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Screening of enzyme system for specific degradation of hexenuronosyl-xylotriose

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To screen for hexenuronic acid-degrading enzymes, hexenuronosyl-xylotriose was prepared from the enzymatic hydrolysate of kraft pulp xylan with commercial cellulases. One bacterial strain, namely 07-G-dH which had the ability to utilize hexenuronosyl-xylotriose as a carbon source, was isolated from soil, and was identified as *Paenibacillus* sp. on the basis of its 16S rDNA sequence. Reaction products of hexenuronosyl-xylotriose were obtained using crude intracellular enzymes from *Paenibacillus* sp. strain 07-G-dH and followed by thin-layer chromatography and high-performance anion-exchange chromatography with a pulsed amperometric detector. The enzyme produced from a strain 07-G-dH that capable to hydrolyze xylosidic linkages at the reducing-end side of hexenuronosyl-xylotriose. The resulting hexenuronosyl-xylobiose was further degraded to hexenuronic acid and xylose. These results suggested that the crude enzyme system from strain 07-G-dH contained a hexenuronic acid-degrading enzyme.

Key word: Biobleaching; Hexenuronic acid; Hexenuronic acid-degrading enzyme; Hexenuronosyl-xylotriose; Xylanase

Introduction

During the kraft pulping process, the 4-*O*-methyl-D-glucuronic acid residues present in xylans are partly converted into hexenuronic acid (4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid, HexA) under the condition of high alkalinity and high temperature (Buchert *et al*., 1995; Clayton, 1963; Danielsson *et al*., 2006; Johansson and Samuelson, 1977). HexA has received much attention in recent years because its presence affects pulp properties after bleaching. These effects include a decrease of brightness, an increase of

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brightness reversion, high consumption of bleaching reagents, and the retention of metal ions in pulp (Buchert *et al*., 1996).

Removal of HexA can be accomplished by the addition of acid or an appropriate enzyme. The addition of acid results in degradation of the HexA group structure, affording two furan derivatives. Enzymatic treatment with xylanase also decreases the HexA content (Buchert *et al*., 1997) and confers several advantages over acid treatment, such as mild reaction conditions and specific catalysts for the hydrolysis of target glycosyl residues in the pulp. Xylanase treatment can also directly improve the brightness and decrease the kappa number of oxygen delignified pulps (Wong *et al*., 1996). Commercial lipase which contains high feruloyl esterase activity has also been shown to be useful for bleaching and the prevention of brightness reversion of kraft pulps (Nguyena *et al*., 2008). However, bleaching with xylanase causes a loss of pulp yield due to the excessive hydrolysis of pulp. An alternative method of removing HexA has recently been proposed which involves in enzyme that reacts specifically to the HexA portion of pulp. However, information on the enzymatic removal of HexA remains limited. There are no reports regarding the use of specific HexA-degrading enzymes for biobleaching. In this study, therefore, hexenuronic acid-substituted xylotriose (hexenuronosyl-xylotriose, Δ-X_3) was prepared from enzymatic hydrolysates of kraft pulp xylan (a model compound) and used as a carbon source for screening Δ-X_3-degrading bacterials. The mechanism of Δ-X_3-degradation in the isolated bacterial was also investigated.

Materials and methods

Preparation of Δ-X_3 from an enzymatic hydrolysate of kraft pulp xylan

Total chlorine-free kraft pulp from hardwood (Hokuetsu Kisyu Paper, 2.5 kg) was soaked in 15% (25 l) for 24 h. After filtration through cotton cloth, the filtrate was neutralized with sulfuric acid, and then centrifuged at 8,500 × g for 30 min. The precipitate was suspended in distilled water, and dialysed against distilled water to remove any salts and then dried.

The obtained kraft pulp xylan (100 g), it was hydrolysed with 2 l of an enzyme solution containing 8 g of cellulase “Onozuka” 3S (Yakult, Tokyo, Japan) at pH 4.0 and 55°C for 48 h. The reaction mixture was heated at 100°C for 20 min to stop the reaction. After cooling, the reaction mixture was filtered through Advantec No. 2 and No. 5C filter papers (Advantec). The filtrate was applied to a column (4.6 × 62 cm) activated carbon (Wako Pure Chemicals) for chromatography at a flow rate of 93 ml/h. After washing with 12 l of distilled

water, oligosaccharides in the column were eluted with 12.5 l of 40% ethanol. The eluate was concentrated to 500 ml on a rotary vacuum evaporator.

Part of the concentrate (100 ml containing 5.2% total sugar as xylose) was hydrolysed with 100 ml of an enzyme solution containing cellulase "Onozuka" R-10 (Yakult) at pH 4.0 and 40°C for 48 h. The mixture was heated at 100°C for 20 min, and then filtered through Advantec No. 2 and No. 5C filter papers. The filtrate was applied to a column (3.1 × 29 cm) packed with activated carbon for chromatography at a flow rate of 30 ml/h. After washing with distilled water, oligosaccharides were eluted with 1.5 l of 40% ethanol. The eluate was concentrated on a rotary vacuum evaporator.

The oligosaccharide concentrate was further purified by silica gel 60 column chromatography. One milliliter of the concentrate (7.2% of total sugar) was applied to a column (2.2 × 56 cm) packed with silica gel 60 (230–400 mesh, Merck) equilibrated with a solvent system at a ratio of 1-butanol - acetic acid -water of 2:1:1 (v/v), at a flow rate of 30 ml/h and was then eluted with the same solvent system. The structure of the purified oligosaccharide was determined according to the method of Teleman *et al*. (1996). Fast atom bombardment-mass spectrometry (FAB-MS) spectra results were recorded with Jeol HX1010A mass spectrometer (JEOL) operating in negative-ion mode with an accelerating voltage of 10 kV. A portion (1 µl) of oligosaccharide in water was mixed with 1 µl of glycerol and thioglycerol (1:1, v/v) on the probe tip.

Isolation and identification of the bacterial strain

Isolate liquid medium containing 0.5% Δ-X_3, 0.1% yeast extract (Difco), 0.1% polypeptone (Difco), 0.1% yeast nitrogen base (Difco), 0.1% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ and pH 7.0 was used for screening bacterials. For solid medium, 15 g/l of agar (Oxoid) was added to the above medium.

The collected soil samples from different sites in Ibaraki and Fukushima Prefecture were used as a source of bacterials. About 50 mg of each soil sample was suspended in 1 ml of sterilized water. One drop of the suspension was transfered into 10 ml of Δ-X_3 medium in an L-form tube and incubated at 30°C for 3–4 days on a Monod shaker at 60 oscillations per min. After centrifugation at 12,000 × g for 10 min, the culture supernatant was subjected to thin-layer chromatography (TLC) analysis to determine the utilization of Δ-X_3. One drop of culture broth, in which the Δ-X_3 disappeared that was inoculated into new medium. Microorganisms that could grow on Δ-X_3 as a carbon source were accumulated by repeating subculture (>10 times). The accumulated culture broth was spread on an agar plate containing Δ-X_3 and the Δ-X_3 utilization

ability of individual colonies on the plate was tested using the same procedure described above.

For microbial identification, the 16S rDNA fragments of isolates were amplified using primers: 9F, 339F, 785F, 1099F, 536R, 802R, 1242R and 1510R. The sequences of PCR products were determined on an automatic DNA sequencer (ABI PRISM 3200). Phylogenetic analysis was conducted based on 16S rDNA sequence. The sequence similarity searches was performed by BLAST algorithm and aligned to other sequences of *Paenibacillus* sp. from GenBank. Phylogenetic trees were generated by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method by using CLC Main Workbench 6. Bootstrap analysis was based on 1000 resamplings.

Preparation of intracellular enzyme

The isolated bacterial strain was cultivated in 100 ml of enzyme production medium containing 0.5% birchwood xylan (Sigma), 0.1% yeast extract, 0.1% polypeptone, 0.1% yeast nitrogen base, 0.1% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$, pH 7.0 in a 500-ml shaking flask at 30°C for 24 h on a reciprocal shaker (125 oscillations/min). The culture was harvested by centrifugation at 7,000 × g for 30 min, and 1 g of precipitate (wet cell paste) was suspended in 10 ml of 20 mM sodium phosphate buffer, pH 6.5. The suspension was disrupted by ultrasonication and then centrifuged at 12,000 × g for 30 min. The precipitate (cell debris) was suspended in 20 mM sodium phosphate buffer (pH 6.5) containing 1% Triton X-100, stirred for 1 h at 4°C, and then centrifuged at 12,000 × g for 30 min. The supernatant was concentrated by ultrafiltration with Amicon YM10 membrane (Millipore). The concentrate was dialysed against 20 mM sodium phosphate buffer, pH 6.5 and used as an intracellular crude enzyme.

Enzymatic reaction to hexenuronic acid-substituted xylooligosaccharides

The reaction mixture (200 µl) consisting of equal volumes of 2 mM Δ-X_3 in 20 mM sodium phosphate buffer, pH 6.5 and the crude enzyme solution that was incubated at 35°C for 30 min. The reaction was stopped by heating at 100°C for 5 min. Cold acetone (800 µl) was added to the reaction mixture and then centrifuged at 12,000 × g for 10 min. The supernatant was dried and then dissolved in 2 ml of distilled water and subjected to high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) analysis. Similarly, 20 µl of 2 mM substrate (Δ-X_3 or hexenuronosyl-xylobiose,

Δ-X_2) was added to 20 µl of the crude enzyme solution and incubated at 35°C. The reaction mixture was heated at 100°C for 5 min, passed through a SAIKA-SPE C18-30 cartridge (AiSTI Science) and then spotted onto TLC plates.

Analysis of products by enzymatic reaction

In preparation of Δ-X_3, total sugar was measured by the orcinol-hydrochloric acid method (Fernell and King, 1953) using D-xylose as a standard. The sugar content of polysaccharide was determined by the method of Kusakabe *et al.* (1977). β-Xylanase and α-glucuronidase activities were assayed by the previously described method (Park *et al*., 2001).

Sugars in the reaction mixture of Δ-X_3 and crude enzyme were analyzed by HPAEC-PAD (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex). The column was equilibrated with 0.1 M NaOH at 30°C with a flow rate of 1 ml/min. The saccharides were eluted in a linear-gradient from 0 to 0.3 M sodium acetate in 0.1 M NaOH within 15 min.

TLC was performed on silica gel 60 TLC plates (Merck) using the solvent system (I), 1-butanol - acetic acid - water (2:1:1, v/v) or (II) 1-butanol - formic acid - water (2:1:1, v/v). The sugars on the plate were detected by heating at 100°C for 10 min after spraying with sulfuric acid. Unsaturated saccharides were detected with either thiobarbituric acid (TBA) reagent reported by Warren (1996) or *o*-phenylenediamine dihydrochloride reagent reported by Moran *et al*. (1968).

Results and discussion

Preparation of Δ-X_3 from an enzymatic hydrolysate of kraft pulp xylan

Kraft pulp xylan (134.3 g) was obtained from 2.5 kg of kraft pulp by alkaline extraction and the sugar content of the xylan was 88.1% xylose. Then, 100 g of kraft pulp xylan was hydrolyzed with cellulase "Onozuka" 3S and the resulting hydrolysate contained 68.5 g of total sugar. Figure 1 shows the sugar composition in the hydrolysate. TLC (lane 1 in Fig. 1) showed that the hydrolysate was composed of xylose, xylooligosaccharides and several kinds of acidic oligosaccharides. Xylose and xylobiose were effectively removed from the hydrolysate by the first charcoal column chromatography and a sugar solution containing acidic oligosaccharides was obtained (lane 2 in Fig. 1, 26.0 g of total sugar). The obtained oligosaccharides were treated with cellulase "Onozuka" R-10 to hydrolyse oligosaccharides composed of xylose and 4-*O*-methyl-D-glucuronic acid to their corresponding monomers. As both the α-glucuronidase and β-xylosidase activities in cellulase "Onozuka" R-10 were

higher activity than those in cellulase “Onozuka” 3S, 4-*O*-methyl-D-glucuronic acid-substituted xylooligosaccharides which were hydrolysed and the unknown saccharide located between xylotriose and xylotetraose on TLC remained (lane 3 in Fig. 1). After the second charcoal column chromatography, an unknown oligosaccharide-rich fraction containing 1.6 g of total sugar was obtained (lane 4 in Fig. 1). The major oligosaccharide in this fraction was further purified by silica gel in 60 column chromatography and 0.4 g of oligosaccharide which gave a single spot on TLC (lane 5 in Fig. 1). The purified saccharide afforded the m/z 571 corresponding to the molecular ion peak [M-H] on FAB-MS analysis. The purified Δ-X_3 was used as a substrate only in the enzyme reactions because large scale preparation of Δ-X_3 proved difficult and the Δ-X_3 fraction obtained after the second charcoal chromatography was used as a carbon source for the screening of bacterial.

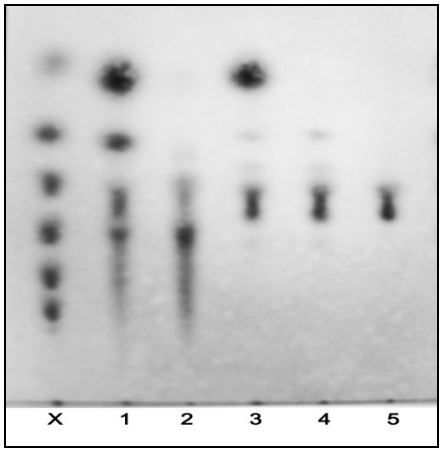

Fig. 1. TLC of hexenuronosyl-xylooligosaccharides isolated from the enzymatic hydrolysate of kraft pulp xylan. Lane X, authentic xylose to xylohexaose; lane 1, enzymatic hydrolysate of kraft pulp xylan; lane 2, xylose and oligosaccharides eluted from charcoal column; lane 3, sugar solution after treatment with cellulase "Onozuka" R-10; lane 4, oligosaccharides eluted from 2nd charcoal column; lane 5, isolated hexenuronosyl-xylooligosaccharide

Isolation and identification of bacterial strains

Microorganisms from soil were subjected for screening Δ-X_3-degrading enzymes. There were many cultures that expressed the ability to utilize Δ-X_3 in early stage of screening. The repeated subculture confirmed gradually decreased utilization or lost the ability to utilize Δ-X_3. It was found only four isolates that maintained the ability to utilize Δ-X_3. These four isolates were cultured in broth that diluted with sterilized water and spread on Δ-X_3 agar

plates to show the ability of individual colony to utilize Δ-X_3. One isolate that maintained high ability to utilize Δ-X_3 designated as a strain namely 07-G-dH.

Physiological and biochemical characteristics of strain 07-G-dH were determined. The isolated strain 07-G-dH was gram-positive, rod-shaped, catalase-positive and oxidase-positive. The strain 07-G-dH was able to grow at 3% NaCl, though no cell growth could be observed when NaCl was present at a final concentration of 5%. The details of the physiological and biochemical characteristics are presented in Table 1 and Table 2. Acorrding to the recommended identification scheme of Bergey's manual of determinative bacteriology (Holt, 1994), the strain 07-G-dH is classified into the genus *Paenibacillus.*

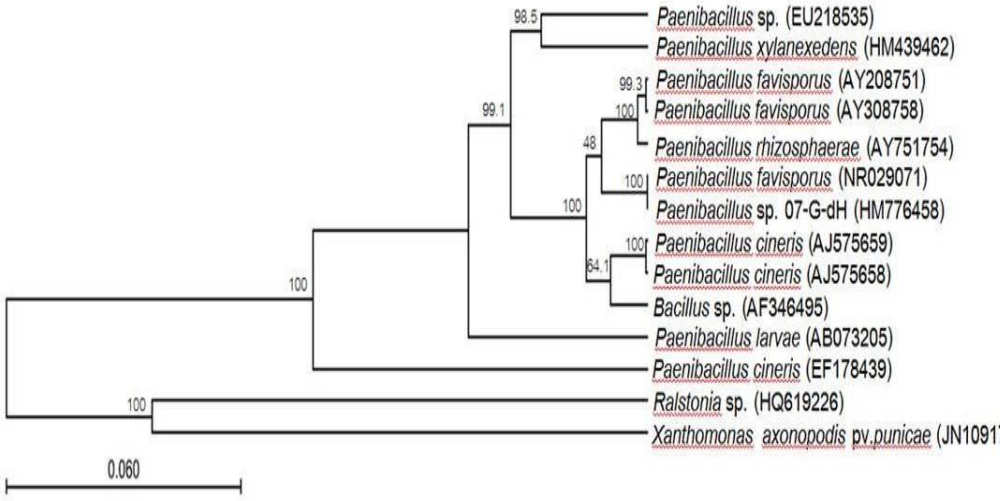

Fig. 2. UPGMA phenogram showing the phylogenetic relationship among the species of *Paenibacillus* sp. strain 07-G-dH based on 16S rDNA sequences and representative strains of the genus *Paenibacillus*. Significance of each branch is indicated by a bootstrap value calculation for 1000 replications. Bar, 6 nt substitutions per 100 nt.

Table 1. Characteristics of Strain 07-G-dH

Properties		**07-G-dH**
Culture temperature (℃)		30
Cell form		Rod-shaped (0.7-0.8×1.5-2.5 μm)
Gram staining		+
Spore formation		+
Mobility		+
Colony form		Medium : nutrient agar Cultivation time : 48 h Diameter : 1.0－2.0 mm Color : cream Form : circle Bulge : lenticular Edge : entire Surface : smooth Clarity : unclarity Consistency : butter-like
Growth temperature (℃)	37	+
	45	+
	50	+
Catalase reaction		+
Oxidase reaction		+
Acid/gas production from glucose (acid production/gas production)		－/－
O/F test (oxidation/fermentation)		－/－
Growth at 3% NaCl		+
Growth at 5% NaCl		－
Casein hydrolysis		－
Utilization of citrate		－
H_2S production		－
Acetoin production		－
Gelatin hydrolysis		－
Nitrate reduction		－

+ : positive, － : negative

Table 2. Fermentation Testof Strain 07-G-dH

Substrates	07-G-dH	Substrates	07-G-dH
Glycerol	−	Esculin	+
Erythritol	−	Salicin	+
D-Arabinose	−	Cellobiose	+
L-Arabinose	+	Maltose	+
Ribose	+	Lactose	+
D-Xylose	+	Melibiose	+
L-Xylose	−	Sucrose	+
Adonitol	−	Trehalose	+
β-Methyl-xyloside	+	Inulin	+
D-Galactose	+	Melezitose	+
D-Glucose	+	Raffinose	+
D-Fructose	+	Starch	+
D-Mannose	+	Glycogen	−
L-Sorbose	+	Xylitol	−
Rhamnose	−	Gentiobiose	+
Galactitol	−	D-Turanose	+
Inositol	+	D-Tagatose	−
Mannitol	+	D-Fucose	−
Sorbitol	−	L-Fucose	−
α-Methyl-D-mannoside	−	D-Arabitol	−
α-Methyl-D-glucoside	−	L-Arabitol	−
N-Acetyl-glucosamine	+	Gluconate	−
Amygdalin	+	2-Keto-gluconic acid	−
Arbutin	+	5-Keto-gluconic acid	−

＋ : positive, － : negative

The species of strain 07-G-dH, the sequence of its 16S rDNA was identified and compared by BLAST analysis to sequences in the GenBank//DDBJ/EMBL database. Figure 2 shows the phylogenetic tree obtained by the UPGMA method. The full-length 16S rDNA sequence of strain 07-G-dH (1,513 bp, GenBank accession number HM776458) showed the closest match to the corresponding sequences from *Paenibacillus cineris* LMG18439 (99.7%, AJ575658) and *Paenibacillus favisporus* GMP03 (99.6%, AY308758). However, several characteristics of strain 07-G-dH were different from those of *P. favisporus* and *P. cineris*, i.e., strain 07-G-dH was able to grow at 50°C and negative for nitrate reduction. These results are differented from the typical characteristics of *P. favisporus* (Vela´zquez *et al.,* 2004). On the

other hand, strain 07-G-dH utilized sorbose and did not utilize α-methyl-D-mannoside and α-methyl-D-glucoside. The strain 07-G-dH was not able to reduce of nitrate. These characteristics were different from those of *P. cineris*. (Logan *et al.,* 2004). Thus, we classified the isolated strain 07-G-dH into the genus *Paenibacillus* sp. and proposed that strain 07-G-dH is a new strain which is closely related to *P. favisporus* and *P. cineris*.

Enzymatic reaction to Δ-X_3

The mechanism of the enzyme system of *Paenibacillus* sp. strain 07-G-dH was examined using Δ-X_3 as a substrate. TLC analysis of the reaction products is shown in Fig.3. The enzyme of *Paenibacillus* sp. strain 07-G-dH rapidly degraded Δ-X_3 and yielded two kinds of products. The major product showed an R_f value that slightly higher than xylotriose and afforded a red-color spot on TLC using TBA reagent (Fig. 3B), whereas the minor product showed the same R_f value as D-xylotriose (Kubata *et al.,* 1994) and did not react with TBA reagent. After 30 min, the reaction products were subjected to HPAEC-PAD analysis (Fig.4). The peaks of D-xylose and unknown products were observed in the reaction medium with a decrease in the peak corresponding to Δ-X_3. In addition, the enzyme system of *Paenibacillus* sp. strain 07-G-dH expressed high xylanase activity. These results suggested that the unknown product, with an R_f value slightly higher than Δ-X_3 was hexenuronosyl-xylobiose (Δ-X_2). To confirm the chemical structure, the unknown product was purified by preparative TLC. FAB-MS spectra of the purified unknown product afforded [M-H] peak of Δ-X_2 at m/z 439. The unknown product was identified as a product of endo-β-xylanase or the reducing end xylose-releasing exo-oligoxylanase in the enzyme system of *Paenibacillus* sp. strain 07-G-dH, which degraded the first xylosidic linkage from the reducing-end side of Δ-X_3 and produced D-xylose and Δ-X_2 as the report of Honda and Kitaoka (2004).

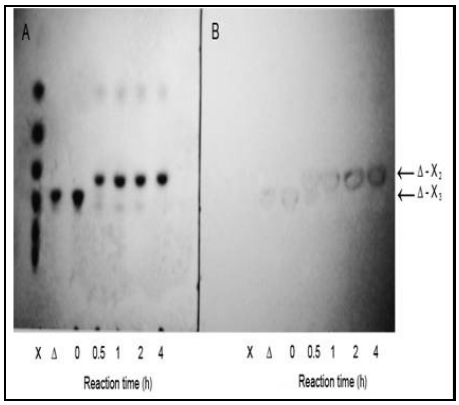

Fig. 3. Time-course of enzymatic hydrolysis of Δ-X_3 with the crude enzyme of *Paenibacillus* sp. strain 07-G-dH. X, authentic xylose to xylohexaose; Δ, authentic hexenuronosyl-xylotriose (Δ-X_3). TLC was performed with the solvent system I, 1-butanol/acetic acid/distilled water (2:1:1, v/v). Sugars on the plates were detected with sulfuric acid (A) and TBA (B)

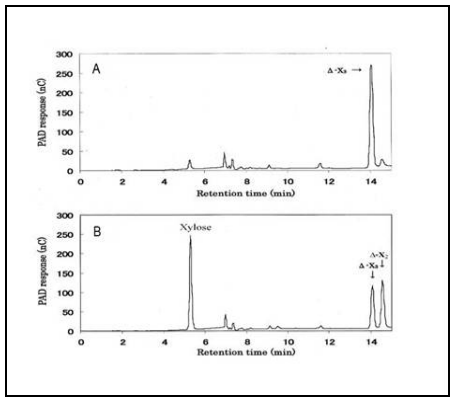

Fig. 4. HPAEC chromatograms of the enzymatic hydrolysate. (A), control; (B), reaction product after 30 min of enzymatic reaction

The resulting Δ-X_2 was incubated with the enzyme from *Paenibacillus* sp. strain 07-G-dH. Δ-X_2 was degraded by the enzyme after 6 h and yielded two kinds of products. One of the products showed the same R_f value as D-xylose on TLC (Fig. 5A), and did not react with TBA reagent. On HPAEC-PAD analysis, a peak corresponding to D-xylose was observed (data not shown). These results suggested that the above product was D-xylose. The other product showed an R_f value which slightly higher than D-xylose and galacturonic acid and afforded a red-colored spot on TLC using TBA reagent (Fig. 5B). An attempt to purify the above product by column chromatographes was unsuccessful as the product proved unstable. However, the R_f value of the unknown product was almost the same as that of HexA reported previously by Moran *et al.* (1968). Additionally, the product reacted with *o*-phenylenediamine dihydrochloride reagent for the detection of keto-deoxy-gluconic acid, and a bright lemon-yellow spot on TLC was observed (Fig. 6). These results suggested that the later product was HexA (4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid).

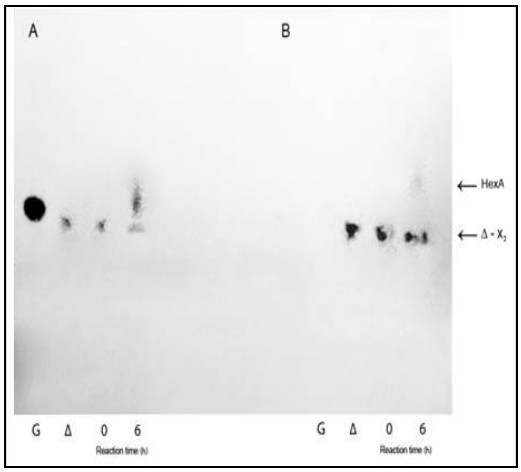

Fig. 5. Time-course of enzymatic hydrolysis of Δ-X_2 with the crude enzyme of *Paenibacillus* sp. strain 07-G-dH. G, galacturonic acid; Δ, authentic hexenuronosyl-xylobiose (Δ-X_2). TLC was performed with the solvent system II, 1-butanol - fortic acid - distilled water (2:1:1, v/v). Sugars on the plates were detected with sulfuric acid (A) and TBA (B)

Fig. 6. Detection of keto-deoxy-gluconic acid by the *o*-phenylenediamine dihydrochloride reagent. G, galacturonic acid; Δ, authentic hexenuronosyl-xylobiose (Δ-X_2); HexA, reaction product from Δ-X_2.

Uchida *et al.* (1992) stated that α-Glucuronidase from *Aspergillus niger* could not hydrolyse Δ-X_3, even though the structure of hexenuronic acid had high similarity to that of glucuronic acid. These phenomen suggested that HexA-degrading enzyme is distinguished from α-glucuronidase, and we screened HexA-degrading enzyme by using Δ-X_3 as a carbon source.

Based on the results described above, we propose that the degradation mechanism of Δ-X_3 occurs as follows: endoxylanase or exo-oligoxylanase in the enzyme system of *Paenibacillus* sp. strain 07-G-dH degraded Δ-X_3, producing Δ-X_2 and D-xylose. The HexA residue in the resulting Δ-X_2 is then degraded via the action of the HexA-degrading enzyme, and xylobiose. The degradation product of the second reaction is further hydrolysed by β-xylosidase, indicating that the HexA-degrading enzyme is essential for the complete degradation of Δ-X_3. This is the first report, to the knowledge, on the specific degradation enzyme of the HexA group in hexenuronic acid-substituted xylooligosaccharides. However, the HexA-degrading activity of the enzyme system in *Paenibacillus* sp. strain 07-G-dH was insufficient to study the enzymatic properties, especially the substrate specificity for various kinds of substrates containing HexA. The cloning and expression of the gene encoding the HexA-degrading enzyme of *Paenibacillus* sp. strain 07-G-dH in future

studies would aid our understanding of this enzyme and its potential applications.

On the process of bio-pulping, many enzymes, such as xylanases and cellulases, are used to treat pulps before applying the standard chemical bleaching sequences (Bajpai *et al*., 2006). However, the bleaching with xylanase causes loss of pulp yield due to the excessive hydrolysis of pulp. To accomplish an increase of brightness, a decrease of brightness reversion and an increase of pulp yield, specific removal of HexA residues from xylan main-chain is essential. HexA-degrading enzyme of *Paenibacillus* sp. strain 07-G-dH, therefore, may play an important role as a key enzyme for the use on bio-pulping.

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