in the intracellular fraction than in the extracellular fraction.

The intracellular fraction completely degraded the substrate within 2 h. According to the HPAEC-PAD data, the $\Delta X2$ levels (indicated by peaks in the chromatogram) increased with increasing hydrolysis time, and the $\Delta X3$ peaks disappeared after 3 h hydrolysis (Fig 3). This finding is consistent with a previous study conducted by Winyasuk *et al.* (2012). The intracellular enzyme fraction contained an exo-oligoxyylanase that degrades the first xylosidic linkage from the reducing-end site of $\Delta X3$.

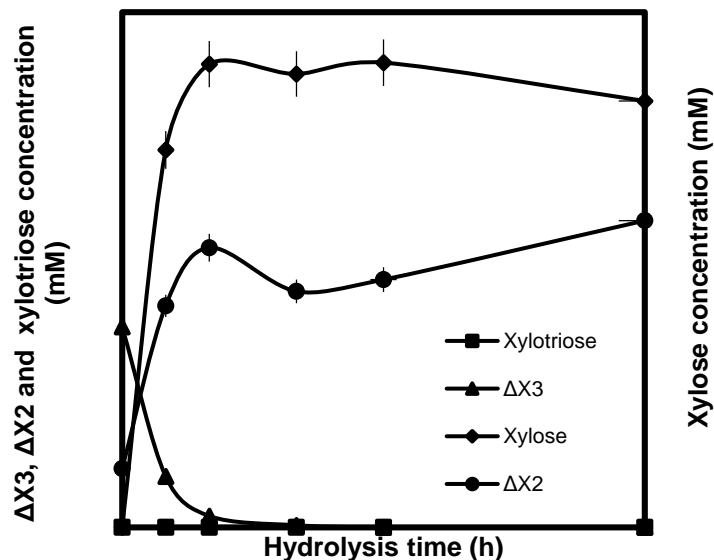


Fig. 2. Biological degradation of $\Delta X3$ by the intracellular enzyme fraction of *Paenibacillus* sp. strain 07. Concentrations of $\Delta X3$ (triangles), $\Delta X2$ (circles), xylose (diamonds), and xylotriose (squares) were measured by HPAEC–PAD.

Glycoside hydrolases belonging to families 8 (Honda and Kitaoka 2004) and 5/30 (Tenkanen *et al.* 2013) are also known to hydrolyze XOs, releasing xylose from their reducing ends. However, these hydrolases lack endo- β -1,4-xylanase activities and cannot act on polymeric substrates such as chitosan, lichenan, and carboxymethylcellulose, which provide unique properties (Honda and Kitaoka 2004; Tenkanen *et al.* 2013). These enzymes also cleave the XOs in intracellular xylan metabolism (Honda and Kitaoka 2004; Tenkanen *et al.* 2013). It is proposed here that a similar glycoside hydrolase may play an important $\Delta X3$ degradation role in the intracellular xylan metabolism of *Paenibacillus* sp. strain 07.

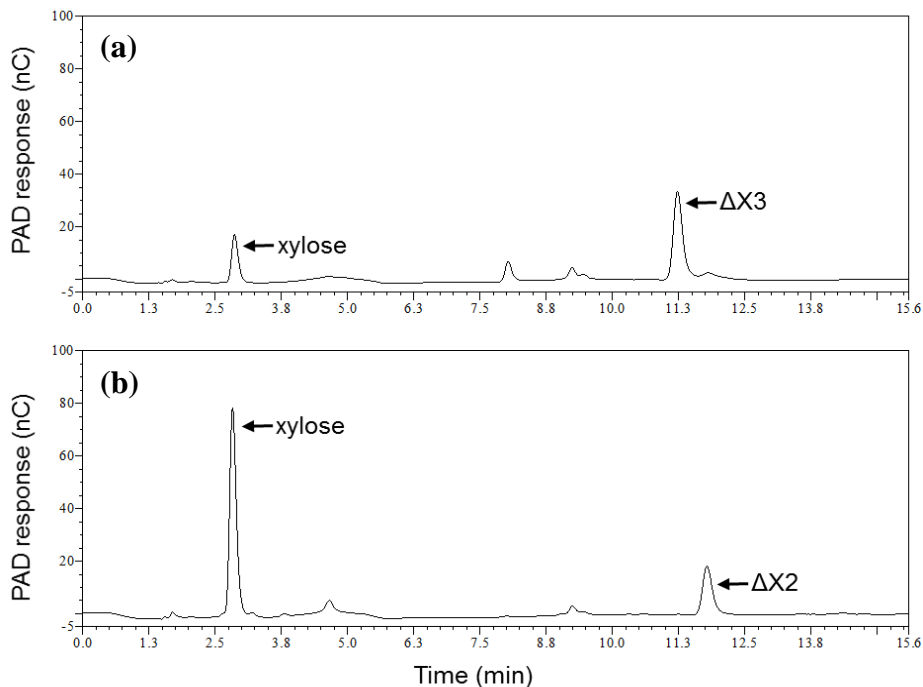


Fig. 3. HPAEC chromatograms of $\Delta X3$ before (a) and after (b) hydrolysis by the intracellular fraction of *Paenibacillus* sp. strain 07. The x- and y- axes represent the retention time (min) and PAD response (nC), respectively. The standard was authentic $\Delta X3$.

The crude extracellular fraction was incubated with $\Delta X3$, and the reaction yielded different product patterns (Figs. 4 and 5). The chromatogram data revealed one major hydrolysis product (xylose) and two minor products (xylotriose and $\Delta X2$; Fig. 5).

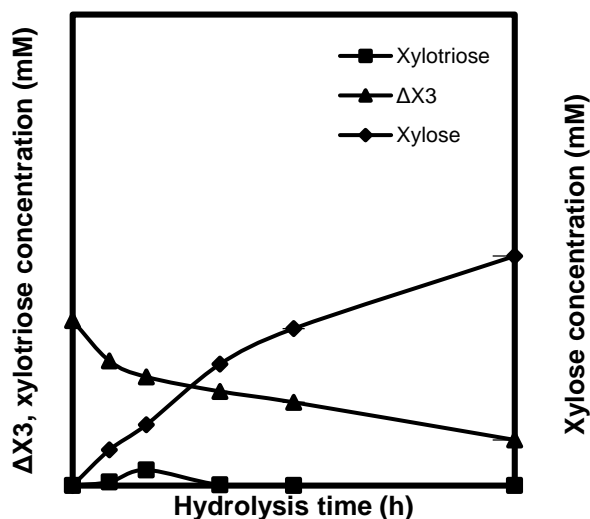


Fig. 4. Biological degradation of $\Delta X3$ by the extracellular enzyme fraction of *Paenibacillus* sp. strain 07. Concentrations of $\Delta X3$ (triangles), xylose (diamonds), and xylotriose (squares) were measured by HPAEC–PAD analysis.

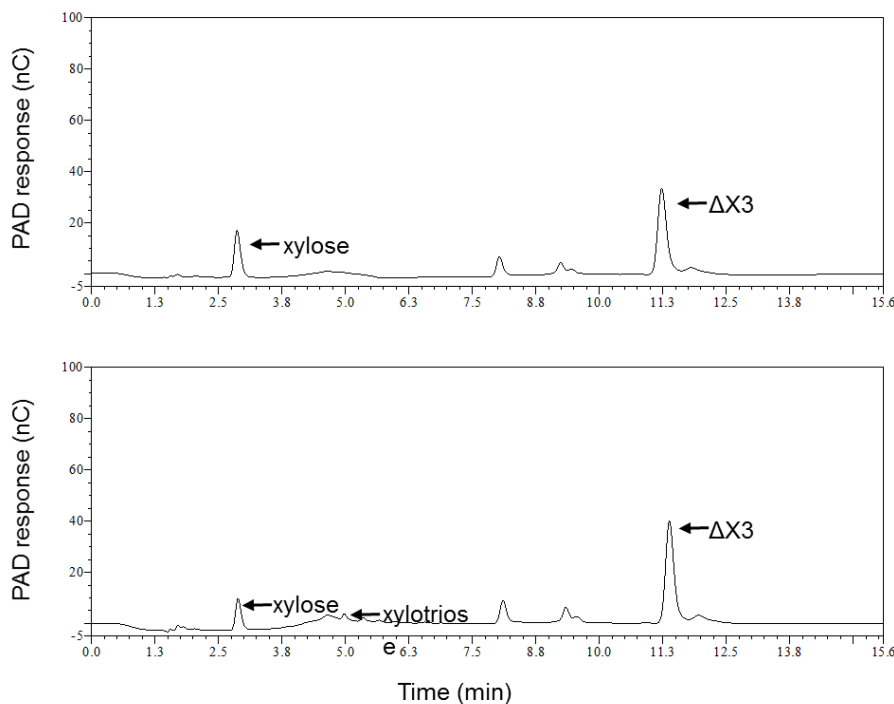


Fig. 5. HPAEC chromatograms of hexenuronosyl xylotriose ($\Delta X3$) before (a) and after (b) hydrolysis by the extracellular fraction of *Paenibacillus* sp. strain 07. The x- and y- axes represent the retention time (min) and PAD response (nC), respectively. The standard was authentic $\Delta X3$.

As the hydrolysis time increased, more xylose was liberated in the hydrolysis reaction. However, despite the xylose liberation from $\Delta X3$, the $\Delta X2$ concentration was not significantly increased. Additionally, the xylotriose concentration increased during the first hour of hydrolysis and then decreased (Fig. 4), indicating that the released xylotriose was quickly converted to xylose monomers by an extracellular β -xylosidase.

These phenomena imply the presence of two enzymes in the extracellular fraction: a) an enzyme that specifically degrades HexA from $\Delta X3$ and releases xylotriose; and b) a β -xylosidase that hydrolyzes xylotriose to yield xylose. Recently, we confirmed that a α -glucuronidase from *P. curdlanolyticus* B-6 can remove the HexA side group from $\Delta X3$ (Septiningrum *et al.* 2015). Interestingly, α -glucuronidase activity was not detected in the extracellular fraction of *Paenibacillus* sp. strain 07 (Table 1), suggesting that the HexA-liberating enzyme produced by this strain is novel.

Table 1. Enzymatic Properties of Extracellular and Intracellular Fractions from *Paenibacillus* sp. Strain 07

Enzymatic activity	Specific activity (U/mg protein)	
	Extracellular fraction	Intracellular fraction
α -Glucuronidase ^a	ND ^d	0.023±0.001
β -Xylosidase ^b	0.005±0.003	0.034±0.005
β -Glucosidase ^c	0.005±0.001	0.034±0.002
Xylanase	1.79 ±0.14	0.30±0.01

Values are the means of triplicate experiments \pm standard deviations.

^aCultured on adouronic acid substrate.

^bCultured on 4-nitrophenyl β -D-xylopyranoside substrate.

^cCultured on 4-nitrophenyl- β -D-glucopyranoside substrate.

^dND; not detected.

The new HexA-liberating enzyme cleaves the α -1, 2-linkages between the xylose unit of the xylan chain and the carboxylic acid side groups (HexA). Thus, it could potentially be exploited in applications. According to the activity data, this new enzyme is quite distinct from α -glucuronidase (Table 1). The Δ X3-degrading enzyme system of *Paenibacillus* sp. strain 07 is summarized in Fig. 6. The enzyme activity profiles of the intracellular and extracellular fractions of *Paenibacillus* sp. strain 07 revealed two or more unidentified enzymes. The first is a reducing-end, xylose-releasing exo-oligoxylanase, similar to the glycoside hydrolases in families 8 and 5/30; the second is a HexA-liberating enzyme. To characterize the newly discovered HexA liberating enzyme, the enzyme must be purified from the extracellular fraction and/or its gene must be cloned from *Paenibacillus* sp. strain 07. These investigations will be undertaken in our future work. The enzyme activities of the uncharacterized enzymes are potentially applicable to the pulp bleaching process.

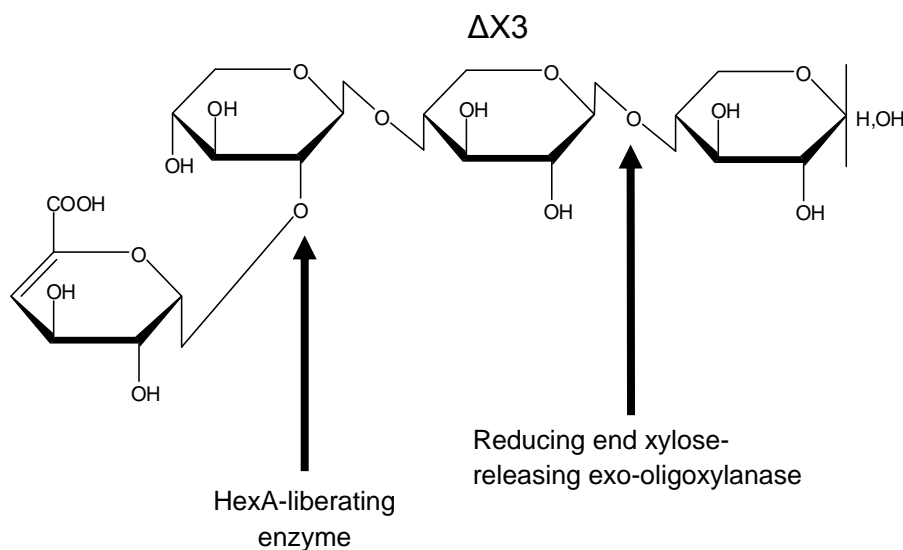


Fig. 6. Δ X3-degrading enzymes in the extracellular and intracellular enzyme fractions of *Paenibacillus* sp. strain 07

CONCLUSIONS

1. The crude intracellular enzyme fraction obtained from *Paenibacillus* sp. contained an enzyme that released xylose residues from the reducing ends of Δ X3.
2. The crude extracellular enzyme fraction obtained from *Paenibacillus* sp. strain 07 contained two important enzymes: a HexA-liberating enzyme (indicated by xylotriose production), and a reducing-end xylose that releases exo-oligoxylanase; and β -xylosidase.

ACKNOWLEDGMENTS

The authors are grateful for the support extended by Dr. S. Yoshida and Mr. K. Yoon, University of Tsukuba, and Dr. E. Wang, Researcher & Division Chief, Taiwan Forestry Research Institute, Chairman of the Organizing Committee of the 2014 Pan Pacific Conference of the Technical Association of the Pulp and Paper Industry (TAPPI). Krisna Septiningrum acknowledges support from the Directorate General of Industrial Resilience and International Access Development, Ministry of Industry, Indonesia.

REFERENCES CITED

- Aracri, E., and Vidal, T. (2011). "Xylanase- and laccase-aided hexenuronic acids and lignin removal from specialty sisal fibres," *Carbohydr. Polym.* 83, 1355-1362. DOI: 10.1016/j.carbpol.2010.09.058
- Biely, P., Vrsanska, M., Tenkanen, M., and Kluepfel, D. (1997). "Endo-beta-1,4-xylanase families: Differences in catalytic properties," *J. Biotechnol.* 57, 151-166. DOI: 10.1016/S0168-1656(97)00096-5
- Gellerstedt, G., and Li, J. (1996). "An HPLC method for the quantitative determination of hexenuronic acid groups in chemical pulps," *Carbohydr. Res.* 294, 41-51. DOI: 10.1016/S0008-6215(96)90615-1
- Honda, Y., and Kitaoka, M. (2004). "Enzyme catalysis and regulation: A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxyylanase," *J. Biol. Chem.* 279, 55097-55103. DOI: 10.1074/jbc.M409832200 PMID:15491996
- Jiang, Z.-H., Van Lierob, B., and Berry, R. (2000). "Hexenuronic acid groups in pulping and bleaching chemistry," *TAPPI J.* 83, 167-175.
- Kuwabara, E., Koshitsuka, T., Kajiyama, M., and Ohi, H. (2011). "Impact on the filtrate from bleached pulp treated with peroxymonosulfuric acid for effective removal of hexenuronic acid," *Japan TAPPI J.* 65, 1071-1075. DOI: 10.2524/jtappij.65.1071
- Kuwabara, E., Zhou, X., Homma, M., Takahashi, S., Kajiyama, M., and Ohi, H. (2012). Relationship between hexenuronic acid content of pulp and brightness stability in accelerated aging," *Japan TAPPI J.* 66, 743-757. DOI: 10.2524/jtappij.66.743
- Lyytikäinen, K., Saukkonen, E., Kajanto, I., and Käyhkö, J. (2011). "The effect of hemicellulose extraction on fiber charge properties and retention behavior of kraft pulp fibers," *BioResources* 6(1), 219-231. DOI: 10.15376/biores.6.1.219-231
- Milner, Y., and Avigad, G. (1967). "A copper reagent for the determination of hexuronic acids and certain ketohexoses," *Carbohydr. Res.* 4, 359-361. DOI: 10.1016/S0008-6215(00)80191-3
- Nelson, N. (1944). "A photometric adaptation of the Somogyi method for determination of glucose," *J. Biol. Chem.*, 153, 375-380.
- Park, N. H., Yoshida, S., Kawabata, Y., Sun, H. J., and Kusakabe, I. (2001). "Screening test for xylanolytic activities of commercially available enzymes and release of arabinose from arabinoglucuronoxylan by the enzymes," *J. App. Glycosci.* 48, 253-262. DOI: 10.5458/jag.48.253

- Rantanen, H., Virkki, L., Tuomainen, P., Kabel, M., Schols, H., and Tenkanen, M. (2007). "Preparation of arabinoxylobiose from rye xylan using family 10 *Aspergillus aculeatus* endo-1,4- β -D-xylanase," *Carbohydr. Polym.* 2(21), 350-359. DOI: 10.1016/j.carbpol.2006.11.022
- Septiningrum, K., Ohi, H., Waeonukul, R., Pason, P., Tachaapaikoon, C., Ratanakhanokchai, K., Sermsathanaswadi, J., Deng, L., Prawitwong, P., and Kosugi, A. (2015). "The GH67 α -glucuronidase of *Paenibacillus curdlanolyticus* B-6 removes hexenuronic acid groups and facilitates biodegradation of the model xylooligosaccharide hexenuronosyl xylotriase," *Enzyme. Microb. Technol.* 71, 28-35. DOI: 10.1016/j.enzmictec.2015.01.006 PMID:25765307
- Sevastyanova, O., Li, J., and Gellerstedt, G. (2006). "Influence of various oxidizable structures on the brightness stability of fully bleached chemical pulps," *Nord. Pulp. Pap. Res. J.* 21, 49-53. DOI: 10.3183/NPPRJ-2006-21-01-p049-053
- Sun, H. J., Yoshida, S., Park, N., and Kusakabe, I. (2002). "Preparation of (1-4)- β -D-xylooligosaccharides from an acid hydrolysate of cotton-seed xylan: Suitability cotton-seed xylan as a starting material for the preparation of (1-4)- β -D-xylooligosaccharides," *Carbohydr. Res.* 337, 657-661. DOI: 10.1016/S0008-6215(02)00031-9
- Takahashi, S., Nakagawa-izumi, A., and Ohi, H. (2011). "Differential behavior between acacia and Japanese larchwoods in the formation and decomposition of hexenuronic acid during alkaline cooking," *J. Wood. Sci.*, 57, 27-33. DOI: 10.1007/s10086-010-1143-0
- Tavast, D., Brännvall, E., Lindström, M. E., and Henriksson, G. (2011). "Selectiveness and efficiency of combined peracetic acid and chlorine dioxide bleaching stage for kraft pulp in removing hexenuronic acid," *Cell. Chem. Technol.* 45, 89-95.
- Teleman, A., Hausalo, T., Tenkanen, M., and Vuorinen, T. (1996). "Identification of the acidic degradation products of hexenuronic acid and characterization of hexenuronic acid-substituted xylooligosaccharides by NMR spectroscopy," *Carbohydr. Res.* 280, 197-208. DOI: 10.1016/0008-6215(95)00309-6
- Tenkanen, M., Luonteri, E., and Teleman, A. (1996). "Effect of side groups on the action of β -xylosidase from *Trichoderma reesei* against substituted xylo-oligosaccharides," *FEBS Lett.* 399, 303-306. DOI: 10.1016/S0014-5793(96)01313-0
- Tenkanen, M., Vršanská, M., Siika-aho, M., Wong, D. W., Puchart, V., Pentillä, M., Saloheimo, M., and Biely, P. (2013). "Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity," *FEBS J.* 280, 285-301. DOI: 10.1111/febs.12069 PMID:23167779
- Thakur, V. V., Jain, R. K., and Mathur, R. M. (2012). "Studies on xylanase and laccase enzymatic prebleaching to reduce chlorine-based chemicals during CEH and ECF bleaching," *BioResources* 7, 2220-2235. DOI: 10.15376/biores.7.2.2220-2235
- Winyasuk, W., Gomi, S., Shimokawa, T., Satake, T., and Yoshida, S. (2012). "Screening of enzyme system for specific degradation of hexenuronosyl-xylotriase," *Int. J. Agri. Tech.* 8, 103-116.

Article submitted: September 17, 2015; Peer review completed: December 2, 2015;
Revised version received and accepted: January 10, 2016; Published: February 1, 2016.
DOI: 10.15376/biores.11.1.2756-2767