Applied Biochemistry and Biotechnology (2021) 193:1042–1055
https://doi.org/10.1007/s12010-020-03451-2

Substrate Specificities of GH8, GH39, and GH52 β-xylosidases from *Bacillus halodurans* C-125 Toward Substituted Xylooligosaccharides

Koji Teramoto, et al. *[full author details at the end of the article]*

Received: 9 September 2020 / Accepted: 8 November 2020 /
Published online: 4 January 2021

Abstract

Substrate specificities of glycoside hydrolase families 8 (Rex), 39 (*Bh*Xyl39), and 52 (*Bh*Xyl52) β-xylosidases from *Bacillus halodurans* C-125 were investigated. *Bh*Xyl39 hydrolyzed xylotriose most efficiently among the linear xylooligosaccharides. The activity decreased in the order of xylohexaose > xylopentaose > xylotetraose and it had little effect on xylobiose. In contrast, *Bh*Xyl52 hydrolyzed xylobiose and xylotriose most efficiently, and its activity decreased when the main chain became longer as follows: xylotetraose > xylopentaose > xylohexaose. Rex produced *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-α-L-arabinofuranosyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose (Ara^2Xyl_3) and *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-4-*O*-methyl-α-D-glucuronopyranosyl-(1 → 2)]-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose ($MeGlcA^2Xyl_3$), which lost a xylose residue from the reducing end of *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-α-L-arabinofuranosyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose (Ara^3Xyl_4) and *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-4-*O*-methyl-α-D-glucuronopyranosyl-(1 → 2)]-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose ($MeGlcA^3Xyl_4$). It was considered that there is no space to accommodate side chains at subsite −1. *Bh*Xyl39 rapidly hydrolyzes the non-reducing-end xylose linkages of $MeGlcA^3Xyl_4$, while the arabinose branch does not significantly affect the enzyme activity because it degrades Ara^3Xyl_4 as rapidly as unmodified xylotetraose. The model structure suggested that *Bh*Xyl39 enhanced the activity for $MeGlcA^3Xyl_4$ by forming a hydrogen bond between glucuronic acid and Lys265. *Bh*Xyl52 did not hydrolyze Ara^3Xyl_4 and $MeGlcA^3Xyl_4$ because it has a narrow substrate binding pocket and 2- and 3-hydroxyl groups of xylose at subsite +1 hydrogen bond to the enzyme.

Keywords β-D-xylosidase · Glycoside hydrolase family 8 · Glycoside hydrolase family 39 · Glycoside hydrolase family 52 · Arabinoxylooligosaccharide · Aldouronic acid · Glucronoxylooligosaccharide

Key Points
- *Rex produced Ara^2Xyl_3 and $MeGlcA^2Xyl_3$ from Ara^3Xyl_4 and $MeGlcA^3Xyl_4$, respectively.*
- *BhXyl39 selectively hydrolyzed $MeGlcA^3Xyl_4$.*
- *BhXyl52 did not hydrolyze Ara^3Xyl_4 and $MeGlcA^3Xyl_4$.*

Introduction

The term "bioeconomy" refers to economic activities associated with the use of bioproducts. The improved utilization of unused resources is becoming increasingly important in the context of global warming and the energy crisis. Plant cell walls are the most abundant resource on earth, being mainly composed of cellulose, hemicellulose, and lignin, at proportions of approximately one-third each [1]. Xylan, the major hemicellulose, is the second most abundant biomass resource on earth next to cellulose [2]. Xylan consists of a β-1,4-linked xylose main chain with the substitution of α-1,3-linked arabinofuranose and an α-1,2-linked 4-*O*-methyl-glucuronic acid/glucuronic acid [3]. Because xylan is composed mainly of pentose, having a complex structure composed of several constituent sugars, and is more easily decomposed than cellulose by pretreatments, changing it into a fermentation inhibitor, research about hemicellulose lags behind that on cellulose and lignin and no effective methods to utilize xylan have been developed. However, it is important to use xylanolytic enzymes for the application of xylan because enzymes are molecules that can precisely discriminate the structures of substrates, even for substrates with heterogeneous and complex structures.

For the decomposition of xylan, xylanolytic enzymes such as β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72), are needed [2]. Many microorganisms possess numerous different xylanolytic enzymes and are involved in the degradation of biomass in nature. To apply these enzymes for the utilization of xylan, it is essential to elucidate the substrate recognition mechanism of these enzymes; however, the substrate specificity of these enzymes, especially for branches in xylan, remains unclear.

We have studied the structure–function relationship and substrate specificity of xylanolytic enzymes such as β-xylanases [4–13], α-L-arabinofuranosidases [14, 15], and α-glucuronidases [16]. In this work, we focused on β-xylosidases and analyzed how branches in xylan affect their activity. Carbohydrate-active enzymes are classified in the CAZy database (http://www.cazy.org/) based on their amino acid sequences [17]. β-Xylosidases are classified into glycoside hydrolase (GH) families 1, 3, 5, 30, 39, 43, 51, 52, 54, 116, and 120.

Even substrate specificities of GH3 xylosidases from *Aspergillus awamori* and *Trichoderma reesei* toward branched xylooligosaccharides were analyzed [18–20], there are still a lot of unknown things regarding the substrate specificity of β-xylosidases.

Many β-xylosidases have been reported to have α-L-arabinofuranosidase activity as a side activity, which is considered to be advantageous for xylan degradation. However, such side activity is disadvantageous in preparing branched oligosaccharides. In this study, since the correlation between the β-xylosidase family and substrate specificity is poorly understood, GH39 and GH52 β-xylosidases from *Bacillus halodurans* C-125, a useful bacterium for xylan degradation [21], were characterized to clarify the mechanism of their substrate recognition for branches in xylan. The specificity of reducing-end xylose-releasing exo-oligoxylanase (Rex; EC3.2.1.156) belonging to GH8 [22] was also investigated.

Materials and Methods

Protein Expression and Purification

The genes encoding putative β-xylosidases such as *rex* (GH8), *bhxyl39* (GH39), and *bhxyl52* (GH52) were cloned from the genome of *B. halodurans* C-125. The genes were amplified by PCR with KOD-Plus-Neo (Toyobo, Osaka, Japan), using the following primer pairs: (for GH8: forward: CATATGAAGAAAACGACAGAAGGTGC ATTTTG, reverse: AAGCTTGTGCCCCTTTG; for GH39: forward: CCATGGAAAC AGTAGTTGTAAATGATCGTTC, reverse: AAGCTTATACGAAGGAATCAGCCGAT C; and for GH52: forward: CATATGAATCGAGGAGAAACTGGTTATGAAACATG, reverse: GCGGCCGCTATCGGATAAATGGTT). The underlined sequences represent restriction sites. Before insertion of the PCR products into the expression vector, the amplified fragment was sub-cloned by Target Clone-Plus- (Toyobo, Osaka, Japan) and was confirmed by sequencing with a 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). *rex*, *bhxyl39*, and *bhxyl52* were cloned between the *Nde*I and *HindIII* sites of pET30a (Novagen, Darmstadt, Germany), *Nco*I and *Hind*III sites of pET28a (Novagen), and *Nde*I and *Not*I sites of pET28a, respectively. The obtained pET30-*rex*, pET28-*bhxyl39*, and pET28-*bhxyl52* plasmids were transformed into *Escherichia coli* BL21(DE3) (Merck KGaA, Darmstadt, Germany) by electroporation. The transformants were grown in Luria–Bertani medium containing kanamycin at 37 °C with shaking (200 rpm). Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM when the optical density at 600 nm reached 0.2, and the culture was then shaken (200 rpm) at 25 °C for 22 h. The cells were collected by centrifugation (6000 rpm, 15 min, 4 °C), suspended in 50-mM phosphate buffer (pH 7.0), sonicated for 5 min, and then centrifuged (10,000 rpm, 30 min at 4 °C) to remove insoluble materials. The recovered supernatant was applied to HisTrap HP (GE Healthcare, USA) for purification using a 6× histidine tag at the C-terminus. The active fraction was collected and dialyzed against distilled water. The purity of the obtained purified enzyme was confirmed by SDS-PAGE [23].

Substrates

PNP-glycosides and xylans from beechwood, birchwood, and oat spelts were purchased from Sigma Chemical Company (St. Louis, MO, USA). Xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5), xyloheptaose (X_6), and wheat arabinoxylan (low viscosity; 2cSt.) were obtained from Megazyme International (Wicklow, Ireland). In accordance with a previous report [24], branched oligosaccharides such as *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-α-L-arabinofuranosyl-(1 → 3)]-*O*-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose (Ara^3Xyl_4) and *O*-β-D-xylopyranosyl-(1 → 4)-[*O*-4-*O*-methyl-α-D-glucuronopyranosyl-(1 → 2)]-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranose ($MeGlcA^3Xyl_4$) were prepared as follows. The scheme is shown in Fig. 1. From 30.9 g of sugarcane bagasse, 640 mg of Ara^3Xyl_4 and 130 mg of $MeGlcA^3Xyl_4$ were obtained. The structure of the obtained oligosaccharide was determined by NMR, LC-MS, and crystal structure analysis. GH11 xylanase (SoXyn11B) was prepared in accordance with a previously reported method [12]. The amounts of pentose and hexose were determined as previously reported [25].

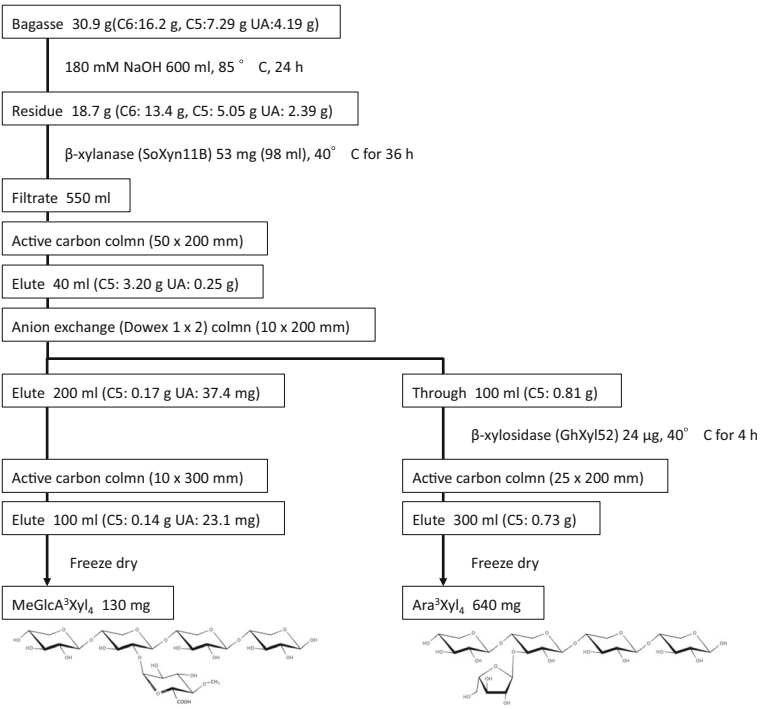

Fig. 1 Preparation of substituted xylooligosaccharides

Enzyme Assay and Substrate Specificity

To evaluate the activity against PNP substrates, a mixture of 25 μL of 2 mM substrate and 20 μL of McIlvaine buffer (pH 5.5 for *Bh*Xyl39, pH 6.2 for *Bh*Xyl52) was preincubated for 5 min, after which 5 μL of enzyme was added to the solution. The enzyme reaction was performed at 50 °C for 10 min, after which 50 μL of 0.2 M Na_2CO_3 was added to stop the reaction. Color development was measured by determining absorbance at 405 nm. To evaluate the substrate specificity of *Bh*Xyl39 and *Bh*Xyl52 to polysaccharides, the reactions were performed in McIlvaine buffer (pH 5.5 for *Bh*Xyl39, pH 6.2 for *Bh*Xyl52) containing 1% (w/v) substrate and 1-μM enzyme at 50 °C for 10 min. The reaction was stopped by heating the solutions at 100 °C for 20 min. The hydrolytic activity was determined by measuring the amounts of reducing sugars using the Somogyi–Nelson method [26]. The activity against xylooligosaccharides was measured as follows. A reaction mixture containing 30 μL of 100-μM substrate, 35 μL of 0.005% (w/v) L-fucose, 100 μL of McIlvaine buffer (pH 5.5 for GH39, pH 6.2 for GH52), 735 μL of DW, and 100 μL of enzyme (final concentrations of 400 nM for GH39 and 5.5 nM for GH52) was incubated at 30 °C for 0, 10, 20, 40, 60, 80, 100, 150, and 200 min, followed by inactivation by heating at 100 °C for 10 min. The hydrolysis products were analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detection (HPAEC-PAD) system and a CarboPac PA1 column (4 × 250 mm) (Dionex Corp., Sunnyvale, CA) as described previously [6]. The decrease in the peak of oligosaccharide was quantified and the degradation rate was calculated. The substrate specificity of GH8 for branched oligosaccharides was determined as follows. The reaction mixture containing 500 μL of 100-μM substrate, 100 μL of McIlvaine buffer at pH 7.0, 200 μL of

DW, 100 μL of 200-μM L-fucose, and 100 μL of 5-μM enzyme was incubated at 30 °C for 24 h. The reaction products were analyzed by Dionex HPLC. Hydrolysis products were also analyzed by MALDI-TOF MS on a REFLEX II (Bruker Daltonics) or a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) in positive-ion mode.

Homology Modeling and Ligand-Docking Modeling

The homology modeling was conducted by the program MODELER 9v12 [27]. The models of *Bh*Xyl39 and *Bh*Xyl52 were calculated in single-template mode using structural models of *Thermoanaerobacterium saccharolyticum* B6A-RI β-xylosidase [XynB, PDB ID 1PX8, 62.0% amino acid identity with *Bh*Xyl39 [28]] and *Parageobacillus thermoglucosidasius* NBRC 107763 β-xylosidase [PDB ID 4C1P, 73.3% amino acid identity with *Bh*Xyl52 [29]], respectively.

MeGlcA^{3}Xyl$_4$ and Ara^{3}Xyl$_4$ binding models of *Bh*Xyl39 were constructed manually using COOT [30]. The ligand models were set in the catalytic pocket of the homology-modeled *Bh*Xyl39 so that the non-reducing xylose moiety was located at subsite −1, with reference to the position of xylose (X_1) in the *T. saccharolyticum* β-xylosidase structure (XynB, PDB ID 1PX8). The X_3 binding model of *Bh*Xyl52 was similarly constructed, with reference to the position of X_2 in the *P. thermoglucosidasius* β-xylosidase structure (XynB, PDB ID 4C1P). These docking models were subjected to structure optimization using the program REFMAC5 [31].

X_1 and X_2 binding models of Rex were constructed by superimposition of the X_2 model bound at subsites −1 and −2 from the X_2 complex Rex structure (PDB ID 1WU6) onto the X_1 complex Rex structure (PDB ID 1WU6) [32]. Structural drawings were prepared using the Cuemol2 program (http://cuemol.osdn.jp/en/).

Results

Expression, Purification, and Properties of *Bh*Xyl39

The construct in which *bhxyl*39 was cloned into pET28 was expressed in IPTG-inducible *E. coli* BL21(DE3). The recombinant enzyme was purified by affinity chromatography using the His-Tag encoded on the C-terminal of the protein expression product. The purified protein showed a single band on SDS-PAGE, with the expected molecular mass of 42 kDa (Fig. 2a).

The effects of pH and temperature on *Bh*Xyl39 activity and stability were determined using PNP-Xyl as the substrate. Maximal enzyme activity was detected at pH 5.5 and 50 °C upon a reaction lasting 10 min. *Bh*Xyl39 was stable between pH 5.0 and 8.0 at 30 °C for 30 min and was also stable up to 40 °C during 30 min of incubation at pH 5.5 (Fig. 3).

Expression, Purification, and Properties of *Bh*Xyl52

The construct in which *bhxyl52* was cloned into pET28 was expressed in IPTG-inducible *E. coli* BL21(DE3). *Bh*Xyl52 was purified by metal affinity chromatography and subjected to SDS-PAGE (Fig. 2b). *Bh*Xyl52 was observed as a single band with a molecular mass of approximately 76 kDa, which was in agreement with the molecular mass expected from the amino acid sequence (78,580). The effects of pH and temperature on the activity and stability of the obtained purified enzyme were investigated. The properties of this enzyme are shown in

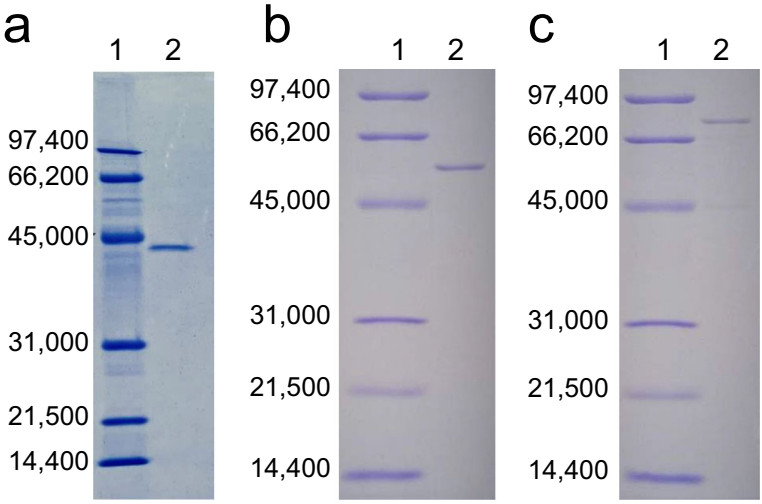

Fig. 2 SDS-PAGE of purified enzymes **a** Rex, **b** *Bh*Xyl39, and **c** *Bh*Xyl52. Lane 1, molecular mass marker (1 μg each band); lane 2, purified enzyme (1 μg)

Fig. 3b. The optimal pH of this enzyme was pH 6.2 and the optimal temperature was 50 °C. *Bh*Xyl52 was stable between pH 6.0 and 8.0 at 30 °C for 30 min and was also stable up to 40 °C during 30 min of incubation at pH 6.0.

Substrate Specificities of Rex

The activities of Rex for branched oligosaccharides such as Ara^3Xyl_4 and $MeGlcA^3Xyl_4$ were examined (Fig. 4). Rex showed hydrolytic activity for both substrates. MALDI-TOF MS analysis of Ara^3Xyl_4 and $MeGlcA^3Xyl_4$ hydrolysis products detected m/z 569 and m/z 627 as a sodium adduct ion $[M + Na]^+$, respectively, suggesting that Rex released only reducing-end

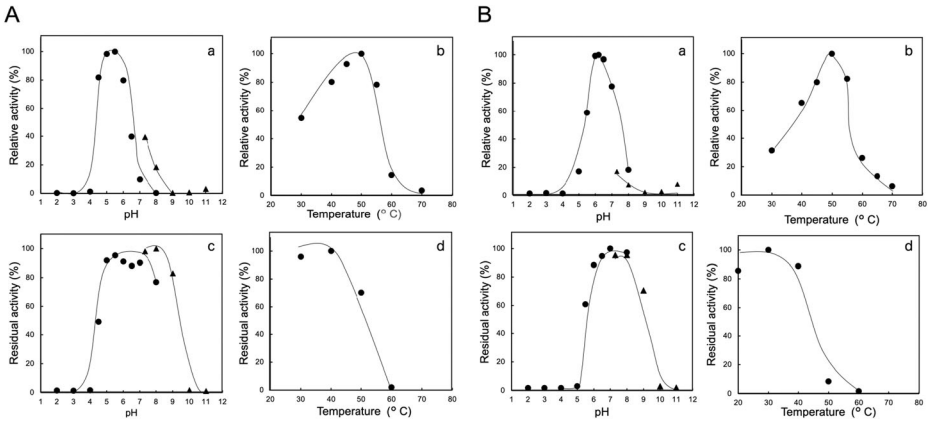

Fig. 3 Enzymatic properties of *Bh*Xyl39 and *Bh*Xyl52 A: *Bh*Xyl39, B: *Bh*Xyl52. a: The effect of pH on enzyme activity. b: The effect of temperature on enzyme activity. c: The effect of pH on enzyme stability. d: The effect of temperature on enzyme stability. Open circle, McIlvaine buffer; closed triangle, Atkins–Pantin buffer

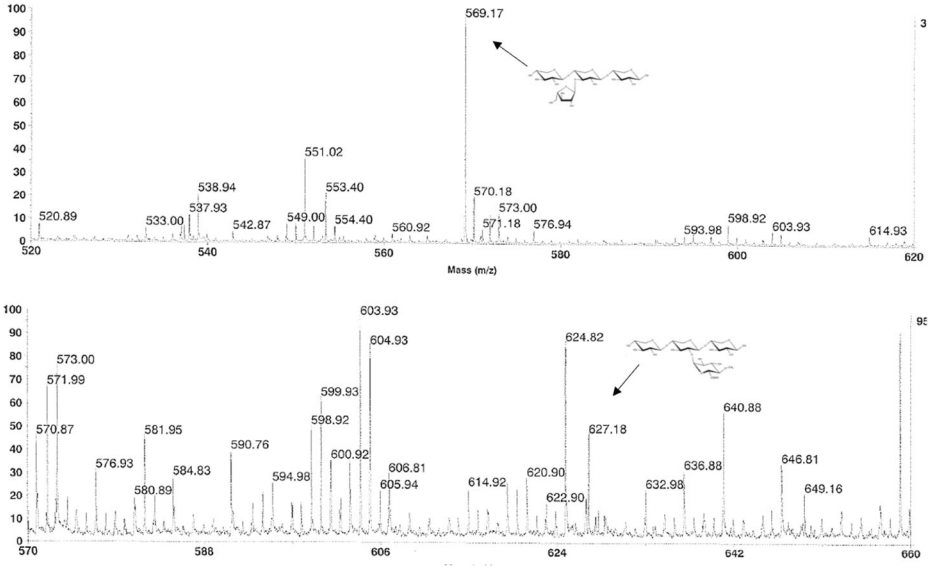

Fig. 4 Substrate specificities of Rex for substituted xylooligosaccharides A: MALDI-TOF MS analysis of Ara^3Xyl_4 hydrolysate with Rex. B: MALDI-TOF MS analysis of $MeGlcA^3Xyl_4$ hydrolysate with Rex

xylose from these oligosaccharides. m/z 437, which corresponds to an oligosaccharide having one arabinose and two xyloses, was also detected among the hydrolysis products of Ara^3Xyl_4, but this would be derived from contamination of oligosaccharides having branches at different positions. Because the excess amount of enzymes such as X_4 was immediately degraded, it is considered that the reaction is very slow even if the enzyme cleaves a xylose linkage next to branched xylose.

Substrate Specificities of *Bh*Xyl39

*Bh*Xyl39 hydrolyzed only PNP-Xyl among PNP substrates and it had little effect on polysaccharides. When the enzyme acted on linear xylooligosaccharides, it hardly hydrolyzed X_2. The enzyme hydrolyzed X_3 at the highest hydrolysis rate, followed by $X_6 > X_5 > X_4$ (Table 1). When the enzyme acted on branched oligosaccharides, the enzyme hydrolyzed $MeGlcA^3Xyl_4$ to xylose and $MeGlcA^3Xyl_3$ very rapidly. The hydrolysis rate was significantly higher than

Table 1 k_{cat}/K_m of *Bh*Xyl39 and *Bh*Xyl52 for various xylooligosaccharides

	*Bh*Xyl39 (min^{-1}•μM)	*Bh*Xyl52 (min^{-1}•μM)
X_2	n.d.	5.8
X_3	3.5	5.8
X_4	1.1	3.1
X_5	1.1	1.6
X_6	2.0×10^{-2}	7.9×10^{-1}
Ara^3Xyl_4	1.2×10^{-2}	n.d.
$MeGlcA^3Xyl_4$	7.5	n.d.

n.d. not detected

that of X_3, which had the highest hydrolysis rate among the linear oligosaccharides. In contrast, the activity toward Ara^3Xyl_4 to release non-reducing-end xylose was almost the same as that of X_4.

Substrate Specificities of *Bh*Xyl52

*Bh*Xyl52 also only acted on PNP-Xyl among the PNP substrates. No increase in reducing power was observed when the enzyme was incubated with various xylans (data not shown). When comparing the hydrolysis rates of linear xylooligosaccharides, the enzyme most rapidly hydrolyzed X_2 and X_3 at almost the same rate. The rates gradually decreased with increasing oligosaccharide length as follows: $X_2 = X_3 > X_4 > X_5 > X_6$ (Table 1). Moreover, *Bh*Xyl52 did not hydrolyze the branched oligosaccharides, even when the amount of enzyme was increased 1000 times (data not shown).

Discussion

B. halodurans C-125 has been reported as an alkaline xylanase-producing bacterium [21]. Sequence analysis of genomic DNA of this strain has been completed and its xylanolytic enzyme system has been clarified. In this study, we focused on β-xylosidases because the substrate specificity of these enzymes against substituted oligosaccharides was poorly understood. Despite *B. halodurans* C-125 possessing β-xylosidase genes classified into GH1, 8, 39, 43, and 52 as candidates for study, GH1 and 43 were excluded from the targets because they are multi-functional enzymes and hydrolyze the linkages of both main and side chains.

To date, 13 GH39 xylosidases, including *Bh*Xyl39, have been characterized. As for *Bh*Xyl39, there are three reports of the characterization of recombinant *Bh*Xyl39 from three different research groups. In a report by Smaali et al. [33], the optimal pH of the enzyme was 7.5 at 55 °C. The enzyme hydrolyzed only PNP-Xyl among the tested PNP-glycosides and had no activity for birchwood xylan (the activity for PNP-Glc was detected, but it was 0.32% of that for PNP-Xyl). The activity of the enzyme for GH11 xylanase hydrolysate of wheat bran arabinoxylan was also investigated, but the hydrolysis rate and specificity for the contained individual oligosaccharides were not clarified. According to a report by Wagschal et al. [34], *Bh*Xyl39 showed optimal activity at pH 6.5, and the enzyme cleaved PNP-Ara*f* and PNP-Ara*p* in addition to PNP-Xyl. It was also described that only the release of xylose was observed by *Bh*Xyl39 from xylans from rye, wheat, oat-spelt, beech, and birch. In addition, Liang et al. [35] reported that the optimal activity of *Bh*Xyl39 was observed at pH 7.0 and 35 °C–45 °C, and it hydrolyzed PNP-Xyl, as well as ONP-Gal, PNP-Ara*f*, PNP-Man, and PNP-Glc. A report has also described that a small amount of xylose was produced from beechwood xylan. In our experiments, we obtained results that differed from those in these previous reports. The optimal pH of *Bh*Xyl39 was 5.5 and the optimal temperature was 50 °C (Fig. 2). The optimal pH may shift significantly depending on the temperature at which measurement is performed. Regarding substrate specificity, synthetic substrates other than PNP-Xyl and polysaccharides were not hydrolyzed by

*Bh*Xyl39. Since the activities on the substrates except for PNP-Xyl were extremely low in all cases, if activity was recognized at all, it is considered that this enzyme prefers a low-molecular-weight substrate and has almost no side activity other than β-xylosidase activity. Similar results were obtained with other characterized GH39 enzymes; therefore, the properties of not acting on a polymer substrate and hydrolyzing only xylosyl linkages in oligomeric substrates are considered to be properties common to the GH39 family. However, no members of the GH39 family of enzymes have had their substrate specificity tested for linear xylooligosaccharides of different lengths and branched oligosaccharides.

On the other hand, GH52 β-xylosidases have hardly been characterized. According to the CAZy database, to date only nine GH52 β-xylosidases have been reported. A β-xylosidase from *Aeromonas caviae* ME-1, one of the characterized enzymes, has been shown to degrade only PNP-Xyl among PNP substrates and it hydrolyzed a mixture of X_1–X_3 into X_1. The enzyme showed optimal activity at pH 6.0 and 50 °C, and was stable in the range of pH 5–8 and below 40 °C [36]. The other characterized enzyme GH52 β-xylosidase from *Geobacillus stearothermophilus* had optimal activity at pH 5.5 and 70 °C and was stable in the range of pH 5–6.5 and at temperatures below 65 °C, and it was reported not to produce X_1 from beechwood xylan [37]. The GH52 xylosidase from *B. stearothermophilus* T-6 showed maximal activity at 65 °C and pH 5.6–6.3. Catalytic amino acids were identified by sequence alignment and site-directed mutagenesis, but only activity against PNP-Xyl has been investigated [38]. *Bh*Xyl52 had an optimal pH of 6.2 and an optimal temperature of 50 °C. The enzyme did not hydrolyze chromogenic substrates other than PNP-Xyl and did not act on polysaccharides. Such substrate specificity was considered to be a property common to GH52 β-xylosidases.

Throughout the study of β-xylosidases, hydrolysis rates for limited linear oligosaccharides have been reported. However, only a few studies have been performed about the substrate specificity of a series of linear xylooligosaccharides and for branched oligosaccharides [39]. As described above, no studies have been performed of the substrate specificity for a series of linear xylooligosaccharides and branched oligosaccharides on GH39 and GH52. It is known that xylan has side chains of α-1,3-linked arabinofuranose and α-1,2-linked 4-*O*-methyl-glucuronic acid/glucuronic acid [3]. This difference has a significant effect on the three-dimensional structure. Since arabinose extends in the same direction as the main chain, the arabinose side chain and the glucuronic acid side chain have different effects regarding the recognition of xylose adjacent to branched xylose. In fact, GH10 xylanase produced oligosaccharides with different branched structures in the case of an arabinose side chain and a glucuronic acid side chain [9, 24]. With the exception of Rex, β-xylosidases generally recognize the non-reducing end of the substrates. Therefore, recognition of non-reducing-end xylose adjacent to branched xylose is undisturbed by the glucuronic acid side chain, but arabinose interferes with the recognition of the xylose residue. In fact, it has been reported that the mold enzyme (GH3) does not hydrolyze the linkage of xylose adjacent to a branch in oligosaccharides having an arabinose side chain [18]. However, since the GH3 enzyme has α-L-arabinofuranosidase activity as a side activity, it is unclear whether this enzyme does not really cleave such a linkage. We have confirmed that GH3 β-xylosidases icluding *Trichoderma reesei* enzyme released non-reducing terminal

xylose then arabinose side chain when the substrates were incubated with large amount of enzyme that instantly degrades linear xylooligosaccharides (unpublished data). This problem of side activity makes it difficult to analyze the properties of β-xylosidase with respect to branched oligosaccharides, and the substrate specificity of β-xylosidase for branched oligosaccharides has yet to be clarified. Therefore, in the present study, the characteristics of GH39 and GH52 β-xylosidases for branched oligosaccharides were clarified in detail for the first time.

As shown in this paper, β-xylosidase activity changes with variation in the length of the main chain. Therefore, hydrolysis activities were compared using the same length of main chain. This led to some interesting results. For example, substrates with glucuronic acid side chains were preferred in GH39, but arabinose side chains did not affect the activity. Moreover, since it does not hydrolyze X_2, it was considered that the GH39 enzyme recognizes three or more longer sugars. We used a structure model of *Bh*Xyl39 to understand how the enzyme discriminates substrates. The binding structures of *Bh*Xyl39 with MeGlcA$^3X_4$ and Ara$^3Xyl_4$ are shown in Fig. 5a and b, respectively. The hydroxyl groups at the 2- and 3-positions of the subsite +1 xylose face the solvent surface surrounded by a large pocket-like structure. It was thought that the arabinose residue fits in a large pocket structure, does not cause steric hindrance, and can be hydrolyzed the same as X_4. In contrast, the carboxyl group of the glucuronic acid residue appears to form a hydrogen bond with the amino group of Lys235. The

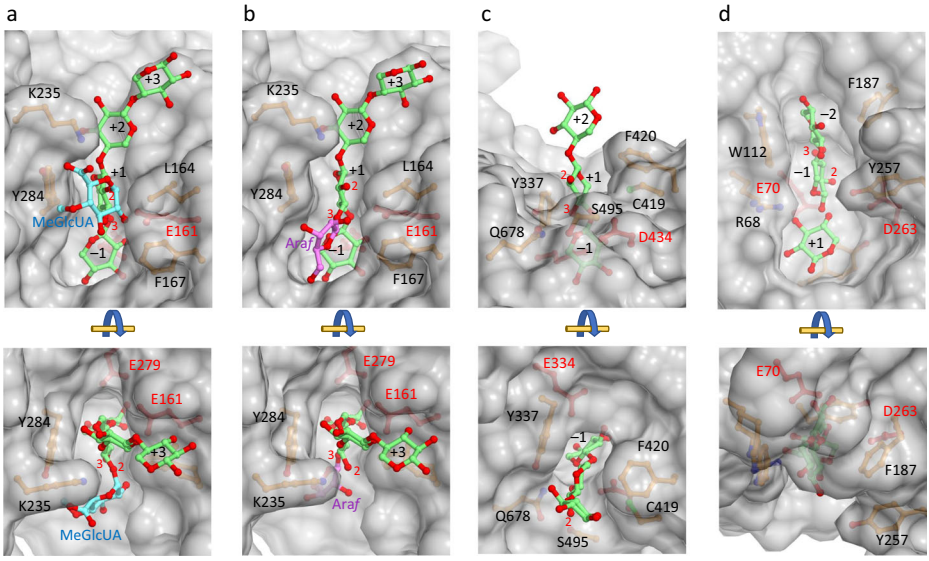

Fig. 5 Sugar binding structures of Rex, *Bh*Xyl39, and *Bh*Xyl52 Xylooligosaccharide binding surface structural models at the catalytic site of β-xylosidases. **a** Xylose and xylobiose binding structure of Rex. **b** MeGlcA$^3Xyl_4$ binding structure of *Bh*Xyl39A. **c** Ara$^3Xyl_4$ binding structure of *Bh*Xyl39A. **d** Xylotriose binding structure of *Bh*Xyl52A. Xylose, MeGlcUA, and arabinofuranose moieties are drawn with green, cyan, and magenta ball-and-stick models. Catalytic residues and substrate binding residues (partial) are drawn with red and orange ball-and-stick models. Subsites are indicated by −2, −1, +1, +2, and +3. Oxygen atoms of C-2 or C-3 hydroxyl groups of xylose moiety at subsite −1 (+1 in Rex) are indicated by red numbers

interaction between the carboxyl group of glucuronic acid and the amino group of basic amino acids is often observed in xylan-degrading enzymes [40, 41]. GH30 xylanase was shown to significantly decrease the hydrolytic activity of xylan when the interaction was removed by amino acid mutation [11]. Therefore, *Bh*Xyl39 shows higher affinity for oligosaccharides having a glucuronic acid side chain and selectively hydrolyzes them more than other oligosaccharides (Table 1).

On the other hand, the hydroxyl groups at the 2- and 3-positions of subsite +1 xylose were buried in the substrate binding pocket of *Bh*Xyl52 (Fig. 5c), suggesting there are no space subsite +1 xylose accommodate side chains. Thus, the linkage of non-reducing-end xylose with substituted xylose could not be cleaved by *Bh*Xyl52 (Table 1).

Rex has already been studied in detail [22, 32], but its specificity for branched oligosaccharides remained unknown. As such, the degradation products of X_4, MeGlcA^{3}Xyl$_4$, and Ara^{3}Xyl$_4$ by Rex were analyzed by HPLC and MS. It was confirmed that only one xylose was released from the reducing end as MALDI-TOF MS analysis detected m/z 627 from MeGlcA^{3}Xyl$_4$ and m/z 569 from Ara^{3}Xyl$_4$. When the enzyme acted on Ara^{3}Xyl$_4$, m/z 437, which corresponds to the loss of two xylose residues, was detected, but no significant peak was observed in the HPLC analysis, suggesting that this reaction was absent or occurred at a significantly low level. Valenzuela et al. [42] reported that Rex-like enzyme released only one xylose from the reducing end of MeGlcA^{3}Xyl$_4$. Our results are the same as in this report. According to the sugar binding structure of Rex mutant with X_2, the hydroxyl group at the 2-position of subsite −1 xylose was hydrogen-bonded to the hydroxyl group of Arg266, the hydroxyl group at the 3-position hydrogen-bonded to Ala70 and Thr69 via water, and there was no space to accommodate the side chains (Fig. 5d). The hydroxyl groups at the 2- and 3-positions of subsite −2 xylose are both hydrogen-bonded to Tyr244 via water, but both face the solvent side and there is space to accommodate side chains. It was suggested that Rex produces oligosaccharides retaining one xylose from the branch to the reducing end by accommodating arabinose and glucuronic acid side chains at subsite −2.

In conclusion, we investigated the substrate specificity of β-xylosidases belonging to different GH families and found that the characteristics were significantly different depending on the particular family. Xylan is acetylated in nature and the acetyl modification at the hydroxyl groups at the 2- and 3-positions of the xylan backbone cause steric hindrance to affect xylanolytic enzyme activity [43]. Since the effect of steric hindrance by acetyl groups were the same as that by arabinose or glucuronic acid modifications, it is considered that GH39 β-xylosidases are not affected by the acetylation of the second xylose residue from the non-reducing end, in contrast GH52 enzymes could not hydrolyze such substrate. These findings are useful for understanding how microorganisms use these enzymes to decompose biomass in nature, and are also useful for utilizing xylan-degrading enzymes. Although hemicellulose is shunned for its heterogeneity, our results strongly suggest that microorganisms use enzymes to strictly discriminate between hemicellulose structures in nature. Since GH52 has extremely strict substrate specificity for branches in xylan, it is considered to be a useful tool for preparing branched oligosaccharides from natural materials and for analyzing the structures of oligosaccharides. We would like to emphasize once again that it is very important to understand the properties of hemicellulases for branched structures in hemicelluloses to expand the range of applications of hemicellulose, which is a breakthrough in the utilization of biomass.

Acknowledgments The authors wish to thank Mitsui Sugar Co., Ltd., for providing us with sugarcane bagasse. The authors would also like to acknowledge funding from Okinawa Science & Technology Promotion Center.

Authors' Contributions SK conceived and designed the research. KT, ST, and TS conducted experiments. ZF contributed to the molecular modeling. KT and SK analyzed the data. SK wrote the manuscript. All authors read and approved the final manuscript.

Funding This study was funded in part by the Innovation System Construction Program of Okinawa Science & Technology Promotion Center.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflicts of interest.

Ethical Approval This article does not describe any studies with human participants or animals performed by any of the authors.

References

1. Aspinall, G. O. (1980). Chemistry of cell wall polysaccharides. In J. Preiss (Ed.), *The biochemistry of plants (a comprehensive treatise), vol 3. Carbohydrates: Structure and function* (pp. 473–500). New York: Academic Press.
2. Saha, B. C. (2003). Hemicellulose bioconversion. *Journal of Industrial Microbiololgy and Biotechnology, 30*(5), 279–291.
3. Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual Review of Plant Biology, 61*, 263–289.
4. Kaneko, S., Kuno, A., Fujimoto, Z., Shimizu, D., Machida, S., Sato, Y., Yura, K., Go, M., Mizuno, H., Taira, K., Kusakabe, I., & Hayashi, K. (1999). An investigation of the nature and function of module 10 in a family F/10 xylanase FXYN of *Streptomyces olivaceoviridis* E-86 by module shuffling with the Cex of *Cellulomonas fimi* and site-directed mutagenesis. *FEBS Letters, 460*(1), 61–66.
5. Kaneko, S., Iwamatsu, S., Kuno, A., Fujimoto, Z., Sato, Y., Yura, K., Go, M., Mizuno, H., Taira, K., Hasegawa, T., Kusakabe, I., & Hayashi, K. (2000). Module shuffling of a family F/10 xylanase: Replacement of modules M4 and M5 of the FXYN of *Streptomyces olivaceoviridis* E-86 with those of the Cex of *Cellulomonas fimi*. *Protein Engineering, Design & Selection, 13*(12), 873–879.
6. Kaneko, S., Ichinose, H., Fujimoto, Z., Kuno, A., Yura, K., Go, M., Mizuno, H., Kusakabe, I., & Kobayashi, H. (2004). Structure and function of a family 10 β-xylanase chimera of *Streptomyces olivaceoviridis* E-86 FXYN and *Cellulomonas fimi* Cex. *Journal of Biological Chemistry, 279*(25), 26619–26626.
7. Kaneko, S., Ito, S., Fujimoto, Z., Kuno, A., Ichinose, H., Iwamatsu, S., & Hasegawa, T. (2009). Importance of interactions of the α-helices in the catalytic domain N- and C-terminals of the family 10 xylanase from *Streptomyces olivaceoviridis* E-86 to the stability of the enzyme. *Journal of Applied Glycoscience, 56*(3), 165–171. https://doi.org/10.5458/jag.56.165.
8. Kaneko, S., Ichinose, H., Fujimoto, Z., Iwamatsu, S., Kuno, A., & Hasegawa, T. (2009). Substrate recognition of a family 10 xylanase from *Streptomyces olivaceoviridis* E-86: A study by site-directed mutagenesis to make an hindrance around the entrance toward the substrate-binding cleft. *Journal of Applied Glycoscience, 56*(3), 173–179.
9. Fujimoto, Z., Kaneko, S., Kuno, A., Kobayashi, H., Kusakabe, I., & Mizuno, H. (2004). Crystal structures of decorated xylooligosaccharides bound to a family 10 xylanase from *Streptomyces olivaceoviridis* E-86. *Journal of Biological Chemistry, 279*(10), 9606–9614.
10. Ichinose, H., Diertavitian, S., Fujimoto, Z., Kuno, A., Lo Leggio, L., & Kaneko, S. (2012). Structure-based engineering of glucose specificity in a family 10 xylanase from *Streptomyces olivaceoviridis* E-86. *Process Biochemistry, 47*(3), 358–365.
11. Maehara, T., Yagi, H., Sato, T., Ohnishi-Kameyama, M., Fujimoto, Z., Kamino, K., Kitamura, Y., St John, F., Yaoi, K., & Kaneko, S. (2018). GH30 glucuronoxylan-specific xylanase from *Streptomyces turgidiscabies* C56. *Applied and Environmental Microbiology, 84*(4), e01850–e01817.

12. Yagi, H., Takehara, R., Tamaki, A., Teramoto, K., Tsutsui, S., & Kaneko, S. (2019). Functional characterization of the GH10 and GH11 xylanases from *Streptomyces olivaceoviridis* E-86 provide insights into the advantage of GH11 xylanase in catalyzing biomass degradation. *Journal of Applied Glycoscience, 66*(1), 29–35.
13. Tsutsui, S., Sakuragi, K., Igarashi, K., Samejima, M., & Kaneko, S. (2020). Evaluation of ammonia pretreatment for enzymatic hydrolysis of sugarcane bagasse to recover xylooligosaccharides. *Journal of Applied Glycoscience, 67*(1), 17–22.
14. Fujimoto, Z., Ichinose, H., Maehara, T., Honda, M., Kitaoka, M., & Kaneko, S. (2010). Crystal structure of an exo-1,5-α-L-arabinofuranosidase from *Streptomyces avermitilis* provides insights into the mechanism of substrate discrimination between exo- and endo-type enzymes in glycoside hydrolase family 43. *Journal of Biological Chemistry, 285*(44), 34134–34143.
15. Maehara, T., Fujimoto, Z., Ichinose, H., Michikawa, M., Harazono, K., & Kaneko, S. (2014). Crystal structure and characterization of the glycoside hydrolase family 62 α-L-arabinofuranosidase from *Streptomyces coelicolor*. *Journal of Biological Chemistry, 289*(11), 7962–7972.
16. Yagi, H., Maehara, T., Tanaka, T., Takehara, R., Teramoto, K., Yaoi, K., & Kaneko, S. (2017). 4-*O*-methyl modifications of glucuronic acids in xylans are indispensable for substrate discrimination by GH67 α-glucuronidase from *Bacillus halodurans* C-125. *Journal of Applied Glycoscience, 64*(4), 115–121.
17. Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal, 280*(2), 309–316.
18. Kormelink, F. J. M., Gruppen, H., Viëtor, R. J., & Voragen, A. G. J. (1993). Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans. *Carbohydrate Research, 249*(2), 355–367.
19. Tenkanen, M., Luonteri, E., & Teleman, A. (1996). Effect of side groups on the action of β-xylosidase from Trichoderma reesei against substituted xylo-oligosaccharides. *FEBS Letters, 399*(3) 303–306.
20. Herrmann, M. C., Vršanská, M., Milada Jurickova, M., Hirsch, J., Biely, P., & Kubicek, C. P. (1997). The β-D-xylosidase of Trichoderma reesei is a multifunctional β-D-xylan xylohydrolase. *Biochemical Journal, 321*, 375–381.
21. Honda, H., Kudo, T., & Horikoshi, K. (1985). Molecular cloning and expression of the xylanase gene of alkaliphilic *Bacillus* sp. strain C-125 in *E. coli*. *Journal of Bacteriology, 161*(2), 784–785.
22. Honda, Y., & Kitaoka, M. (2004). A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. *Journal of Biological Chemistry, 279*(53), 55097–55103.
23. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.
24. Kusakabe, I., Ohgushi, S., Yasui, T., & Kobayashi, T. (1983). Structures of the arabinoxylo-oligosaccharides from the hydrolytic products of corncob arabinoxylan by a xylanase from *Streptomyces*. *Agricultural and Biological Chemistry, 47*(12), 2713–2723.
25. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1986). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology, 118*, 3–40.
26. Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry, 195*, 19–23.
27. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., & Sali, A. (2006). Comparative protein structure modeling using Modeller. *Current Protocols in Bioinformatics, 15*, 5.6.0–5.6.30.
28. Yang, J. K., Yoon, H. J., Ahn, H. J., Lee, B. I., Pedelacq, J. D., Liong, E. C., Berendzen, J., Laivenieks, M., Vieille, C., Zeikus, G. J., Vocadlo, D. J., Withers, S. G., & Suh, S. W. (2004). Crystal structure of β-D-xylosidase from *Thermoanaerobacterium saccharolyticum*, a family 39 glycoside hydrolase. *Journal of Molecular Biolology, 335*(1), 155–165.
29. Espina, G., Eley, K., Pompidor, G., Schneider, T. R., Crennell, S. J., & Danson, M. J. (2014). A novel β-xylosidase structure from *Geobacillus thermoglucosidasius*: The first crystal structure of a glycoside hydrolase family GH52 enzyme reveals unpredicted similarity to other glycoside hydrolase folds. *Acta Crystallographica Section D Structural Biology, 70*(5), 1366–1374.
30. Emsley, P., & Cowtan, K. (2004). Coot: Model-building tools for molecular graphics. *Acta Crystallographica Section D Structural Biology, 60*(12), 2126–2132.
31. Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F., & Vagin, A. A. (2011). REFMAC5 for the refinement of macro- molecular crystal structures. *Acta Crystallographica Section D Structural Biology, 67*(4), 355–367.
32. Fushinobu, S., Hidaka, M., Honda, Y., Wakagi, T., Shoun, H., & Kitaoka, M. (2005). Structural basis for the specificity of the reducing end-xylose-releasing exo-oligoxylanase from *Bacillus halodurans* C-125. *Journal of Biological Chemistry, 280*(17), 17180–17186.

33. Smaali, I., Rémond, C., & O'Donohue, M. J. (2006). Expression in *Escherichia coli* and characterization of β-xylosidases GH39 and GH-43 from *Bacillus halodurans* C-125. *Applied Microbiology and Biotechnology, 73*(3), 582–590.
34. Wagschal, K., Franqui-Espiet, D., Lee, C. C., Robertson, G. H., & Wong, D. W. S. (2008). Cloning, expression and characterization of a glycoside hydrolase family 39 xylosidase from *Bacillus halodurans* C-125. *Applied Biochemistry and Biotechnology, 146*(1-3), 69–78.
35. Liang, Y., Li, X., Shin, H., Chen, R. R., & Mao, Z. (2009). Expression and characterization of a xylosidase (Bxyl) from *Bacillus halodurans* C-125. *Chinese Journal of Biotechnology, 25*, 1386–1393.
36. Suzuki, T., Kitagawa, E., Sakakibara, F., Ibata, K., Usui, K., & Kawai, K. (2001). Cloning, expression, and characterization of a family 52 β-xylosidase gene (xysB) of a multiple-xylanase-producing bacterium, *Aeromonas caviae* ME-1. *Bioscience, Biotechnology, and Biochemistry, 65*(3), 487–494.
37. Huang, Z., Liu, X., Zhang, S., & Liu, Z. (2014). GH52 xylosidase from *Geobacillus stearothermophilus*: Characterization and introduction of xylanase activity by site-directed mutagenesis of Tyr509. *Journal of Industrial Microbiology and Biotechnology, 41*(1), 65–74.
38. Bravman, T., Zolotnitsky, G., Shulami, S., Belakhov, V., Solomon, D., Baasov, T., Shoham, G., & Shoham, Y. (2001). Stereochemistry of family 52 glycosyl hydrolases: A β-xylosidase from *Bacillus stearothermophilus* T-6 is a retaining enzyme. *FEBS Letters, 495*(1–2), 39–43.
39. Malgas, S., Mafa, M. S., Mkabayi, L., & Pletschke, B. I. (2019). A mini review of xylanolytic enzymes with regards to their synergistic interactions during hetero-xylan degradation. *World Journal of Microbiology and Biotechnology, 35*(12), 187.
40. Nurizzo, D., Nagy, T., Gilbert, H. J., Gideon J., & Davies, G. J. (2002). The structural basis for catalysis and specificity of the Pseudomonas cellulosa α-glucuronidase, GlcA67A. *Structure, 10*(4), 547–556.
41. Urbániková, L., Vršanská, M., Mørkeberg Krogh, K. B. R, Hoff, T., & Biely, P. (2011). Structural basis for substrate recognition by Erwinia chrysanthemi GH30 glucuronoxylanase. *The FEBS Journal, 278*(12), 2105–2116.
42. Valenzuela, S. V., Lopez, S., Biely, P., Sanz-Aparicio, J., & Pastor, F. I. J. (2016). The glycoside hydrolase family 8 reducing-end xylose-releasing exo-oligoxylanase Rex8A from *Paenibacillus barcinonensis* BP-23 is active on branched xylooligosaccharides. *Applied and Environmental Microbiology, 82*(17), 5116–5124.
43. Biely, P., Westereng, B., Puchart, V., de Maayer, P., & Cowan, D. A. (2014). Recent progress in understanding mode of action of acetylxylan esterases. *Journal of Applied Glycoscience, 61*(2), 35–44.

Affiliations

Koji Teramoto[1] · Sosyu Tsutsui[1,2] · Tomoko Sato[3] · Zui Fujimoto[3] · Satoshi Kaneko[1,2]

✉ Satoshi Kaneko
sakaneko@agr.u-ryukyu.ac.jp

[1] Department of Subtropical Biochemistry and Biotechnology, Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan

[2] The United Graduate School of Agricultural Sciences, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan

[3] Advanced Analysis Center, National Agriculture and Food Research Organization (NARO), 2-1-2 Kannondai, Tsukuba 305-8602, Japan