

Expression and characterisation of a pH and salt tolerant, thermostable and xylose tolerant recombinant GH43 β -xylosidase from *Thermobifida halotolerans* YIM 90462^T for promoting hemicellulose degradation

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Abstract A gene encoding a β -xylosidase (designated as Thxyl43A) was cloned from strain *Thermobifida halotolerans* YIM 90462^T. The open reading frame of this gene encodes 550 amino acid residues. The gene was over-expressed in *Escherichia coli* and the recombinant protein was purified. The monomeric Thxyl43A protein presented a molecular mass of 61.5 kDa. When p-nitrophenyl- β -d-xylopyranoside was used as the substrate, recombinant Thxyl43A exhibited optimal activity at 55 °C and pH 4.0 to 7.0,

being thermostable by maintaining 47% of its activity after 30 h incubation at 55 °C. The recombinant enzyme retained more than 80% residual activity after incubation at pH range of 4.0 to 12.0 for 24 h, respectively, which indicated notable thermostability and pH stability of Thxyl43A. Moreover, Thxyl43A displayed high catalytic activity (> 60%) in presence of 5–35% NaCl (w/v) or 1–20% ionic liquid (w/v) or 1–50 mM xylose. These properties suggest that Thxyl43A has potential for promoting hemicellulose degradation and other industrial applications.

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Keywords *Thermobifida halotolerans* YIM 90462^T · β -xylosidase · pH-stability · Thermostability · Salt-tolerance · Xylose-tolerance

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Introduction

Xylans, as the main component of hemicellulose (which is the second most abundant lignocellulosic biomass in nature), are heteropolysaccharides consisting of a linear β -D-(1,4) linked xylopyranoside backbone (Saha 2003). The complete degradation of xylan to xylose units requires the synergistic actions of endo-xylanase, β -xylosidase, α -L-arabinofuranosidase and esterases (Khandeparker and Jalal 2015). During these processes, β -xylosidases can relieve the end-product inhibition of endo-xylanase by degrading xylo-oligosaccharides. Up to now, various β -xylosidases have been found in bacteria, archaea, fungi, crustaceans and insects. Based on their amino acid sequence similarities (Mustafa et al. 2016), β -xylosidases are classified into different glycoside hydrolase (GH) family, including GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116 and GH120 (Henrissat 1991; <http://www.cazy.org/glycoside-hydrolases.html>).

β -xylosidases have a wide range of applications in the food, feed, paper and pulp, pharmaceutical industries and in the bioconversion of lignocellulosic biomass (Jordan and Wagschal 2010). Nevertheless, the applicability of β -xylosidases in industrial applications is restricted due to their typically low stability at temperatures above 50 °C (Jordan et al. 2013) and low activity in the presence of inhibitors, such as salt, ionic liquids and xylose, for example (Sengupta et al. 2017; Fan et al. 2010). Hence in the present study, we focussed on searching for a thermostable, pH stable, ionic liquid tolerant and xylose tolerant β -xylosidase (Bankeeree et al. 2018). Microorganisms, the main source of β -xylosidases, can inhabit extreme environments, such as hot springs, deep ocean vents, volcanic crater, salt mines, salt lakes and deep ocean (Pikuta et al. 2007). Many investigations have indicated that β -xylosidases from these extremophiles exhibit characteristic properties, such as the β -xylosidase from the hyperthermophile bacterium *Thermotoga maritima*, showing thermostability (Xue and Shao 2004), and a β -xylosidase from a marine-derived fungal endophyte, exhibiting ionic liquid tolerance (Sengupta et al. 2017). The strain *Thermobifida halotolerans* YIM 90462^T, an aerobic, thermotolerant, halotolerant, and cellulolytic actinobacterium, was isolated from a salt mine sample (Yang et al. 2008). To date, two thermostable cellulases (Thcel9A and Thcel5A)

(Zhang et al. 2011, 2015), a halotolerant endoglucanase (Thcel6A) (Yin et al. 2015) and two thermostable xylanases (ThxynA and Thxyn11A) (Zhang et al. 2012a, b), have been identified from *T. halotolerans* YIM 90462^T.

In this study, the gene encoding a new β -xylosidase (designated Thxyl43A) was cloned from *T. halotolerans* YIM 90462^T. To our knowledge, this is the first report of β -xylosidase from a member of the genus *Thermobifida*. Thxyl43A belongs to glycoside hydrolase family 43 (GH43). It was over-expressed in *Escherichia coli* BL21 (DE3) and purified by Ni-nitrilotriacetic acid affinity chromatography. After purification, the enzymatic properties of Thxyl43A were investigated. The enzymes pH, salt, thermostable and xylose tolerant characteristics make it an ideal candidate for promoting hemicellulose degradation and other industrial applications.

Materials and methods

Strains, media, vectors and chemicals

T. halotolerans YIM 90462^T (= KCTC 19123^T = DSM 44931^T) was grown in ISP 4 medium containing 10% (w/v), as described previously (Yang et al. 2008). *E. coli* DH5 α and the plasmid pEASY-T1 (TransGen, Beijing, China) were used for gene cloning. *E. coli* BL21 (DE3) and vector pET-28a (+) were used for gene expression. Recombinant *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) medium containing kanamycin (50 μ g/ml).

Cloning of the *thxyl43a* gene

A GH43 xylosidase gene (named *thxyl43a*) was identified from the genome sequence of *T. halotolerans* YIM 90462^T (NCBI accession No. MG827398). The full-length DNA fragment of *thxyl43A*, without its signal peptide-coding sequence, was amplified using specific primers with restriction sites (Table 1), which were designed based on their DNA sequences by Primer Premier 5.0. The complete ORF of *thxyl43A* was amplified using the TransStarFastPfu Fly DNA Polymerase (TransGen Biotech, China). The amplification was carried out for 34 cycles of 98 °C for 10 s and 68 °C for 2 min, 72 °C for 10 min with initial 2 min denaturation at 98 °C. The PCR product was

Table 1 Oligonucleotide primers used for this work

Primers	Primer sequence (5' → 3')	Size (bp)
Thxyl43A-F	TCAGTGC <u>CATATG</u> ATGACGTCTCCCCGAGTCA	31
Thxyl43A-R	ATCAAGCTTTCAGGAGGGGGACTGAGG	27

Restriction sites incorporated into primers are shown underlined

digested with *Nde* I and *Hind* III and inserted into the vector pET28a, digested with the same sites, to yield the expression plasmid designated pET28a-*thxyl43A*.

Sequence analysis

Sequence assembly was performed using Seqman Vector NTI Advance 10.0 software (Invitrogen). DNA and protein sequences were aligned using BLASTx and BLASTp programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. The signal peptide was predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The primary structure of the amino acid sequence was deduced and analysed using EXPASY tools (<http://web.expasy.org/protparam/>). Multiple alignments with protein sequences of closely related Thxyl43A (retrieved from NCBI database) were carried out using Clustal X (Thompson et al. 1997). Phylogenetic analyses were performed using the MEGA 5 software package (Tamura et al. 2011). Trees were constructed by the neighbor-joining (NJ) method with a Poisson correction model.

The sequence of Thxyl43A from *T. halotolerans* YIM 90462^T was compared with the protein structure data available in Protein Data Bank (<http://www.rcsb.org/>). The structural model of Thxyl43A was generated by the MODELLER package (Sali and Blundell 1993) using *Bacillus halodurans* C-125 β -xylosidase (PDB ID, 1YRZ; sequence identity, 49%), *Geobacillus stearothermophilus* β -xylosidase (PDB ID, 2EXH; sequence identity, 47%) and *Selenomonas ruminantium* β -xylosidase (PDB ID, 3C2U; sequence identity, 46%) as templates. Multiple sequence alignment was performed using clustal W (Larkin et al. 2007) and the figure was produced by using Esript 3 (Gouet et al. 2003).

Heterologous expression and purification of Thxyl43A

The pET28a-*thxyl43A* vector was transformed into *E. coli* BL21 (DE3) and the positive clones were isolated for Thxyl43A expression. The transformants were cultured overnight in LB culture medium with 50 μ g/ml kanamycin at 37 °C and 220 rpm. One milliliter culture was inoculated into 100 ml terrific broth and incubated at 37 °C with 220 rpm. During cultivation, IPTG was added to a final concentration of 0.1 mM at mid-exponential phase ($OD_{600} \approx 0.6$) and followed by further incubation for 8 h at 25 °C and 220 rpm. The cells were harvested by centrifugation at 8000 \times g and re-suspended in 20 ml PBS buffer (pH 8.0).

The cells were disrupted by ultrasonication. The lysate was centrifuged at 12,000 \times g for 30 min at 4 °C. The cell-free extracts were applied on Ni-nitrilotriacetic acid chelating affinity column (GE, USA) as the recombinant protein possessed an N-terminal His-tag. The column was washed with ten column volumes of buffer A (20 mM sodium phosphate, 0.3 mM NaCl, pH 8.0), followed by ten column volumes of buffer A with 40 mM imidazole, pH 8.0 and eluted with two volumes buffer A with 500 mM imidazole, pH 8.0. The eluted protein was collected and analysed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The concentration of protein was determined by Bradford assay kit (Sangon, China) using bovine serum albumin as standard.

Enzyme assays

The β -xylosidase activity of Thxyl43A was determined by using *p*-nitrophenyl β -D-xylopyranoside (pNPX) (Sigma, USA), based on the release of *p*-nitrophenol. The reaction mixture, consisting of 40 μ l pNPX (5 mM), 150 μ l of sodium citrate buffer

(pH 7.0) and 10 μl Thxyl43A enzyme (0.2 mg/ml), was incubated at 55 °C for 10 min in a total volume of 200 μl , and stopped by adding 600 μl of 1 M Na_2CO_3 . 150 μl of the reaction mixture was added to 96 cell culture plates and the absorbance of released *p*-nitrophenol was measured at 405 nm using a microplate reader (SYNERGY H1, BioTek, USA) with pure *p*-nitrophenol (Sigma, USA) as a standard. One Unit (U) of β -xylosidase was specified as the amount of enzyme that produce 1 μmol of *p*-nitrophenol per min.

Influence of temperature, pH and salt on β -xylosidase activity

The optimal temperature was determined by measuring the β -xylosidase activity over a range of 15–80 °C at the optimal pH. The optimal pH was examined at pH range of 3.0–12.0 using the following buffers: sodium citrate buffer (pH 3.0–8.0), borate buffer (pH 7.6–8.6) and glycine–NaOH buffer (pH 8.6–12), under the optimal temperature. The results were expressed as relative activity to the value obtained at either optimum temperature or optimum pH. To estimate thermostability of Thxyl43A, the enzyme was pre-incubated at 45, 50, 55 and 60 °C for various time intervals (from 0 to 72 h). Then the residual activity was measured under the standard conditions (pH 7.0, 55 °C, 10 min). The pH stability of Thxyl43A was investigated by determining residual enzyme activity under the standard conditions after incubation in the buffers mentioned above at 25 °C for 12 h and 24 h.

The effect of salt on Thxyl43A activity was tested by adding NaCl (w/v, 0, 5, 10, 15, 20, 25, 30 and 35%) to sodium citrate buffer (pH 7.0) at 55 °C. The salt tolerance of Thxyl43A was assessed by pre-incubating the enzyme in sodium citrate buffer (pH 7.0) containing different concentration of NaCl (10, 20 and 30%) for 0 to 12 h at room temperature (25 °C), and the residual enzyme activity was measured under the standard conditions.

Substrate specificity and kinetic parameters of Thxyl43A

The substrate specificity of Thxyl43A was investigated using different *p*-nitrophenyl-monosaccharide substrates, including *p*NPX, *p*-nitrophenyl- α -L-arabinofuranoside (*p*NPA) and *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG), and the hydrolysis was carried as

described for the *p*NPX assay above. Xylanase activity was determined using oat spelt xylan and beechwood xylan. Reducing sugars released were analysed by the 3,5-dinitrosalicylic acid (DNS) method, as described previously (Miller 1959).

Furthermore, enzyme activities of Thxyl43A for xylobiose (X2), xylotriose (X3) and xyloetraose (X4) were evaluated by using high performance liquid chromatography (HPLC; Waters, Milford, MA) (Bradford 1976), equipped with a Dionex CarboPac PA20 column (3 mm \times 150 mm; Sunnyvale, CA). The flow rate of the solvent (50 mM EDTA–CaNa2) and the absorption were 0.5 ml/min and 260 nm, respectively. One unit of enzyme activity (U) was defined as the quantity of enzyme required to release one μmol of reducing sugars per min. In these experiments, all the substrates were purchased from Sigma, USA.

The kinetic properties (K_m and V_{max}) of the recombinant Thxyl43A were investigated at 55 °C for 5 min in sodium citrate buffer (pH 7.0) using *p*NPX and *p*NPA as the substrates. The concentration of substrate ranged from 0.25 to 5.0 mM. The Lineweaver–Burk equation was used to calculate K_m and V_{max} , based on the enzymatic reactions. GraphPad prism 5 was used for the nonlinear regression calculation and kinetic parameters determination.

Effect of metal ions, chemical reagents and ionic liquid

The influences of different metal ions, chemical reagents and ionic liquid on Thxyl43A activity were assessed by adding selected metal ions, chemical reagents and 1-Allyl-3-methylimidazolium chloride as ionic liquid (MACKLIN, China) to the reaction mixture under standard conditions. The activity of enzyme without metals or chemicals was set as control with a relative activity of 100%. The experiments were repeated three times independently, each time in triplicate.

D-Xylose inhibition of Thxyl43A acting on *p*NP-X at pH 7.0 and 55 °C

For an inhibition study by D-xylose, initial-rate reactions of Thxyl43A activity were determined by incorporating varying concentrations of D-xylose (0–500 mM) in the assay mixture, which contained

1 mM *p*NPX, under standard conditions. The relative β -xylosidase activity was measured by comparing with control in the absence of xylose (100%).

TLC analysis of hydrolytic products

The reaction mixture, which consisted of 2 mM short xylooligosaccharides and 10 μ g purified enzyme, was incubated at 55 °C for 0, 1, 2, 5, 10, 20 min, respectively. Hydrolytic products were analysed by TLC (thin-layer chromatography) using silica gel 60 plates (Merck, Darmstadt, Germany) developed with 1-butanol/acetic acid/water (2:1:1, by vol.) as described previously (Yin et al. 2016). Sugars were detected by heat treatment at 100 °C for 10 min after spraying of plates with freshly prepared 5% (v/v) H₂SO₄ in ethanol. Xylose (X1), X2, X3 and xylotetraose X4 were used as sugar standards.

Results and Discussion

Cloning, expression, purification and sequence analysis of Thxyl43A

The gene Thxyl43A contains 1653 base pairs encoding a polypeptide of 550 amino acids. The nucleotide sequence of Thxyl43A is deposited in the NCBI GenBank database under the accession number of MG827398 (Protein RefSeq WP_068693357). The theoretical isoelectric point (*pI*) and molecular weight (*M_w*) were calculated to 5.57 and 61.5 kDa. No signal peptide was predicted, suggesting that the enzyme is a cytoplasmic protein. The deduced amino acids sequence of Thxyl43A showed high amino acid sequence identity (89%) to a GH43 protein (GenBank: KUP98632.1) from *Thermobifida cellulositytica* TB100. A phylogenetic analysis of protein sequences revealed Thxyl43A clustered with β -xylosidases (Supplementary Figure S1).

BlastP conserved domain sequence analysis revealed Thxyl43A contains a glycosyl hydrolase family 43 (GH43) domain (Pfam PF04616; COG3507 Beta-xylosidase) in the predicted enzyme. The gene was expressed in *E. coli* BL21 (DE3) and the recombinant protein with a His-tagged N-terminus was purified by Ni-nitrilotriacetic acid affinity chromatography. SDS-PAGE analysis indicated the molecular mass of the recombinant β -xylosidase

protein was in good agreement with the theoretical one (Supplementary Figure S2). Glycosyl hydrolase family 43 members with β -xylosidase activity are widely distributed in nature. They have been identified from bacteria, archaea and fungi, including *Lactobacillus rossiae* DSM 15814^T, *Penicillium purpurogenum* and *Thermotoga thermarum* (Pontonio et al. 2016; Ravanal et al. 2013; Hao et al. 2013).

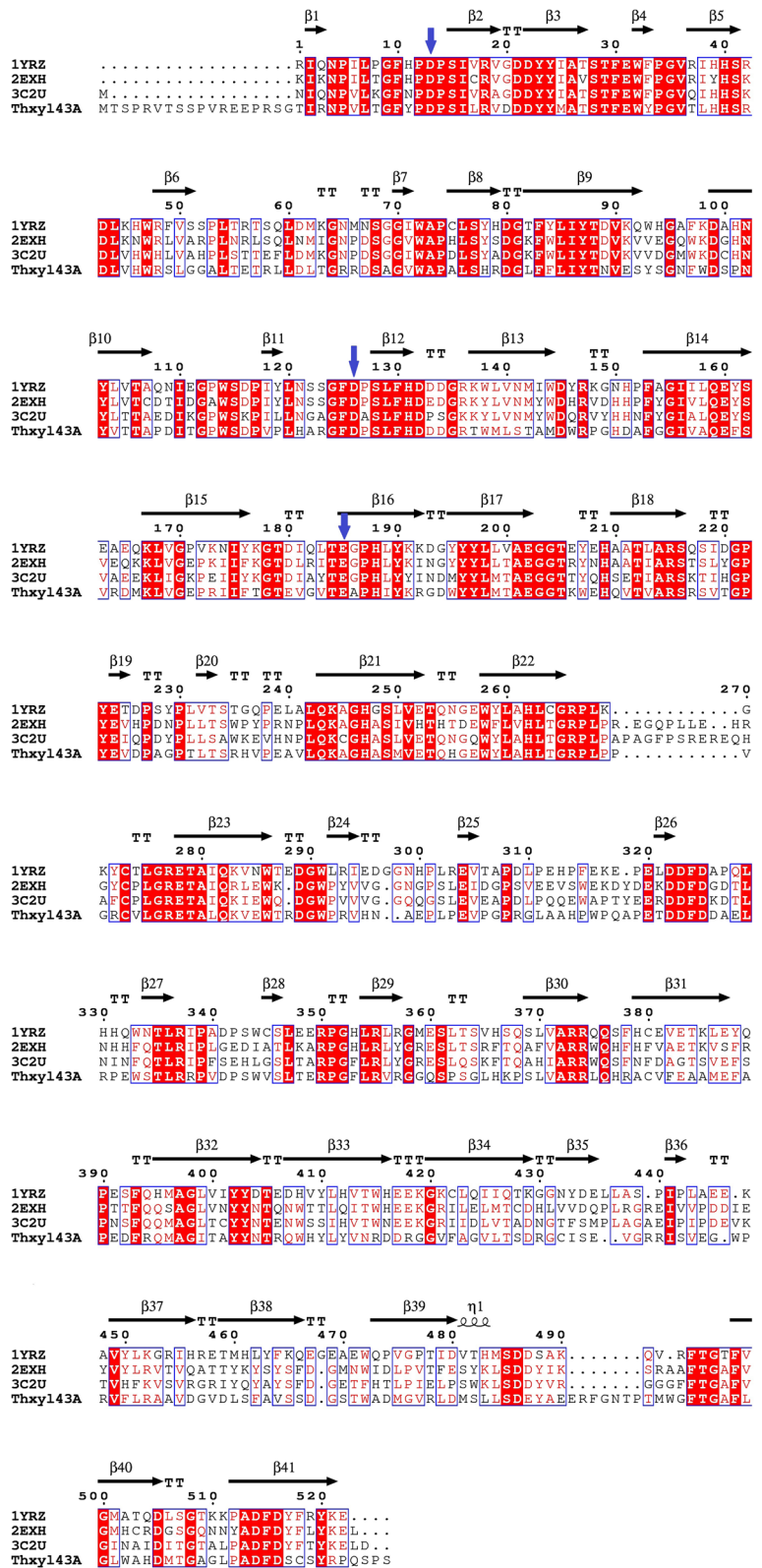
As observed in other GH43 enzymes, Thxyl43A has two domains, an N-terminal β -propeller catalytic domain (residues 1–314) and a C-terminal β -sandwich domain (residues 338–547) (Supplementary Figure S3). Residues 315–337 provide an inter-domain loop, while the 3 residues beyond 547 are at the C-terminus. Multiple sequence alignment of Thxyl43A with the closest structure-resolved β -xylosidases were performed (Fig. 1). Like other GH43 β -xylosidases, three putative catalytic residues (D31, D144 and E203) were found in Thxyl43A (Rohman et al. 2018). The predicted Thxyl43A structure was compared to reference structures in PDB: 1YRZ from *B. halodurans* C-125 (258/531 = 49% amino acid identity), 2EXH from *G. stearothermophilus* (Brüx et al. 2006; 245/538 = 46% amino acid identity) and 3C2U from *S. ruminantium* (Brunzelle et al. 2008; 253/543 = 47% amino acid identity). The structure of Thxyl43A was very similar to 1YRZ, 2EXH and 3C2U (Supplementary Figure S4).

Biochemical characterisation of the purified recombinant ThXyl43A

The effects of temperature and pH on ThXyl43A activity were examined. The optimal temperature for the recombinant enzyme was 55 °C (Fig. 2a). ThXyl43A not only exhibited relatively high activity at lower temperatures but also showed thermostability at temperatures up to at least 60 °C. About 27% and 40% β -xylosidase activity was retained at 15 °C and 25 °C respectively, and more than 60% relative activity at 60 °C. In contrast, other thermostable β -xylosidases showed low relative activities at temperatures under 40 °C (Hao et al. 2013; Bhalla et al. 2014).

As shown in Fig. 2c, more than 80% enzyme activity after incubation for 66 h at 45 or 50 °C and more than 90% residual activity was retained after incubation at 55 °C for 24 h. The *t*_{1/2} values of inactivation were 29.7 h at 55 °C and 4.8 h at 60 °C.

Fig. 1 Multiple sequence alignment of Thxy143A with some similar β -xylosidases. The secondary structural elements of Thxy143A are indicated above. The PDB entries are: 1YRZ from *B. halodurans* C-125; 2EXH from *G. stearothermophilus*; 3C2U from *S. ruminantium*. Identical residues are shaded in red. The blue arrow symbol indicates three catalytic residues (two aspartates and a glutamate) of the enzymes. (Color figure online)



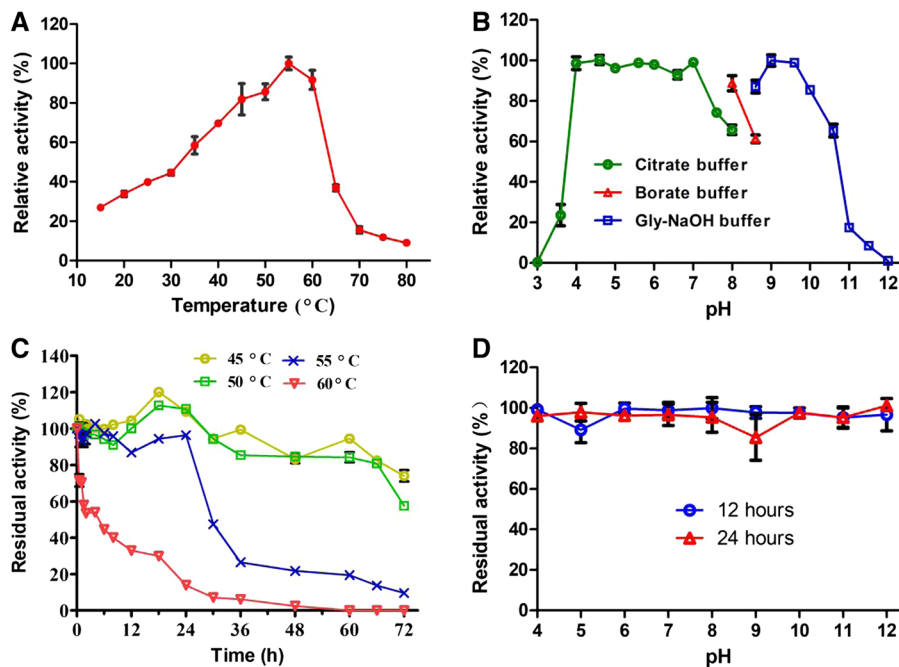


Fig. 2 Effects of temperature and pH on the activity and stability of recombinant Thxyl43A. **a** Temperature effect on the activity of Thxyl43A. **b** pH effect on the activity of Thxyl43A. **c** The effect of temperature on stability at different temperatures (45, 50, 55 and 60 °C) for 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h. **d** The effect of pH on stability. The pH stability test

was performed by pre-incubating the enzyme in different buffer systems at 25 °C for 12 h and 24 h, and then residual activity was determined under standard conditions (55 °C, pH 7.0). The primary activity was taken as 100%. Each value in the Figure represents the mean ± SD (n = 3). 100% = 46.1 U/mg

In comparison, the $t_{1/2}$ values of GH43 β -xylosidases from *Bifidobacterium animalis* subsp. lactis BB-12, *T. thermarum* and *Geobacillus thermoleovorans* IT-08 were less than 1 h at their optimal reaction temperature (Hao et al. 2013; Viborg et al. 2013; Wagschal et al. 2009). These data suggest that Thxyl43A is a highly thermostable xylosidase.

The optimal pH for ThXyl43A activity was found to be pH 7 (Fig. 2b). The enzyme was notably active even at pH 4–6 and 9–10. ThXyl43A exhibited different activities in different buffers: at pH 8.0 about 65% and 90% relative activity was detected in Citrate buffer and Borate buffer respectively, and at pH 8.6 and 9.0 less than 65% relative activity was observed in Borate buffer, while more than 85% was found in Gly-NaOH buffer. ThXyl43A not only showed high activity in acid buffer, but also exhibited high activity in alkaline buffers. More than 60% relative activity was noted at pH 10.6. In addition, ThXyl43A also showed pH stability. It exhibited more than 90% residual activity after incubation for 12 or 24 h at pH 4–12 (Fig. 2d). Pre-treatments, such as steam

exposure, acid or alkaline pre-treatment, are typically needed before generating bioethanol from plant biomass such wheat straw (Eisenhuber et al. 2013). Therefore, the widely pH range and pH stability of ThXyl43A suggests that Thxyl43A may be suitable for hydrolysing acid or alkaline pre-treated lignocellulose.

Determination of substrate specificity and kinetic parameters

The substrate specificity was analysed and the results are presented in Table 2. The recombinant ThXyl43A was able to hydrolyse several β -linked glycosidic substrates. The specific activity followed the order pNPX > xylobiose > xylotetraose > xylotriose > pNPA > pNPG. However, no activity was found for beechwood xylan and cellobiose. GH43 enzymes which have the ability to degrade pNPX, pNPA and xylo-oligosaccharides have been identified as β -xylosidase/ α -arabinofuranosidase (Ravanel et al.

Table 2 Substrate specificities of ThXyl43A

Substrates	Specific activity (U/mg)
pNP-X	46.1
pNP-A	3.1
pNP-G	0.1
Beechwood Xylan	0
xylobiose	11.4
xylotriose	5.6
xylotetraose	9.6
Cellobiose	0

2013; Viborg et al. 2013). This suggests Thxyl43A may be a bifunctional xylosidase/arabinofuranosidase.

Kinetic constants for *p*NPX and *p*NPA were determined using Lineweaver–Burk plots. The apparent K_m , V_{max} , k_{cat} and k_{cat}/K_m values for *p*NPX and *p*NPA are given in Table 3. The K_m values of Thxyl43A for *p*NPX and *p*NPA were 0.78 mM and 0.42 mM, respectively. The K_m values of fungal GH43 xylosidases for *p*NPX were higher, ranging from 1 to 5 mM (Yan et al. 2007; Zanoelo et al. 2004) and K_m values of a bacterial GH43 β -xylosidase/ α -arabinofuranosidase, from a rumen metagenome, for *p*NPX and *p*NPA were 3.43 mM and 2.23 mM, respectively (Zhou et al. 2011). The lower K_m values of Thxyl43A suggests it has higher affinity for substrates such as *p*NPX and *p*NPA.

Effect of metal ions, chemical reagents, salt and ionic liquid

The effect of various metal ions (1 mM) and chemical reagents (1%) on ThXyl43A activity are shown in Supplementary Table S1. ThXyl43A activity was not affected by K^+ , Mg^{2+} , Fe^{2+} , Fe^{3+} , Ca^{2+} , Co^{2+} , Ba^{2+} ,

Table 3 Kinetic parameters of Thxyl43A

Parameters	Value	Value
Substrate	<i>p</i> NP-X	<i>p</i> NP-A
K_m (mM)	0.78	0.42
V_{max} ($\mu\text{mol}/\text{min}/\text{mg}$)	43.9	3.67
k_{cat} (S^{-1})	50	3.8
k_{cat}/K_m ($\text{mM}^{-1} S^{-1}$)	64.1	9

Ag^{2+} , Al^{3+} , methyl alcohol, ethyl alcohol, isopropyl alcohol, Tween 20, Tween 60, Tween 80 and DTT; weakly inhibited by Zn^{2+} , EDTA and PFMS; highly inhibited by Cu^{2+} and completely inhibited by Pb^{2+} and SDS. The enzyme activity was slightly enhanced by Ni^{2+} and Mn^{2+} . Other β -xylosidases were also strongly inhibited by Cu^{2+} , Pb^{2+} and SDS, and improved by Mn^{2+} (Bhalla et al. 2014; Ye et al. 2017), whilst a *Geobacillus* β -xylosidase was inhibited by Co^{2+} and Ni^{2+} (Bhalla et al. 2014). These data suggested that ThXyl43A was stable in most ions and chemical reagents.

The influence of NaCl on recombinant Thxyl43A is shown in Fig. 3. Slightly greater than 100% relative activity was observed in the presence of NaCl (5%–35%). Moreover, the enzyme activity was improved to 120% and 140% after incubation in 10% NaCl for 0.5 h and 20% NaCl for 0.25 h, respectively (Fig. 3). This indicated that Thxyl43A is a salt tolerant β -xylosidase. Yin et al. (2015) reported a novel halotolerant, thermostable, and alkali-stable GH6 endoglucanase (Thcel6A) from *T. halotolerans* YIM 90462^T also displayed salt tolerance (Yin et al. 2015).

ThXyl43A exhibited high β -xylosidase activity in the presence of ionic liquid (Fig. 4). It retained more than 90% relative activity in concentrations of 1 to 10% ionic liquid (w/v) and 40% activity in 25% ionic liquid. Recent research has shown that lignocellulose pretreatment with ionic liquids has great promise for reducing lignocellulose recalcitrance to enzymatic hydrolysis (Piccolo and Bezzo 2009). However, most commercial cellulolytic enzymes are inhibited by residual ionic liquids in the substrate (Amaike et al. 2017). Therefore, xylosidases with high specific activity in the presence of ionic liquid have significant potential in bioethanol production from lignocellulose. Sengupta et al. (2017) reported an ionic liquid-tolerant xylosidase which was isolated from a marine-derived fungal endophyte (Sengupta et al. 2017). Thxyl43A was isolated from a salt mine microorganism (Yang et al. 2008). These observations suggest that salt environments may be a potential resource for mining ionic liquid tolerant enzymes.

Effect of xylose concentration on β -xylosidase activity

As shown in Supplementary Figure S5, 85% relative activity was retained in the presence of 25 mM xylose

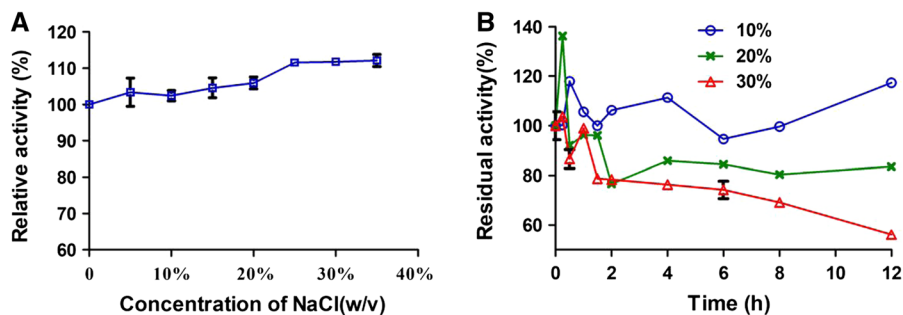


Fig. 3 Influence of NaCl on recombinant Thxyl43A. **a** Salt effect on the activity of Thxyl43A. **b** Salt-tolerance of Thxyl43A at different concentration of NaCl (0, 5, 10, 15, 20, 25, 30, and 35%) for 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 12 h

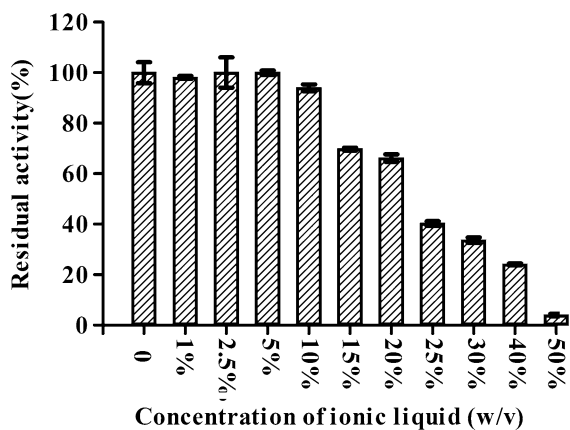


Fig. 4 Influence of ionic liquid on the activity of recombinant Thxyl43A

and more than 60% relative activity was observed in the presence of 50 mM xylose. Xylose, as the end-product of xylan hydrolysis, strongly inhibits some β -xylosidases (Kumar and Ramón 1996). Many fungal β -xylosidases show inhibited enzyme activities (< 50%) in the range of 2 to 25 mM xylose (Yan et al. 2007; Kumar and Ramón 1996). Hence, as the key enzyme for hydrolysis of xylo-oligosaccharides, xylose tolerance is a required property for β -xylosidase. The K_i value of ThXyl43A was 43.8 mM, whereas K_i values of xylosidases from microorganisms are typically as low as 2–10 mM (Yang et al. 2014). These data clearly indicated that ThXyl43A is a xylose tolerant β -xylosidase.

TLC analysis of hydrolytic products

The hydrolytic products of xylo-oligosaccharides by Thxyl43A were analysed by TLC. After incubation at 55 °C for different times, the products of X2 were

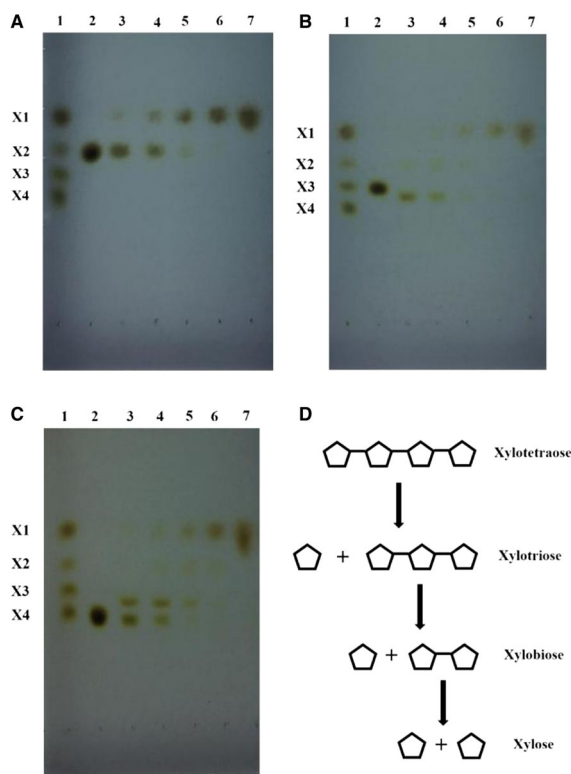


Fig. 5 Thin-Layer Chromatography of hydrolysis products of xylo-oligosaccharides by Thxyl43A. **a** Products of xylobiose. **b** Products of xylotriose. **c** Products of xylotetraose. **d** Hydrolysis of xylo-oligosaccharides by Thxyl43A. Lane 1, standards: Xylose (X1), xylobiose (X2), xylotriose (X3) and xylotetraose (X4); lane 2–7, were enzyme reactions for 0, 1, 2, 5, 10 and 20 min, respectively. TLC plate was developed with 1-butanol/ acetic acid/water (2:1:1, v/v/v). Sugars were detected by heat treatment at 120 °C for 10 min after spraying of plates with freshly prepared 5% (v/v) H₂SO₄ in ethanol

xylose (Fig. 5a), the products of X3 were X2 and xylose (Fig. 5b) and the products of X4 were X3, X2

and xylose (Fig. 5c). Xylose was the end-product of sequential hydrolysis of xylo-oligosaccharides by ThXyl43A (Fig. 5d). These results indicate that ThXyl43A cleaves xylose from one end of the chain of xylo-oligosaccharides, consistent with an exoglycolytic mode of action. This finding is consistent with the mechanism of β -xylosidases from family GH43, which hydrolyse the non-reducing ends of xylooligomers using an inverting mechanism (Barker et al. 2010).

Conclusions

In summary, a GH43 β -xylosidase named ThXyl43A was identified from *T. halotolerans* YIM 90462^T. Purified recombinant ThXyl43A was characterised as a pH and salt tolerant thermostable enzyme, with interesting tolerance to ionic liquid and xylose. Thus ThXyl43A may promote hemicellulose degradation and have potential industrial applications, such as in lignocellulosic ethanol production and as an animal feed additive.

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Author contributions Y.R.Y., M.X. and W.J.L. conceived the study. W.D.X. performed the culture of strains and collection of *E. coli* biomass. Y.R.Y. and M.X.H. performed the protein separation. E.M.Z., L.L. and D.H.M.A. performed the measurement of enzyme activity. W.N.H. and M.X. performed the data analysis. Y.R.Y., W.D.X., M.X. and W.J.L. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no indirect or direct conflict of interest.

Ethical approval This article does not contain any studies related to human participants or animals.

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