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Characterization of a novel salt-, xylose- and alkali-tolerant GH43 bifunctional β-xylosidase/α-L-arabinofuranosidase from the gut bacterial genome

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A GH43 bifunctional β-xylosidase encoding gene (*XylRBM26*) was cloned from *Massilia* sp. RBM26 and successfully expressed in *Escherichia coli*. Recombinant XylRBM26 exhibited β-xylosidase and α-L-arabinofuranosidase activities. When 4-nitrophenyl-β-D-xylopyranoside was used as a substrate, the enzyme reached optimal activity at pH 6.5 and 50°C and remained stable at pH 5.0–10.0. Purified XylRBM26 presented good salt tolerance and retained 96.6% activity in 3.5 M NaCl and 77.9% initial activity even in 4.0 M NaCl. In addition, it exhibited high tolerance to xylose with Ki value of 500 mM. This study was the first to identify and characterize NaCl-tolerant β-xylosidase/α-L-arabinofuranosidase from the gut microbiota. The enzyme's salt, xylose, and alkali stability and resistance to various chemicals make it a potential biocatalyst for the saccharification of lignocellulose, the food industry, and industrial processes conducted in sea water.

[**Key words:** β-Xylosidase; α-L-Arabinofuranosidase; Gut; *Massilia* sp.; Salt tolerance]

As an important component of hemicelluloses in plant cell walls, xylan is a large reproducible biological resource. Its complete degradation requires the synergistic effect of a xylanolytic system that mainly includes endo-1,4-β-D-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-D-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), and α-L-arabinofuranosidase (EC 3.2.1.55) because of its complex structure (1). β-D-Xylosidase can reduce the concentration of xylan hydrolysis products during synergistic hydrolysis to relieve the inhibitory effects of the products on xylanase (2). β-D-Xylosidase is a rate-limiting enzyme during xylan hydrolysis. α-L-Arabinofuranosidase plays a key role in the catalytic hydrolysis of xylose chain substituents and synergistically promotes xylan degradation with xylanase, xylosidase, and other enzymes (3,4).

Based on the amino acid sequence similarity, glycoside hydrolase (GH) families with β-xylosidase activity include GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116, and GH120 (http://www.cazy.org/). GH43 β-xylosidases are greatly promising candidates of high-efficiency β-xylosidases for the degradation of plant biomass compared with enzymes from other families (5). Many GH43 β-xylosidases have been identified, purified, and characterized from various microorganisms, such as *Humicola grisea* var. *thermoidea* (6), *Humicola insolens* (7), *Aspergillus oryzae* (8), *Penicillium oxalicum* (9), *Penicillium purpurogenum* (10), *Phanerochaete chrysosporium* (11), *Bacteroides ovatus* (12), *Weissella* sp. strain 92 (13), *Selenomonas ruminantium* (14), *Enterobacter* sp. (15), and *Geobacillus thermoleovorans* IT-08 (16).

β-Xylosidases are important xylanolytic enzymes that are used in many biotechnological processes (17). Its practical applications, such as marine product processing, involve high-salinity environments. However, only a few salt-tolerant β-xylosidases have been reported (18–20). In addition, xylose is an enzymatic product of xylan and xylooligosaccharides degraded with β-xylosidases and a strong inhibitor of β-xylosidase.

High tolerance to xylose is essential for the commercial application of β-xylosidase. However, most β-xylosidases, especially those from fungi, are strongly inhibited by low concentrations of xylose (K_i of xylose at 2–10 mM) (6,7,21,22).

Influenced by diet, environment, inheritance, and disease, animals have adapted gastrointestinal microorganisms through evolutionary processes. These animals possess the corresponding gene-encoding enzymes to cope with their living states. Various methods, including microbial culture isolation and metagenomics, have been used to obtain xylosidases from gastrointestinal microorganisms in animals, such as termite, yak, cow, and humans (23–29). β-Xylosidases from gut microbiomes of different animals play prominent roles in nutrition and dietary fiber utilization and display various enzymatic characteristics and functions.

The Yunnan snub-nosed monkey (*Rhinopithecus bieti*) is a typical herbivorous primate. Its gastrointestinal tract harbors a dense assemblage of microbial enzyme gene resources that are essential for lignocellulose digestion (30). Recently, a xylanase gene named *XynRBM26* is obtained and heterogeneously expressed through the

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genomic sequencing and functional annotation of *Massilia* sp. RBM26 (31). The enzyme presented good salt tolerance, which could maintain 86% activity in the presence of 5M NaCl. In this paper, we report for the first time the identification, recombinant expression, and characterization of β-xylosidases Xy1RBM26 from *Massilia* sp. RBM26, which is isolated from *R. bieti* feces.

MATERIALS AND METHODS

Bacterial strain, vectors, and reagents *Massilia* sp. RBM26 was isolated and identified from fecal microorganisms of *R. bieti* (31). DNA polymerases, dNTPs, and LA Taq were purchased from Takara (Dalian, China). Genomic DNA extraction and plasmid DNA purification were conducted using kits acquired from Tiangen (Beijing, China). The pEASY-E2 expression kit and *Escherichia coli.* BL21(DE3) procured from TransGen were used to determine the expression of the enzyme-encoding genes. The substrates used for enzymatic tests, including beechwood xylan, 4-nitrophenyl-β-D-xylopyranoside (pNPX), 4-nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-α-D-galactopyranoside (pNPGal), 4-nitrophenyl-α-D-glucopyranoside (pNPGα), and 4-nitrophenyl-α-L-arabinofuranoside (pNPA), were purchased from Sigma–Aldrich (St Louis, MO, USA). Other reagents were of analytical grade and were commercially available.

Sequence analysis and phylogenetic analysis GeneMark.hmm (version 2.4; http://exon.gatech.edu/GeneMark/gmhmm2_prok.cgi) was used for open reading frame prediction. Comparative analysis of nucleotide and amino acid sequences were obtained by conducting BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ExPASy proteomics server (http://expasy.org) was employed for protein identification and analysis, and SignalP (http://www.cbs.dtu.dk/services/SignalP/) was utilized for signal peptide estimation. InterPro (http://www.ebi.ac.uk/interpro/) was used for classifying the Xy1RBM26 glycoside hydrolase family. Tertiary structure prediction was performed using the online SWISS-MODEL server (http://swissmodel.expasy.org). Vector NTI 10.3 (InforMax, Gaithersburg, MD, USA) was used to examine the assembly and alignments of multiple sequences. A phylogenetic tree was generated by the neighbor-joining (NJ) method on MEGA 6.0. Bootstrap analysis was conducted based on 1000 resamplings.

Recombinant expression and protein purification Full-length GH43 xylosidase gene *Xy1RBM26* was identified from the *Massilia* sp. RBM26 genome sequence (31). The DNA fragment of *Xy1RBM26* was amplified from *Massilia* sp. RBM26 genomic DNA by using the primers Xy1RBM26F (5′-ATGATCCACAACCCGATCCTGC-3′) and Xy1RBM26R (5′-CAGCCGGCTGAGGTAGGGCC-3′). Touchdown-PCR amplification comprised an initial denaturation at 94°C for 5 min, followed by 20 touchdown cycles at 94°C for 30 s, 72°C for 30 s (decreasing by 1°C each cycle), and 72°C for 1.5 min. Amplification was then performed via 10 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 7 min. The purified PCR product was cloned into pEASY-E2 and then expressed in *E. coli* BL21(DE3). The nucleotide sequences for GH43 of *XylRBM26* were deposited into the GenBank and assigned the accession number KU885391.

Recombined *E. coli* was grown in LB broth added with 100 μg/mL of ampicillin at 37°C. Enzyme expression was induced with a 1 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 h at 37°C. Recombinant Xy1RBM26 (His6-tagged at N terminal) was purified as previously described (31).

The purity and molecular mass of the purified enzyme were determined through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined by Bradford method with albumin from bovine serum as the standard (32). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was applied to verify the purified protein by Tianjin Biochip (Tianjin, China).

Assays for enzymatic activity pNPX was used as a substrate to determine the activity of recombinant xylosidase XylRBM26. pNPX was dissolved in 0.1 M buffer solution until a final concentration of 2 mM was attained. 450 μL of 2 mM substrate was preheated for 5 min under reaction temperature and added with 50 μL of appropriately diluted enzyme solution. After being incubated for 10 min, the reaction was ended with 2 mL of 1 M Na_2CO_3. The released *p*-nitrophenol (pNP) was measured at 405 nm after being cooled to room temperature. One unit of enzymatic activity (U) was defined as the amount of enzyme that was needed to degrade pNPX to release 1 μmol of pNP per minute. The enzyme assays for pNPGα, pNPA, pNPG, and pNPGal were the same as that for pNPX.

Substrate specificity The specificity of the purified enzyme was assessed on oat spelt xylan, beechwood xylan, carboxymethyl cellulose sodium salt, laminarin, barley β-glucan, and microcrystalline cellulose. The release of reducing sugars was measured by 3,5-dinitrosalicylic acid (DNS) method (31). The substrate was dissolved in 0.1 M buffer until a final concentration of 0.5% (w/v) was obtained. The reactions contained 100 μL of enzyme dilution and 900 μL of the substrate. The enzyme solution was added after the substrate was preheated under reaction temperature for 5 min, and the reaction lasted for another 10 min. Then, the reaction was terminated with 1.5 mL DNS and kept boiling for 5 min. The absorption at 540 nm was measured after the mixture had cooled to room temperature. One unit (U) of enzymatic activity was defined as the enzymatic quantity that released reducing groups corresponding to 1 μmol xylose per minute.

Biochemical characterization pNPX was employed as a substrate to determine recombinant xylosidase XylRBM26 activity. The optimum pH for the xylosidase activity of the purified XylRBM26 was measured in 0.1 M buffer solution at pH 3.0–12.0 and 37°C. The pH stability of the enzyme was estimated by identifying the residual enzyme activity after the enzyme solution was incubated at pH 3.0–12.0 for 1 h at 37°C. The untreated enzyme solution was used as the control. Different buffer solutions were utilized for the various pH ranges: 0.1 mol/L Na_2HPO_4/citric acid for pH 3.0–8.0; 0.1 mol/L Tris/HCl for pH 8.0–9.0; and 0.1 mol/L glycine/NaOH for pH 9.0–12.0. To identify the optimum enzyme temperature, enzymatic reactions proceeded under different temperatures (0°C–60°C) at pH 6.5. The thermostability of the enzyme was assessed by subjecting the purified enzyme solution to different temperatures (45°C, 50°C, 55°C, and 60°C) for 60 min. Enzymatic reaction proceeded at pH 6.5 and 50°C, with the untreated enzyme solution as the control.

Salt resistance and enzyme stabilities in NaCl was investigated as previously described at pH 6.5 and 50°C (31). The effects of different metal ions and chemical reagents on XylRBM26 activity were examined under standard assay conditions, with 1.0 and 10.0 mM (final concentration) KCl, $CoCl_2$, NaCl, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)$, AgCl, $HgCl_2$, EDTA, β-mercaptoethanol, and SDS, as well as 0.5% and 1% (v/v; final concentration) Tween-80 and Triton-X100.

K_m, V_{max}, and k_{cat} were obtained under optimum temperature and pH conditions. 0.1–2 mmol/L pNPX or pNPA was used as the substrate, according to Lineweaver–Burk method. Resistance to xylose was determined by using pNPX as the substrate and adding 0–700 mM xylose to the enzymatic reaction system. Enzymatic reaction proceeded at pH 6.5 at 50°C. The inhibition constant K_i was the same as the xylose concentration when 50% residual enzymatic activity was observed (33). Protease resistance was examined as previously described (31). Residual enzymatic activity was determined using pNPX as the substrate at pH 6.5 at 50°C.

Hydrolytic product analysis of xylRBM26 Thin-layer chromatography (TLC) was used to analyze the products after XylRBM26 acted on xylo-oligosaccharide as described previously (31). The reactions contained 900 μL of 0.5% (w/v) xylobiose, xylotriose, and xylotetraose, as well as 100 μL of enzyme solution. The reactions were performed at 45°C and pH 6.5 for 24 h. The same method was used to determine of arabinobiose, arabinotriose, and arabinotetraose.

RESULTS

Gene cloning and sequence analysis The xylosidase coding gene obtained by PCR cloning was 1629 bp. The initiation codon was ATG. The termination codon was TAG. The GC content was 67.8%. Polypeptide XylRBM26 consisting of 542 amino acids was coded. XylRBM26 had no signal peptide. Its theoretical molecular weight was 61.07 kDa, and the isoelectric point was 5.34. According to an alignment with BLASTP from the NCBI, XylRBM26 exhibited the highest identities (92.0%) with a hypothetical protein derived from *Massilia timonae* in the GenBank (WP005666758). This hypothetical protein belonged to GH43; however, its protein activity was not analyzed. The genes that manifested significant similarity to XylRBM26 were β-xylosidase (WP036247629; WP036210340) from *Massilia* sp. BSC265 and *Massilia* sp. LC238, presenting the same identities of 84.0%. XylRBM26 shared 42.4% identities with GH43 β-xylosidase (ADC85541) derived from *Bifidobacterium animalis* subsp. lactis BB-12 (34). The bacterium from a compost metagenomic uncultured source was only 12.7% identities with GH43 β-xylosidase (LC025936) (35). These findings indicated that XylRBM26 was a new glycoside hydrolase.

According to multiple sequence alignments with the GH43 xylosidase in the GenBank (Fig. 1), XylRBM26 had three typical conservative areas, namely, ^{12}PDPSI^{16}, ^{134}GFDP^{137}, and ^{194}TEAPH^{198}. D13, D136, and E195 were assumed to be catalytic sites. These types of conservative areas were also found in other GH43 xylosidase, such as Xyl43A from *Thermobifida fusca* (36) and XynB3 from *Geobacillus stearothermophilus* T-6 (37). These characteristics indicated that XylRBM26 was a GH43 member.

Phylogenetic analysis In recent years, numerous studies have collected many xylosidases from the environment by using

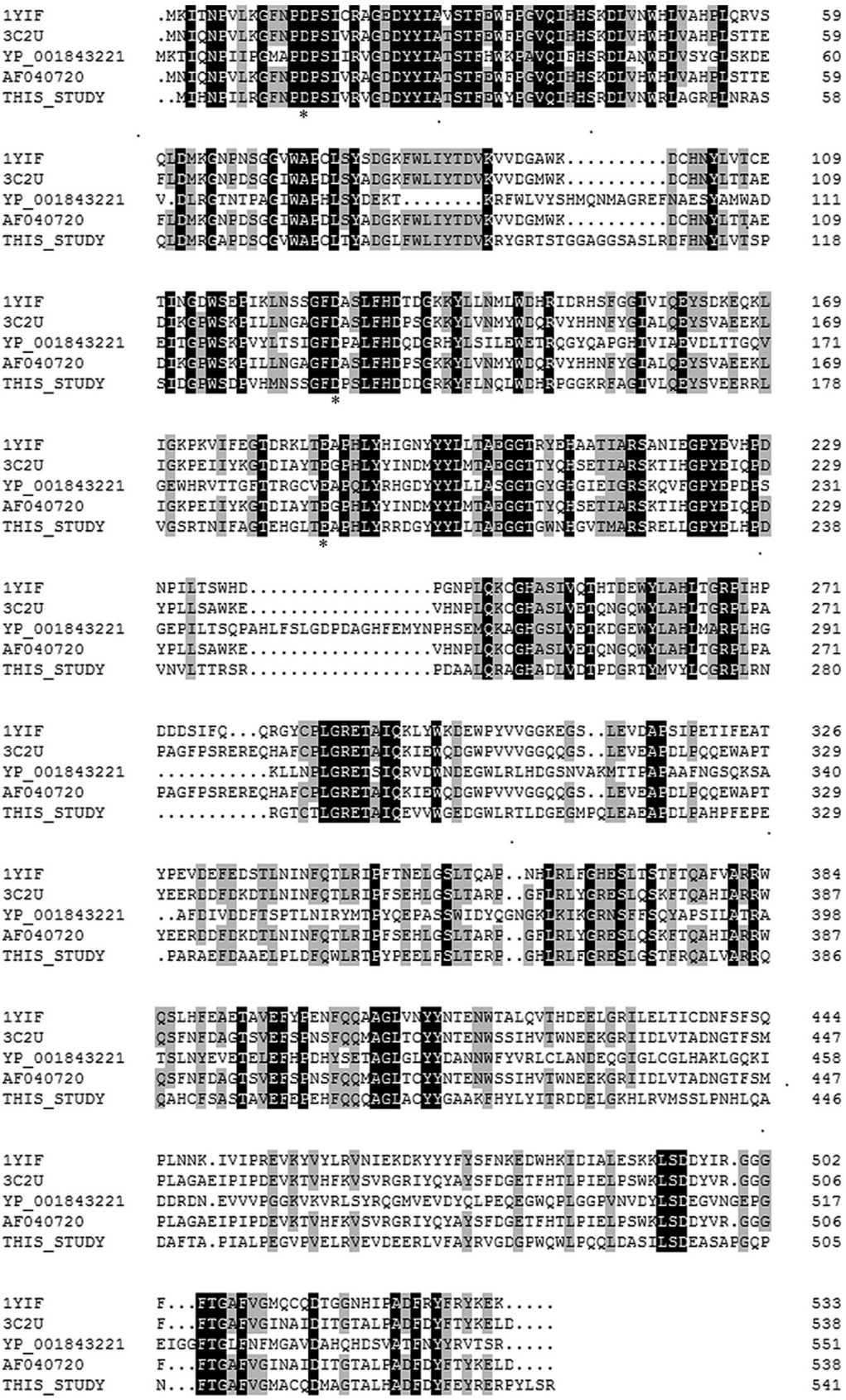

FIG. 1. Multiple sequence alignments of the XylRBM26 protein sequence. Identical and conserved residues are shaded in black and gray, respectively. Asterisks denote putative catalytic residues. 1YIF, β-xylosidase from *Bacillus subtilis*; 3C2U, β-xylosidase from *Selenomonas Ruminantium*; YP_001843221, β-xylosidase from *Lactobacillus fermentum* IFO 3956; AF040720, β-xylosidase from *Selenomonas ruminantium*.

various methods, including isolated microbial culture and metagenomics. XylRBM26 and GH43 xylosidase from other environments in the GenBank were considered to build a phylogenetic tree (Fig. 2). The results indicated that XylRBM26 from *Massilia* sp. RBM26 gathered into one cluster with xylosidases from Firmicutes, and was not related to xylosidases of gut bacteria from similar environment. These results may suggest that XylRBM26 is a novel xylosidase among family 43 glycoside hydrolases.

Heterologous expression and purification Gene coding xylosidase was expressed in *E. coli* BL21(DE3) and purified by Ni^{2+}-NTA metal chelating affinity chromatography. The purified XylRBM26 moved along the SDS-PAGE gel as a single band with the expected molecular weight of approximately 66 kDa (Fig. 3). Three internal peptides from the purified enzyme (Fig. S1), SRPDAALQR, GFNPDPSIVR, and AGHADLVDTPDGR that were randomly selected from the results of MALDI-TOF/MS, matched the deduced amino acid sequence of XylRBM26, confirming that the purified enzyme was indeed XylRBM26.

Substrate specificity of XylRBM26 The specific activities of the purified XylRBM26 toward 2 mmol/L pNPX and pNPA substrates were 2.83 ± 0.23 and 1.28 ± 0.03 U mL^{-1}, respectively, at pH 6.5 and 50°C. However, no XylRBM26 activity was found toward substrates of 0.5% (w/v) beechwood xylan, oat spelt xylan, barley β-glucan, laminarin, microcrystalline cellulose, carboxymethyl cellulose sodium salt, or 2 mmol/L pNPGα, pNPGal, and pNPG.

Biochemical characterization of XylRBM26 The biochemical characteristics of xylosidase were determined using pNPX. The purified XylRBM26 displayed maximum xylosidase activity at pH 6.5 when assayed at 37°C (Fig. 4A). The enzyme retained more than 80% of its activity at a pH range of pH 5.0–10.0 after 60 min of incubation at 37°C (Fig. 4B). The optimum temperature of XylRBM26 was 50°C at pH 6.5, and > 50% activity was retained at 40°C–55°C (Fig. 4C). Thermostability assays revealed that

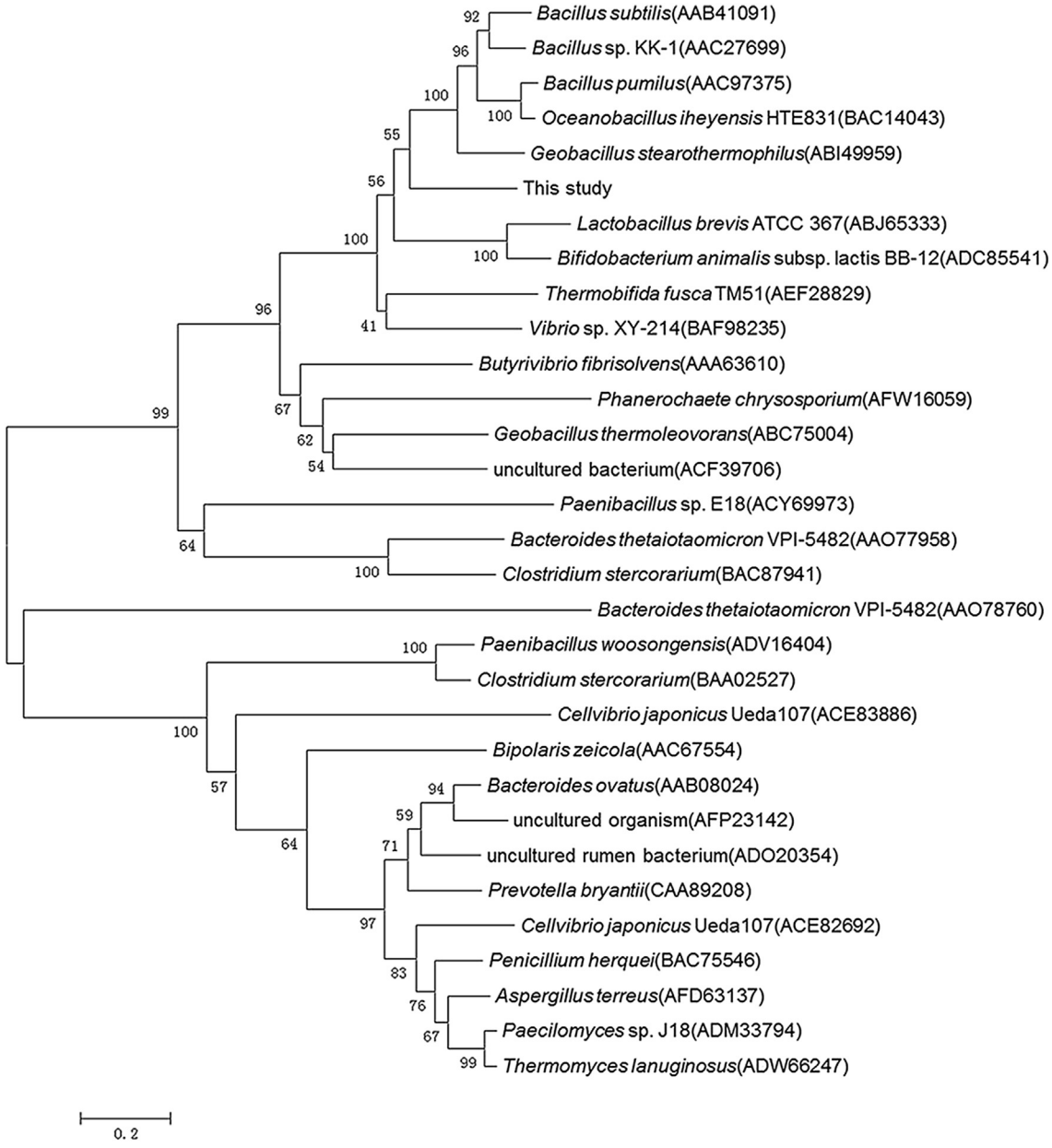

FIG. 2. Phylogenetic analysis based on the amino acid sequences of XylRBM26 with other GH43 family proteins.

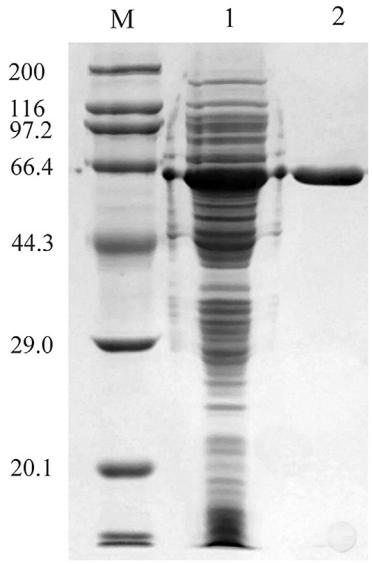

FIG. 3. SDS-PAGE analysis of recombinant XylRBM26. Lanes: M, protein molecular weight marker; 1, induced cytoplasmic total protein; 2, purified XylRBM26.

XylRBM26 was stable at 45°C and 50°C for more than 1 h. However, enzymatic activity was reduced promptly after 10 min incubation when the temperature exceeded 55°C (Fig. 4D). Purified XylRBM26 exhibited good salt tolerance, retaining 134.4%–96.6% activity at concentrations between 1.5 and 3.5 M NaCl and 77.9% initial activity even in 4.0 M NaCl (Fig. 4E). After 1 h incubation at 37°C with 0–4.5 M NaCl, XylRBM26 retained 100%–72.9% of its initial activity (Fig. 4F).

Xylan-degrading enzymes with strong protease resistance have extensive application value in the feed additive industry. To study the protease resistance of XylRBM26, we treated XylRBM26 with 10-fold (w/v) trypsin and proteinase K. After incubation at 37°C for 1 h, the residual enzymatic activities of XylRBM26 treated with trypsin and proteinase K were 74.6% and 35.9%, respectively, suggesting that the recombinant xylosidase exhibited good resistance against trypsin but was weak against proteinase K.

The effects of various reagents on the enzymatic activity were evaluated (Table 1). A complete inhibition of xylosidase activity of the purified XylRBM26 was observed in the presence of 1 mmol/L Hg^{2+}, Ag^{+}, SDS, and 10 mmol/L Zn^{2+}. At a concentration of 10 mmol/L, XylRBM26 activity was strongly inhibited by Ni^{2+} (retaining 28.2% activity), Cu^{2+} (retaining 4.9% activity), and partially inhibited by Fe^{2+} (56.6%). The other tested reagents moderately inhibited or no effect on the enzymatic activity.

The kinetic parameters of XylRBM26 for pNPX were K_m (mM/L): 2.27, k_{cat} (s^{-1}): 2.34, V_{max} (μmol min^{-1} mg^{-1}): 1.60, k_{cat}/K_m (s^{-1} mM^{-1}):1.03. The kinetic parameters of XylRBM26 for pNPA were K_m (mM/L): 4.64, k_{cat} (s^{-1}): 2.04, V_{max} (μmol min^{-1} mg^{-1}): 1.98, k_{cat}/K_m (s^{-1} mM^{-1}): 0.44. These results suggest that XylRBM26 had a greater affinity to pNPX than pNPA. The k_{cat} and V_{max} values of XylRBM26 for pNPX were similar to that of pNPA. The catalytic efficiency constant k_{cat}/K_m for pNPX was greater than that of pNPA.

Effect of xylose on XylRBM26 β-xylosidase activity To analyze the xylose tolerance of XylRBM26, xylose at different concentrations was added to the enzymatic reaction system to a final concentration of 0–700 mM. The results indicated that as the xylose concentration increased, the enzymatic activity of xylosidase presented a decreasing trend (Fig. 5). The inhibition constant *Ki* of this enzyme was calculated as 500. Approximately 39.2% of residual enzymatic activity was observed when the xylose concentration was 700 mM.

Hydrolytic product analysis of XylRBM26 TLC method was used to analyze products after recombinant xylosidase XylRBM26 was used in hydrolyzing 0.5% (w/v) xylobiose, xylotriose, and xylotetraose (Fig. 6). After treatment for 24 h, the hydrolysis products of xylbiose, xylotriose, and xylotetraose with XylRBM26 were xylose. XylRBM26 was used in hydrolyzing 0.5% (w/v) arabinobiose, arabinotriose, and arabinotetraose (Fig. S2). After 24 h, none of the substrates were degraded.

DISCUSSION

The gastrointestinal tract of herbivorous animals contains numerous microorganisms responsible for lignocellulose degradation. Microbial genome sequencing can reveal comprehensive genetic information. With genome sequencing technology, some glycosyl hydrolase genes from *Massilia* species have been found (31,38,39). To our knowledge, no xylosidase from *Massilia* has been functionally characterized.

In this study, a β-xylosidase gene *XylRBM26* was cloned and characterized through the genomic sequencing of *Massilia* sp. RBM26. The highest activity of XylRBM26 was observed around neutral pH (6.5), which was similar to other reported GH43 β-xylosidases (optimal pH range 5.0–7.0) (Table 2). Although XylRBM26 could be classified as a neutral enzyme, it has good pH stability, active over a broad pH range (5.0–10.0) (Table 2). XylRBM26 is an alkali-tolerant enzyme, retaining approximately 100% of activity even at pH 10.0 (Fig. 4B). Generally, the suitable pH of most reported GH43 β-xylosidases is in the pH range of 5.0–9.0 (Table 2). Only Xyl43A from *H. insolens* Y1 showed a similar alkali tolerance characteristic (7). β-Xylosidases with alkaline pH stability can be directly used in the enzymatic hydrolysis process of alkaline-pretreated agro-residues (43).

The optimum temperature of XylRBM26 is 50°C, which is higher than those of β-xylosidases reported from yak rumen (27) and human fecal microorganisms (25). RuXyn1 (27) and XylRBM26 are GH43 xylosidases derived from the gastrointestinal microorganisms of non-human animals. However, the temperature stability of XylRBM26 is substantially higher than that of RuXyn1. Thermostability assays demonstrated that RuXyn1 loses 76% of its activity after 10 min at 45°C and 97% of its activity at 50°C (27). The half-life of XylRBM26 at 45°C and 50°C was longer than 1 h (Fig. 4D).

Most β-xylosidases, particularly β-xylosidases from various fungi, are sensitive to low xylose concentrations, ranging from 2 mM to 10 mM (44). Therefore, β-xylosidases possessing high xylose tolerance show potential for hemicellulose conversion. Compared with other GH43 β-xylosidases, XylRBM26 is highly tolerant to xylose (K_i = 500 mM) (Table 2). The tolerance of XylRBM26 to xylose is only lower than that of β-xylosidases in *H. grisea* var. *thermoidea* (6) but is higher than that of GH43 β-xylosidases from other sources.

Many enzymes from GH43 simultaneously possess β-D-xylosidase and α-L-arabinofuranosidase activities due to the conformational similarity of xylopyranose and arabinofuranose, allowing substrate promiscuity (5). Bifunctional β-xylosidase/α-L-arabinofuranosidase can simultaneously degrade multiple substrates to reduce enzyme dosage and production cost. Thus, it may be important for many biotechnological processes that use enzymes for biomass deconstruction. The recombinant XylRBM26 is similar to most previously reported GH43 xylosidases (6,7,34,45,46), which was able to hydrolyze synthetic substrates pNPX and pNPA. The

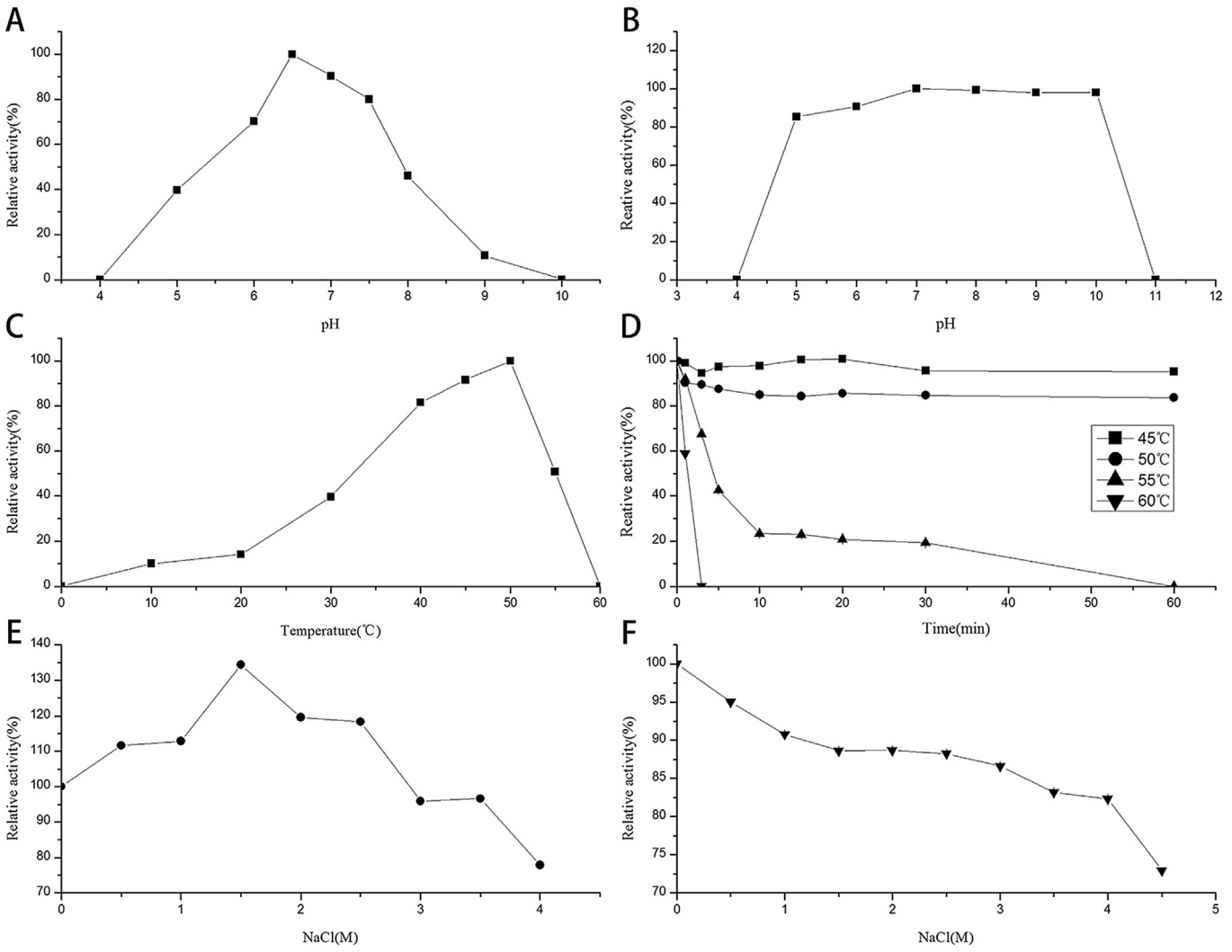

FIG. 4. Characterization of purified XylRBM26. (A) Effect of pH on XylRBM26 activity. Enzymatic activity was determined in a range of pH 3.0–12.0 at 37°C. (B) Effect of pH on enzyme stability. Enzymatic activity was measured under pH 6.5 at 37°C after enzyme pre-incubation in a pH range of 3.0–12.0 at 37°C after 1 h. (C) Effect of temperature on the activity of XylRBM26. Enzymatic activity was determined at pH 6.5 from 0 to 60°C. (D) Effect of temperature on xylosidase thermostability. The thermostability of XylRBM26 was monitored from 45°C to 60°C at pH 6.5 for 0–60 min. (E) Effect of NaCl on XylRBM26 activity. (F) Effect of NaCl on XylRBM26 stability. Enzyme solution was incubated in 0–4.5 M NaCl at 37°C for 60 min, followed by measurement of the residual activity at pH 6.5 and 50°C. Error bars represent the mean ± SD (n = 3).

TABLE 1. Effect of metal ions and chemical reagents on the activity of XylRBM26.

Reagent	Relative activity (%)	
	1 mM	10 mM
None	100	100
Ni^{2+}	86.0 ± 0.05	28.2 ± 0.10
Mg^{2+}	93.1 ± 0.01	95.5 ± 0.04
EDTA	96.1 ± 0.04	96.9 ± 0.06
Cu^{2+}	14.7 ± 0.01	4.9 ± 0.05
Fe^{3+}	93.3 ± 0.05	91.8 ± 0.25
Co^{2+}	95.2 ± 0.10	95.8 ± 0.03
Fe^{2+}	85.6 ± 0.04	56.6 ± 0.14
Mn^{2+}	99.6 ± 0.04	98.2 ± 0.08
β-Mercaptoethanol	96.0 ± 0.01	99.9 ± 0.01
Tween-80	59.6 ± 0.10[a]	112.4 ± 0.10[b]
Na^{+}	94.1 ± 0.04	91.6 ± 0.04
Zn^{2+}	6.3 ± 0.01	0.0
K^{+}	103.1 ± 0.05	103.9 ± 0.06
Triton-100	89.9 ± 0.20[a]	102.3 ± 0.20[b]
Pb^{2+}	100.8 ± 0.04	104.8 ± 0.09
Ag^{+}	0.0	0.0
SDS	0.0	0.0
Hg^{2+}	0.0	0.0

[a] The final concentration is 0.5 % (v/v).
[b] The final concentration is 1.0 % (v/v).

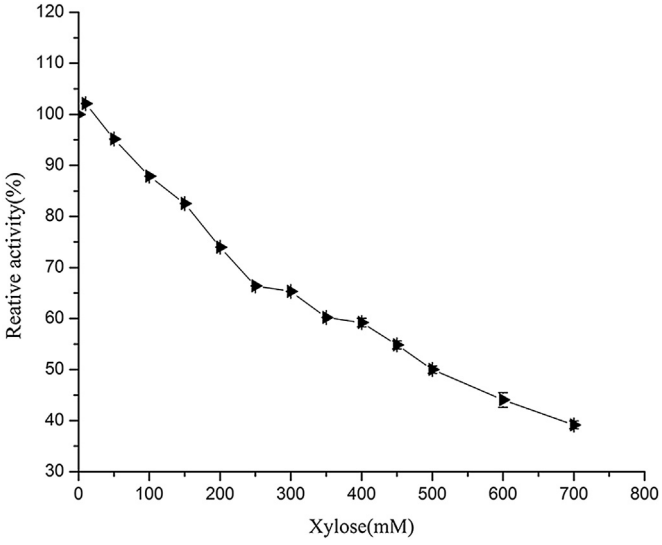

FIG. 5. Effects of xylose on XylRBM26 activity. Residual activity was measured with pNPX as the substrate. The values represent the means of three separate experiments.

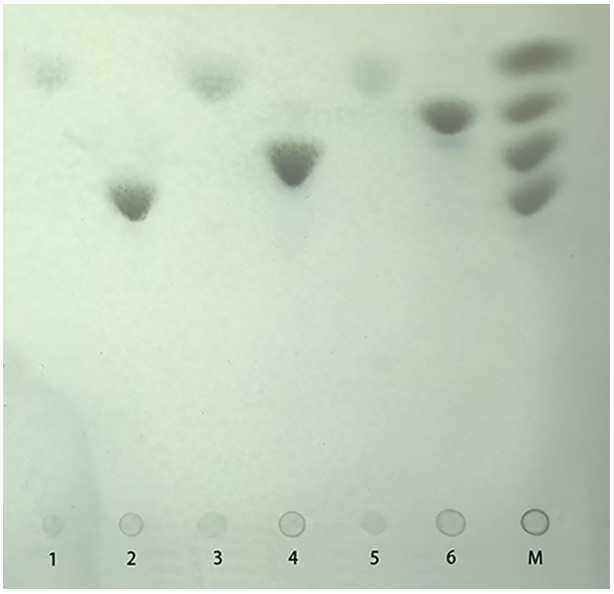

FIG. 6. Thin-layer chromatography of hydrolysis product analysis of xylooligosaccharides after XylRBM26 treatment for 24 h. M, mixture of xylose, xylobiose, xylotriose, and xylotetraose. Lanes 1, 3, 5: samples of xylotetraose, xylotriose, and xylobiose (0.5%, w/v) incubated with XylRBM26 β-xylosidase for 24 h, respectively. Lanes 2, 4, 6: samples of xylotetraose, xylotriose, and xylobiose with inactivated XylRBM26.

results indicated that XylRBM26 is a bifunctional of β-D-xylosidase/α-L-arabinofuranosidase. XylRBM26 is the first enzyme shown to exhibit both xylosidase and arabinofuranosidase functionality in *Massilia* sp.

Investigation of the effects of metal ions and chemical reagents on XylRBM26 activity showed that Pb^{2+} slightly enhances enzymatic activity, which differs from other β-xylosidases. The β-xylosidases from *H. insolens*, *P. oxalicum*, and *Geobacillus thermodenitrificans* were severely inhibited by Pb^{2+} at 5 mM consistency (7,9,47). At 5–40 mM, the purified XylRBM26 exhibited good PbAc tolerance and retained over 96% of xylosidase activity (Fig. S3). However, the xylosidase activity was slightly increased by 10 mM PbAc (110%). XylRBM26 from the gut bacteria was slightly influenced by Ca^{2+} (Fig. S4) compared with known β-xylosidases, whose activities are strongly enhanced by Ca^{2+} (22,35,48).

Salt is one of the oldest food additives, and it has important applications in food safety and preservation. Salt-tolerant enzymes can be used in many industrial processes. Fermentation and material processing under high-salt conditions can reduce costs because sterilization is unnecessary (49). To our knowledge, only three salt-tolerant xylosidases have been reported. Hashimoto et al. (18) described a halotolerant β-xylosidase from *A. oryzae*, which maintained approximately 60% initial activity in 3.0 M NaCl. Wainø and Ingvorsen showed that xylosidase from *Halorhabdus utahensis* (halophilic archaeon) possesses halophilic characteristics, displays optimum activity at 5% NaCl, and retains more than 45% of its maximum activity under salinity range from 0% to 30% (19). Carvalho et al. (20) reported a highly halotolerant β-xylosidase from *Colletotrichum graminicola*, which retained approximately 63% of the control activity in the presence of 2.5 M NaCl. By comparison, the retained activity of XylRBM26 was higher than that of *A. oryzae*, *H. utahensis*, and *C. graminicola* at all salinities tested. More than 77.9% activity of XylRBM26 was maintained at 0–4 M NaCl. All of the halotolerant β-xylosidase genes were characterized from fungi and archaeon. XylRBM26 is the first reported salt-tolerant xylosidase from the gut microbiota.

Hypersaline environments, such as marine and saline soils, harbor the highest number of microbial salt-tolerant genes, and adapting to external environmental stresses is key to microbial survival. Gastrointestinal tract microbes endure numerous stress factors, including osmotic stress (50). However, limited information is available on the diversity of salt-tolerant genes in intestinal microflora, which may be essential for microbial survival. Recently, some new salt-tolerant genes (loci) have been identified from the human gut microbiome, and the cloning and heterologous expression of these genes result in the salt tolerance of transformed cells (51–54). Therefore, the discovery and identification of novel salt-tolerant enzymes and genes and investigations on the structure-function relationship of halotolerant enzymes will provide further insights into the mechanisms by which bacteria adapt to environmental stress in the gut.

In conclusion, this is the first report on the cloning and functional expression of a salt-tolerant GH43 β-xylosidase gene from the gut bacteria of *Massilia* sp. The recombinant XylRBM26 with dual function (β-xylosidase/α-L-arabinofuranosidase) and high tolerance to salt, xylose, and alkali might be used as an enzyme in industrial applications such as enzymatic saccharification of lignocellulose, the food industry, and industrial processes conducted in sea water. The identification of this novel salt-tolerant β-

TABLE 2. Properties of some GH43 β-xylosidases from various sources.

Protein name	Source	Functions	MW (kDa)	Optimum Tm (°C)	Optimum pH	pH stability	K_i of xylose (mM)	Reference
XylRBM26	*Massilia* sp.	Dual function	66[a]	50	6.5	5.0–10.0	500	This study
–	*Bifidobacterium breve* K-110	β-Xylosidase	49[c]	37	5.0	–	–	25
RuXyn1	Yak rumen metagenome	Dual function	42[a]	40	7.0	5.0–6.0	76	27
BXA43	*B. animalis* subsp. *lactis* BB-12	Dual function	62[a]	50	5.5	4.0–8.0	–	34
XylC	*B. adolescentis* LMG10502	β-Xylosidase	62[a]	50	6.0–7.0	–	–	40
BoXA	*Bacteroides ovatus*	β-Xylosidase	–	–	–	–	6.57	12
Xyl43	*Enterobacter* sp.	β-Xylosidase	61.66[a]	40	6.0	–	–	15
GbtXyl43A	*Geobacillus thermoleovorans* IT-08	β-Xylosidase	58.1[a]	–	5.0	–	76	16
HXYLA	*Humicola grisea* var. *thermoidea*	Dual function	37[b]	50	7.0	5.0–7.0	603	6
Xyl43	*Penicillium oxalicum* 114-2	β-Xylosidase	45[b]	50	7.0	6.0–9.0	28.9	9
PcXyl	*Phanerochaete chrysosporium* RP78	Dual function	83[b]	45	5.0	–	76	11
Xyl43A/Xyl43B	*Humicola insolens* Y1	Dual function	37[a]/62[a]	50/50	6.5/7.0	5.0–10.0/6.0–9.0	79/292	7
PtXyl43	*Paecilomyces thermophila*	β-Xylosidase	52.3[a]	55	7.0	–	–	41
XylA	*Aspergillus oryzae*	β-Xylosidase	37.4[a]	30	7.0	7.0–9.0	–	8
TlXyl43	*Thermomyces Lanuginosus* CAU44	β-Xylosidase	51.6[a]	55	6.5	7.0–9.5	63	42

Dual function: β-xylosidase/α-L-arabinofuranosidase.
[a] Expressed in *E. coli*.
[b] Expressed in *P. pastoris*.
[c] Purified from native microorganism.

xylosidase emphasizes the usefulness of genomic sequencing to discover potential novel enzymes related to hemicellulose degradation and help us further understand bacterial salt tolerance mechanisms in the gut.

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