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ORIGINAL ARTICLE

Screening and identification of an *Enterobacter ludwigii* strain expressing an active β-xylosidase

Jiashi Zhang[1] · Tangbing Cui[1] · Xueqing Li[1]

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Abstract

Researchers have expressed increasing interest in the xylanolytic enzymes used in hemicellulose hydrolysis that convert wood and agricultural residues to second-generation biofuels. In our study, 32 isolates showed clear hydrolysis zones on agar plates containing xylan after Congo red staining. Among these isolates, strain LY-62 exhibited the highest β-xylosidase activity (1.29 ± 0.05 U/mL). According to the phylogenetic analysis of the 16S rDNA, strain LY-62 belongs to the *Enterobacter* genus. Using a combination of electron microscopy, Gram-staining, and conventional physiological and biochemical examinations, the strain LY-62 was identified as *Enterobacter ludwigii*. The β-xylosidase gene from *Enterobacter ludwigii* LY-62 was cloned, and the full-length protein was expressed in *Escherichia coli* as an N-terminal or C-terminal His-tagged fusions protein. Optimal β-xylosidase activity was achieved at pH 7.0 and 40 °C. The Michaelis constant K_M values for His-Xyl62 and Xyl62-His were 1.55 and 2.8 mmol/L, respectively. The k_{cat} values for His-Xyl62 and Xyl62-His were 8.51 and 6.94 s^{-1}, respectively. The catalytic efficiencies of His-Xyl62 and Xyl62-His were 5.49 and 2.48 $s^{-1} \times mM^{-1}$, respectively. Thus, Xyl62 is a functional β-xylosidase, and our study represents the first report of a β-xylosidase from *Enterobacter ludwigii*.

Keywords *Enterobacter ludwigii* · Expression · GH43 · β-xylosidase

Introduction

Xylan is the most abundant hemicellulose present in plant and agricultural residues. The complex heteropolysaccharide consists of a linear backbone of β-1,4-linked D-xylopyranosyl residues, which have multiple substitutions. The complete hydrolysis of the heteropolysaccharide requires the coordinated action of various hydrolytic enzymes (Basaran et al. 2001; Beg et al. 2001). The critical hemicellulases are endo-β-1,4-xylanases (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8), which can cleave internal glycosidic bonds from the xylan backbone, and β-xylosidase (β-1,4-D-xylan xylohydrolase, EC 3.2.1.37), which hydrolyze xylooligosaccharides from the non-reducing end to xylose. Xylosidase is very important for xylan degradation because xylan is not completely hydrolyzed by xylanases alone. Xylanolytic enzymes are applied in several industrial applications, such as the conversion of lignocellulosic substances to chemicals and fuels, the preparation of materials for scientific research, animal feed digestion, in the food and textile industries, and as bleaching agents in the biopulp and paper processing industries (Wong et al. 1988; Beg et al. 2001; Subramanian and Prema 2002; Techapun et al. 2003; Polizeli et al. 2005; Moure et al. 2006).

Many bacterial and fungal β-xylosidases have been purified and characterized. Currently, fungi (*Trichoderma reesei* and *Aspergillus niger*) are widely used as enzyme producers and mainly considered as more relevant commercial xylanolytic enzyme producers than bacteria and yeast (Haltrich et al. 1996; Polizeli et al. 2005; Quinlan et al. 2010). β-Xylosidases isolated from different filamentous fungi exhibit marked differences in their physicochemical and biological properties, as well as their modes of action. Most fungal β-xylosidases display the optimal activity at acidic pH values from 4.0 to 5.0 and the optimum temperatures ranging from 40 to 80 °C (Polizeli et al. 2005). Moreover, β-xylosidases from bacteria are relatively stable under weakly acidic or alkaline conditions and display characteristic properties (Corrêa et al. 2012; Graciano et al. 2012; Hao et al. 2013; Jain et al. 2014; Sanyan et al. 2014).

✉ Tangbing Cui
fetbcui@scut.edu.cn

[1] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, Guangdong, China

Although many β-xylosidases and their coding genes have been manipulated and characterized in plants, fungi, bacteria, and archaea, few reports describing the xylanolytic enzyme system of *Enterobacter* are available (Khandeparkar and Bhosle 2006; Changhao et al. 2009; Campos et al. 2013).

In this report, we describe the screening and identification of an *Enterobacter ludwigii* strain LY-62 with β-xylosidase activity (the β-xylosidase was designated Xyl62). Furthermore, the Xyl62 gene was cloned from strain LY-62, and bioinformatics and expression analyses were performed. A detailed description of the characteristics of the recombinant proteins is also researched.

Material and methods

Soil sample pretreatment

Soil samples used in this experiment were collected from the native forest of Wangwu Mountain in Henan Province, China, and stored at 4 °C.

Five grams of soil sample was dissolved in 50 mL of sterile distilled water to prepare a liquid suspension, which was sequentially incubated for 60 min at 37 °C and then centrifuged at 200*g*. Then, the supernatant was removed for enrichment culturing.

Isolation of strains on xylan-agar plates

The prepared supernatant was incubated in enrichment medium which contained 10 g/L beechwood xylan, 5 g/L tryptone, and 5 g/L NaCl and was cultured for 72 h at 37 °C and shaken at 200 g. Aliquots of cultures in enrichment medium were sequentially removed and diluted serially with a 0.9% NaCl solution.

Each serial dilution was spread on a solid medium plate, which contained 0.5 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2$, 0.1 g/L K_2SO_4, 0.2 g/L NaCl, 10 g/L beechwood xylan, and 15 g/L agar (Ammoneh et al. 2014), and incubated for 48 h at 37 °C.

Colonies with identifiable hydrolysis zones were chosen and selected and transferred to a new solid medium plate for further culturing. These plates were then stained with Congo red (1 mg/mL), and colony diameters and hydrolysis zone diameters were measured and recorded.

Isolation of strains with β-xylosidase activity

The isolates derived from the soil collection of Wangwu mountain in Henan province with hydrolysis zone were selected as further screening strains with β-xylosidase activity. The isolates were cultured for 8 h at 37 °C with shaking at 200 g in medium containing 0.5 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.1 g/L K_2SO_4, 0.2 g/L NaCl, 10 g/L beechwood xylan, and 10 g/L tryptone. Then, 30 mL of each culture was removed and centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was discarded (no enzyme activity, data not shown), and the precipitate was washed twice with equal volumes of phosphate buffer (0.01 mol/L, pH 7.0) and centrifuged at 10000*g* for 10 min at 4 °C. The pellet was again suspended in lysis buffer (containing 0.01 mol/L phosphate buffer, pH 7.0, 100 μg/mL lysozyme, and 0.01% Triton X-100). The suspension was ultrasonicated (30 W, 5 s/5 s, 30 min) and then centrifuged at 11000*g* for 10 min at 4 °C; the supernatant was collected as a crude enzyme solution and used to determine β-xylosidase activity.

The procedure for the β-xylosidase activity assay was as follows: 0.1 mL of properly diluted crude enzyme solution was mixed with 0.9 mL of 0.8 mmol/L p-nitrophenyl-β-D-xyloside (pNPX). The mixture was incubated for 10 min at 50 °C; then, the reaction was terminated by adding 1 mL of 2 mol/L sodium carbonate. The absorbance of the reaction solution was determined at 410 nm (Lachke 1988). One unit of β-xylosidase activity was defined as the amount of enzyme that catalyzed the hydrolysis of pNPX to produce 1 μmol of β-nitrophenol per minute. The specific activity of the crude enzyme solution was defined as the enzyme activity per milligram of protein. The protein content was determined using the Bradford method (Bradford 1976).

Strain identification

Cell morphology of strain was observed under light microscope, scanning electronic microscopy, and transmission electron microscopy. Flagella were investigated with transmission electron microscopy. Cells were mounted on copper grids and negatively stained with 1% potassium phosphotungstate (pH 7.0). Then, the grids were examined under a transmission electron microscope.

Total DNA was extracted from strain LY-62 using the TIANamp Bacteria DNA Kit (TIANGEN). Using total DNA as the template and Eubac 27F and 1492R which are universal primers for 16S RNA amplification, the 16S rDNA gene was amplified by PCR. PCR products were visualized on 1% agarose gels with Gelred stain, and the target gene was purified and cloned into the pMD™19-T vector. The recombinant plasmid was transformed into *Escherichia coli* strain DH5a, and positive clones were sequenced with RV-M and M13-47 primers. The phylogenetic tree was constructed using MEGA7.0 software and with the neighbor-joining method (Saitou and Nei 1987) as the tree-building algorithm and a bootstrap analysis (1000 replications) were performed to evaluate the stability of the resulting phylogenetic tree.

Gram staining was conducted using a Gram stain kit according to the manufacturer's instructions. Other carbon-

source utilization tests, acid production tests, and additional physiological tests were performed according to the manufacturer's instructions (Hoffmann et al. 2005).

Cloning and expression of the β-xylosidase gene

The β-xylosidase gene from *Enterobacter cloacae* P101 (accession number CP006580.1) was selected as a reference sequence, and two pairs of primers were designed to clone the complete β-xylosidase gene from *Enterobacter ludwigii* LY-62:

LY-3NdeI: 5'GGAATTCCATATGACAA CGATAACAAACC3'
LY-4XhoI: 5'CCGCTCGAGCTATTTC GCGAGCGGTTCGTAGGTG3'
LY-5NcoI: 5'CATGCCATGGGCATGACAA CGATAACAAACC3'
LY-6XhoI: 5'CCGCTCGAGTTTCGCGAGCGGTT CGTAGGTG3'

The β-xylosidase gene of *Enterobacter ludwigii* LY-62 was amplified by PCR. The amplified products were visualized on 1% agarose gels with Gelred staining, and the target band was retrieved and sequenced. The amplified products were ligated into the pET-28a(+) vector using the NdeI/XhoI cleavage sites for N-terminal fusion proteins and NcoI/XhoI cleavage sites for C-terminal fusion proteins, and transformed into *E. coli* BL21(DE3). The expression of the recombinant proteins was induced with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside. Recombinant proteins were purified using the 6×His-Tagged Protein Purification Kit (CWBIO) (20 mmol/L phosphate buffer, pH 7.0, 500 mmol/L NaCl, and 500 mmol//L imidazole as the elution buffer).

Characterization of enzyme activity

The optimum pH of β-xylosidase was determined by measuring its activity at pH values ranging from 5.0 to 9.0 using sodium citrate (pH 5.0–6.0, 50 mM), phosphate (pH 6.2–8.8, 50 mM), and Tris-HCl (pH 8.4–8.8, 50 mM) buffers. The enzyme activity measured at the optimum pH value was set to 100%, and the relative enzyme activity was defined as a percentage of the optimal enzyme activity at various pH values. The pH stability was determined by preparing enzyme solutions using buffers with different pH values and incubating the resulting mixtures for 12 h at 4 °C. The residual β-xylosidase activity was then determined. The residual enzyme activity was defined as the percentage of enzyme activities measured at various pH values relative to the highest enzyme activity.

The optimum temperature of β-xylosidase was determined at different temperatures (30–65 °C). The enzyme activity measured at the optimum temperature was set as to 100%, and the relative enzyme activity was defined as the percentage of enzyme activities at various temperatures relative to the highest enzyme activity. For the determination of the temperature stability, the crude enzyme solution (phosphate buffer, 50 mM, pH 7.0) was incubated at different temperatures (35–55 °C) for 10, 20, 40, 60, 90, or 120 min. At specified times, sample tubes were withdrawn, cooled on ice and the residual activity was measured.

Different metal ions were added to the pNPX solution to final concentrations was of 2 or 10 mmol/L. The enzyme solution was reacted with the pNPX solution containing different metal ions, and the enzyme activity was measured. The relative enzyme activity was defined as the percentage of enzyme activities in the presence of different metal ions relative to the enzyme activity in the absence of metal ions.

One hundred microliters of enzyme solution (0.11 mg/mL of His-Xyl62 or 0.073 mg/mL of Xyl62-His) was mixed with 900 μL of pNPX solution (0.125–10 mmol/L, pH 7.0) and incubated at 40 °C, and the absorbance at 410 nm was monitored continuously for 30 min to determine the K_M and k_{cat} values for His-Xyl62 and Xyl62-His.

The accession numbers of the 16S rDNA gene and β-xylosidase gene from *Enterobacter ludwigii* LY-62 in the GenBank database are KU867644.1 and KU870672.1, respectively.

Results

Screening of strains with β-xylosidase activity

Solid medium plates were used to isolate microorganisms with xylanolytic enzyme activity, based on the presence of identifiable hydrolysis zones around colonies after Congo red staining. In the present study, 32 strains showing hydrolysis zones were isolated (all of which belong to *Enterobacter*, data not shown). Strain number 62 (designated strain LY-62) produced the largest hydrolysis zone of the bacterial strains isolated (Fig. 1).

Thirty-two strains with hydrolysis zones were selected for further screening to identify strains with intracellular β-xylosidase activity. All 32 strains were measured and confirmed to display β-xylosidase activity, and the results are shown in Table 1. Strain LY-62 exhibited the highest β-xylosidase activity, as the β-xylosidase activity per milliliter of crude lysate and per milligram of total proteins in the extract were 1.29 ± 0.05 U/mL and 1.05 ± 0.16 U/mg, respectively.

Strain LY-62 was selected for further study of its β-xylosidase activity. Values of end-point β-xylosidase activity obtained after a series fixed time of fermentation are shown (Fig. 2). The maximal β-xylosidase activity was observed

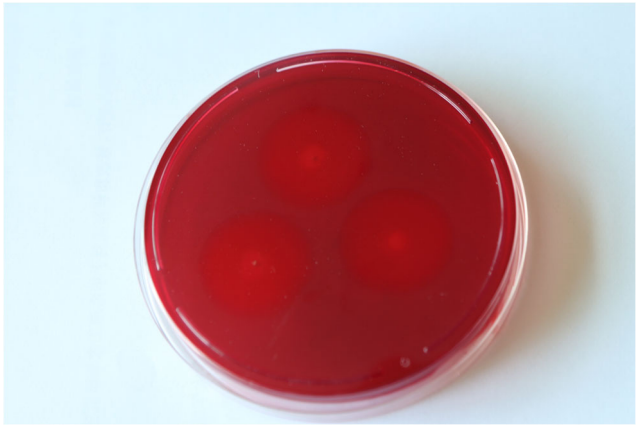

Fig. 1 Hydrolysis zone of *E. ludwigii* LY-62

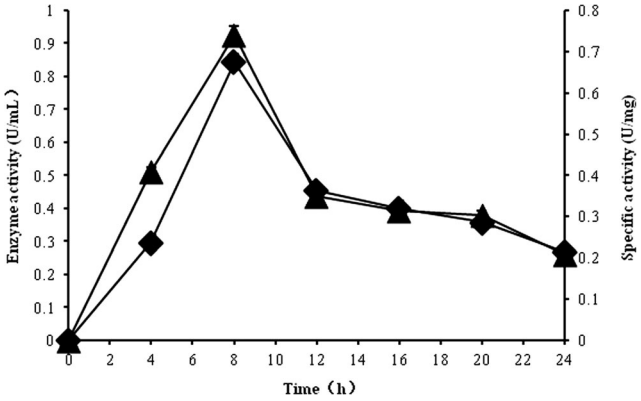

Fig. 2 Time course of β-xylosidase production in *E. ludwigii* LY-62. Black triangle: enzyme activity; black square: specific activity

after 8 h of fermentation, and the result was partially consistent with the bacterial growth.

Identification of strain LY-62

Strain LY-62 was cultured on Lysogeny Broth agar medium for 24 h at 37 °C, after which visible colonies formed, with round (2–3 mm in diameter) and regular edges, a convex surface, a light beige to white, opaque, and glossy appearance. Strain LY-62 is a gram-negative bacterium with flagella (Fig. 3).

Analyzing the alignment of 16SrRNA sequences from LY-62 strain with sequences from the 16SrRNA database from Genbank, a high similarity was observed with sequences from *Enterobacter* genus. The closest relatives of strain LY-62 were *Enterobacter cloacae* UW5, *E. cloacae* P101, *E. cloacae* EcWSU1, and the recently described *Enterobacter ludwigii* strain type EN-119 (99.87, 99.87, 99.87, and 99.93% certainty, respectively). The phylogenetic tree showed that strain LY-62 was verifiably affiliated with the species *E. ludwigii* (Fig. 4).

The physiological and biochemical characteristics of strain LY-62 were investigated in a series of experiments and summarized in Table 2. *E. ludwigii* is distinguished from the other *Enterobacter species* based on its growth on myo-inositol and 3-O-methyl-D-glucopyranose. The physiological and biochemical results further supported the conclusion that strain LY-62 is *E. ludwigii* (Boone et al. 2001; Hoffmann et al. 2005).

Table 1 β-xylosidase activity of 32 strains

Strain number	Enzyme activity (U/mL)	Specific activity (U/mg)	Strain number	Enzyme activity (U/mL)	Specific activity (U/mg)
62	1.29 ± 0.05	1.05 ± 0.16	29	1.08 ± 0.11	0.98 ± 0.18
28	1.25 ± 0.26	0.95 ± 0.04	5	1.08 ± 0.19	1.09 ± 0.31
76	1.23 ± 0.13	1.04 ± 0.17	42	1.07 ± 0.06	0.96 ± 0.16
36	1.21 ± 0.07	1.04 ± 0.10	46	1.06 ± 0.04	0.90 ± 0.06
30	1.19 ± 0.10	0.97 ± 0.05	63	1.05 ± 0.17	0.86 ± 0.07
117	1.18 ± 0.11	0.94 ± 0.08	2	1.05 ± 0.11	0.97 ± 0.14
71	1.18 ± 0.06	0.97 ± 0.03	70	1.05 ± 0.03	1.13 ± 0.09
32	1.16 ± 0.17	0.98 ± 0.04	112	1.04 ± 0.07	0.80 ± 0.02
26	1.16 ± 0.09	0.90 ± 0.06	104	1.03 ± 0.05	0.93 ± 0.01
58	1.12 ± 0.11	0.97 ± 0.05	35	1.03 ± 0.14	0.89 ± 0.08
103	1.14 ± 0.07	0.94 ± 0.07	93	1.02 ± 0.05	1.09 ± 0.11
73	1.12 ± 0.05	0.88 ± 0.08	27	1.01 ± 0.20	0.83 ± 0.13
101	1.12 ± 0.17	0.93 ± 0.10	16	1.01 ± 0.03	0.79 ± 0.06
68	1.12 ± 0.31	1.00 ± 0.20	78	1.01 ± 0.10	0.92 ± 0.07
23	1.10 ± 0.06	0.84 ± 0.03	37	0.91 ± 0.21	0.79 ± 0.15
14	1.10 ± 0.03	0.95 ± 0.07	89	0.81 ± 0.10	0.93 ± 0.13

Data are represented as mean value and standard deviation of three repetitions

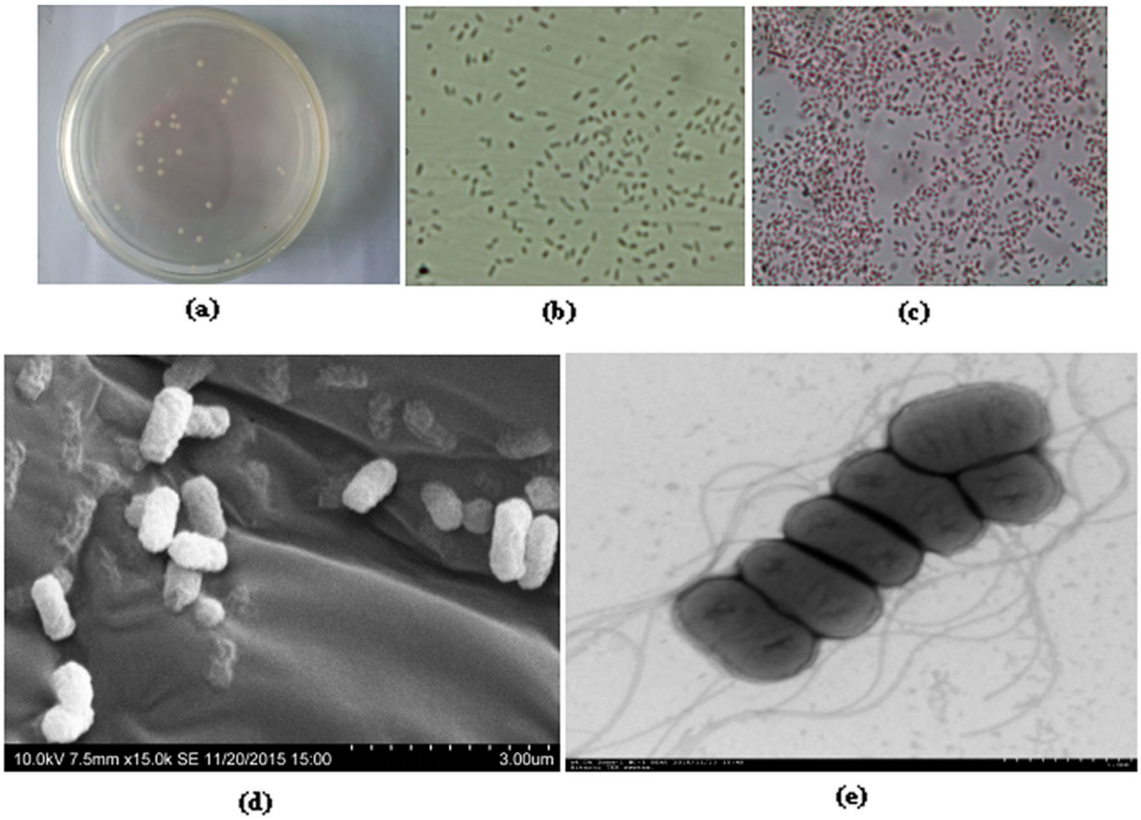

Fig. 3 Morphological characteristics of strain LY-62. **a** Visible colonies of strain LY-62 on Lysogeny Broth agar medium. **b** Morphology of strain LY-62 under a light microscope. **c** Images of strain LY-62 after Gram staining. **d** Morphology of strain LY-62 under a scanning electronic microscope. **e** Morphology of strain LY-62 under a transmission electron microscope

Analysis of the β-xylosidase gene

Simplex band corresponding to amplification products which contain β-xylosidase gene of 1620 bp was exhibited on agarose gel, and purified proteins Xyl62-His and His-Xyl62 were presented using SDS-PAGE (Fig. 5). A homology search was performed for the β-xylosidase gene using BLASTN, which displayed high similarity with the β-xylosidase genes from *E. cloacae* P101, *E. cloacae* EcWSU1, *E. cloacae* UW5, and *E. ludwigii* EN-119 (98.52, 98.40, 98.09, and 98.02% similarity, respectively).

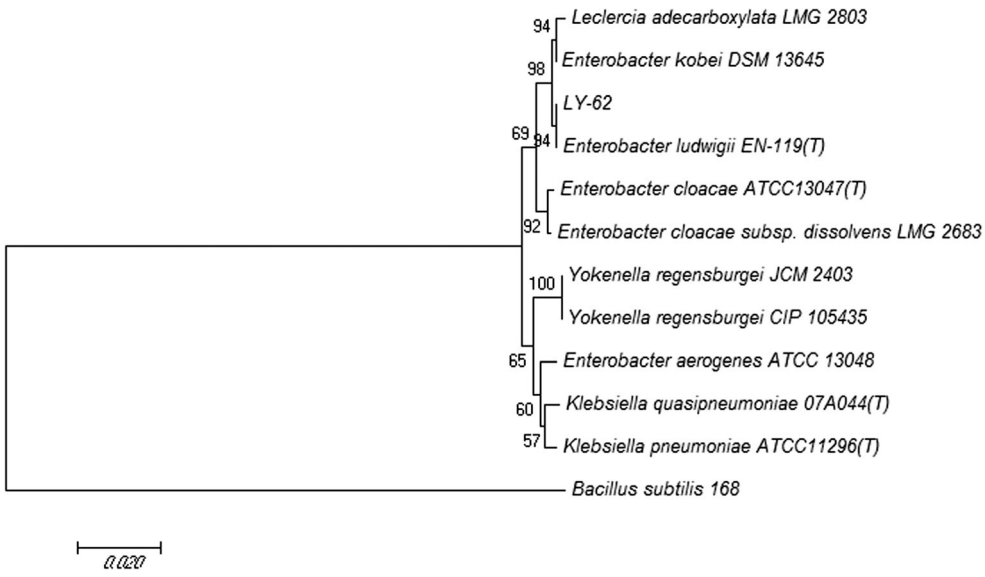

Fig. 4 Phylogenetic tree showing the phylogenetic position of the strain LY-62 using the neighbor-joining method based on 16S rDNA sequences. The numbers at the nodes indicate the levels of the bootstrap support percentages based on neighbor-joining of 1000 replicates. Bootstrap values which were ≥ 50% are indicated at the nodes. The scale bar represents a 0.02 sequence difference. *B. subtilis* 168 was used as an outgroup

Table 2 Physiological and biochemical characteristics of LY-62

Test items	Activity	Test items	Activity
Motility	+	L-Arabinose	+
Catalase	+	Lactose	+
Urease activity	–	Melibiose	+
Indole production	–	Maltose	+
Yellow pigment	–	D-Xylose	+
Methyl red test	–	Raffinose	+
Voges-Prokauer reaction	+	3-0-Methyl-D-glucopyranose	+
Lysine decarboxylase	–	L-Rhamnose	+
Arginine dihydrolase	+	D-Manntiol	+
Ornithine decarboxylase	+	D-Sorbitol	+
Esculin hydrolyzation	+	Myo-inositol	+
Acid from adonitol	–	Gelatin liquefaction	–
Sucrose	+	Malonate utilization	–
Glucose	+	Citrate Simmon's	+

The predicted amino acid sequence of the β-xylosidase (designated as Xyl62) gene from *Enterobacter ludwigii* LY-62 (539 amino acids) was submitted for BLAST analysis, and the protein showed the highest similarities with two GH43 β-xylosidases from *E. cloacae* (99%, gi| 643377338) and *Enterobacter* sp. BIDMC 26 (99%, gi| 695753277). Based on the phylogenetic analysis of the evolutionary relationship of the predicted amino acid sequence of Xyl62, Xyl62 belongs to the GH43 family in the CAZY database (http://www.cazy.org/) (Coutinho and Henrissat 1999), with the closest relation to a β-xylosidase (Xyl43; 98% consensus, 94% identity; NCBI accession AFZ78871) from *Enterobacter* sp. enrichment culture clone nf1B6 (Campos et al. 2013), a beta-xylosidase (YagH; 99% consensus, 94% identity; NCBI accession AMX06955) from *E. asburiae* (Liu et al. 2016), a predicted xylosidase/arabinosidase (YagH; 99% consensus, 93% identity; NCBI accession AGX32436) from synthetic *Escherichia coli* 321.deltaA (Lajoie et al. 2013) and a β-xylosidase from *E. cloacae* subsp. cloacae NCTC 9394 (99% consensus, 94% identity; NCBI accession CBK87506). Thus, the β-xylosidase from *E. ludwigii* LY-62 belonged to the GH43 family. Xyl62 had a theoretical molecular weight (MW) of 61.29 kDa, an isoelectric point (IP) of 5.41 and parameters of high stability (http://web.expasy.org/cgi-bin/protparam/protparam). These properties of Xyl62 are similar to the first reported β-xylosidase (Xyl43) from *Enterobacter* sp. (*E. cloacae*), with an MW 60.84 kDa and an IP of 5.53 (Campos et al. 2013).

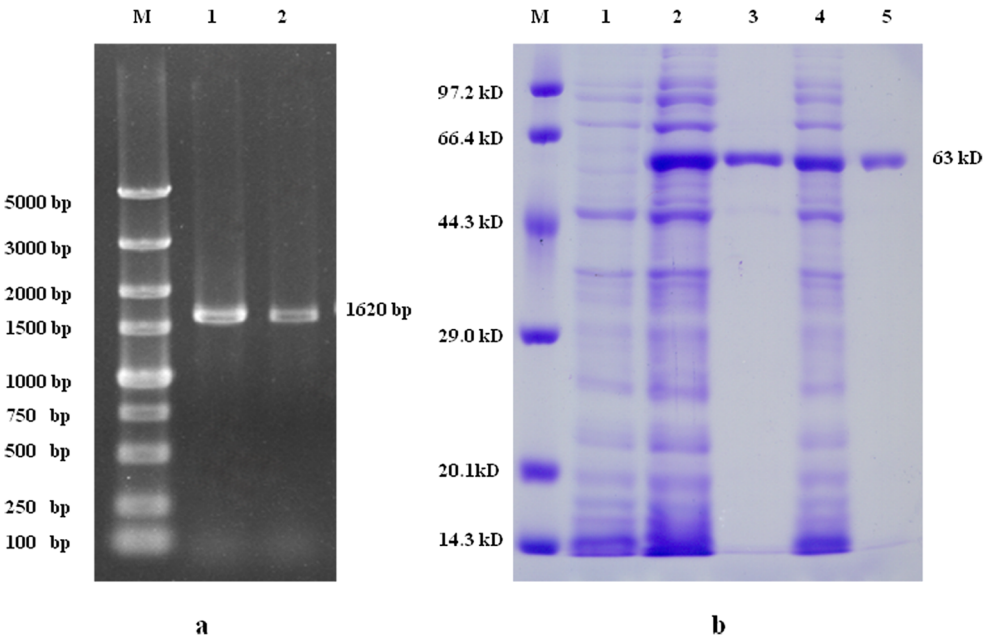

Fig. 5 Agarose electrophoretogram of DNA and SDS-PAGE electrophoretogram of proteins. **a** Lane M: DL 5000 DNA Marker. Lane 1: PCR product using by primers LY-3Nde1 and LY-4Xho1. Lane 2: PCR product using by primers LY-5Nco1 and LY-6Xho1. **b** Lane M: premixed protein marker (low) (TaKaRa). Lane 1: extracellular supernatant of BL21(DE3) containing empty pET-28a(+). Lane 2: extracellular supernatant of BL21(DE3) containing His-Xyl62. Lane 3: purified His-Xyl62. Lane 4: extracellular supernatant of BL21(DE3) containing Xyl62-His. Lane 5: purified Xyl62-His

Expression of β-xylosidase

The gene of β-xylosidase Xyl62 was expressed as either an N- or C-terminal His-tagged fusion protein to confirm that this gene encoded a functional enzyme. The biological function of gene encoded β-xylosidase Xyl62 was verified by the clone and expression. The predicted MW and IP of the recombinant His-Xyl62 (N-terminal His-tagged fusion) and Xyl62-His (C-terminal His-tagged fusion) proteins were MW = 63.45 kDa/IP = 5.76 and MW = 62.80 kDa/IP = 5.54, respectively. In comparison, the predicted MW and IP of the recombinant His-Xyl43 and Xyl43-His proteins were 64.4 kDa/5.85 and 61.66 kDa/5.70, respectively (Campos et al. 2013). Purification of His-Xyl62 and Xyl62-His under native conditions was achieved, and the recombinant proteins were observed by SDS-PAGE (Fig. 5b). Purified solutions were tested for β-xylosidase activity, and the specific activities of His-Xyl62 and Xyl62-His were 74.75 and 77.23 mU/mg, respectively.

Characterization of the recombinant β-xylosidase enzyme activity

The activities of the recombinant proteins were characterized using pNPX as a substrate and measuring β-nitrophenol (pNP) release by monitoring Abs_{410nm}. Both His-Xyl62 (N-terminal His-tagged fusion) and Xyl62-His (C-terminal His-tagged fusion) presented β-xylosidase activity.

As shown in Fig. 6a, His-Xyl62 and Xyl62-His showed optimal activity at pH 7.0. When the pH value was less than 7.0, the β-xylosidase activity increased as the pH value increased. At pH values ranging from 6.8 to 8.0, the relative activities were preserved at levels greater than 80%. His-Xyl43 demonstrated high β-xylosidase activity at pH values ranging between 5.5 and 7, with the highest activity measured at pH 6 at 50 °C (Campos et al. 2013). His-Xyl62 and Xyl62-His showed high stability at pH values ranging from 6.0 to 8.0 (up to 80% activity) (Fig. 6b). The optimum temperature for His-Xyl62 and Xyl62-His was 40 °C (Fig. 6c), and both proteins displayed high activity levels at 35–45 °C (Fig. 6d, e).

The β-xylosidase activity was assayed in the presence of several metal ions, such as Ni^{2+}, Zn^{2+}, K^{+}, Ca^{2+}, Mg^{2+}, and Co^{2+}, at 2 and 10 mmol/L concentrations. Remarkably, Ca^{2+} improved the enzymatic activity up to 150–220%, and Mg^{2+} inhibited the enzymatic activity to 50% of its optimal level. Other metal ions exhibited slight effects on the enzymatic activity (Fig. 7).

The results of kinetic studies of His-Xyl62 and Xyl62-His using pNPX as the substrate are shown in Fig. 8. The K_M and k_{cat} values for His-Xyl62 were 1.55 mmol/L and 8.51 s^{-1}, respectively. The K_M and k_{cat} values for Xyl62-His were 2.80 mmol/L and 6.94 s^{-1}, respectively. The differences of the kinetic parameters between His-Xyl62 and Xyl62-His are likely caused by the position of His tag. In comparison, the catalytic efficiencies of His-Xyl62 and Xyl62-His were better than His-Xyl43, with a K_M of 2.92 mmol/L and a k_{cat} of 1.32 s^{-1}, respectively (Campos et al. 2013). The catalytic efficiency values of the β-xylosidase from *E. ludwigii* LY-62 were 5.49 $s^{-1} \times mM^{-1}$ (His-Xyl62) and 2.48 $s^{-1} \times mM^{-1}$ (Xyl62-His).

Discussion

Strains with identifiable hydrolysis zone on xylan-agar plates displayed with xylanolytic enzyme activity and were selected for further screening for the presence of β-xylosidases. The strain with the highest β-xylosidase activity, LY-62, was confirmed as *E. ludwigii*, which is assigned to the *E. cloacae* complex. Eight species are included in the *E. cloacae* complex: *E. ludwigii*, *E. asburiae*, *E. cloacae*, *E. dissolvens*, *E. hormaechei*, *E. kobei*, *E. nimipressuralis*, and *E. cancerogenus*. The species and genovars of the *E. cloacae* complex are close genetic and phenotypic relatives (Hoffmann et al. 2005).

Xyl62 belongs to the endoenzyme family. Prokaryotic β-xylosidases are intracellular enzymes, whereas fungal β-xylosidases are extracellular or cell wall-binding enzymes (Nieto-Domínguez et al. 2015). Compared to the GH43 β-xylosidase (NCBI accession JX569191) from *Enterobacter* sp., the gene sequence and predicted amino acid sequence of Xyl62 displayed 90 and 94% identity, respectively. GH43 hemicellulolytic enzymes are promising candidates for β-xylosidase activity, as they do not display transglycosylation at high substrate concentrations (Jordan et al. 2007). Commercially applied GH43 β-xylosidases are derived four different bacteria, including *Alkaliphilus metalliredigens*, *Bacillus pumilus*, *Bacillus subtilis* subsp. *subtilis str.* 168, and *Lactobacillus brevis* (ATCC367), and these enzymes have been characterized under different biochemical conditions. They showed functional stability within the range from pH 4.5 to pH 10.5 and displayed thermal stability from 25 to 40 °C (Jordan et al. 2012). The properties of the GH43 β-xylosidases with properties differ from other GH family β-xylosidases and may be suitable for use in enzyme cocktails (Lombard et al. 2014).

The corresponding amino terminal portion of Xyl62 contains a conserved domain of 300 amino acids that is common to β-xylosidases and contains the active site. Another conserved domain of unknown function (classified as DUF1349 superfamily in NCBI database) is located in the 145 amino acids in the carboxy terminus. Further studies are required to explore whether this DUF1349 superfamily conserved domain plays a necessary role in the enzyme activity.

His-Xyl62 and Xyl62-His showed high stability at pH values ranging from 6.0 to 8.0, and both proteins displayed

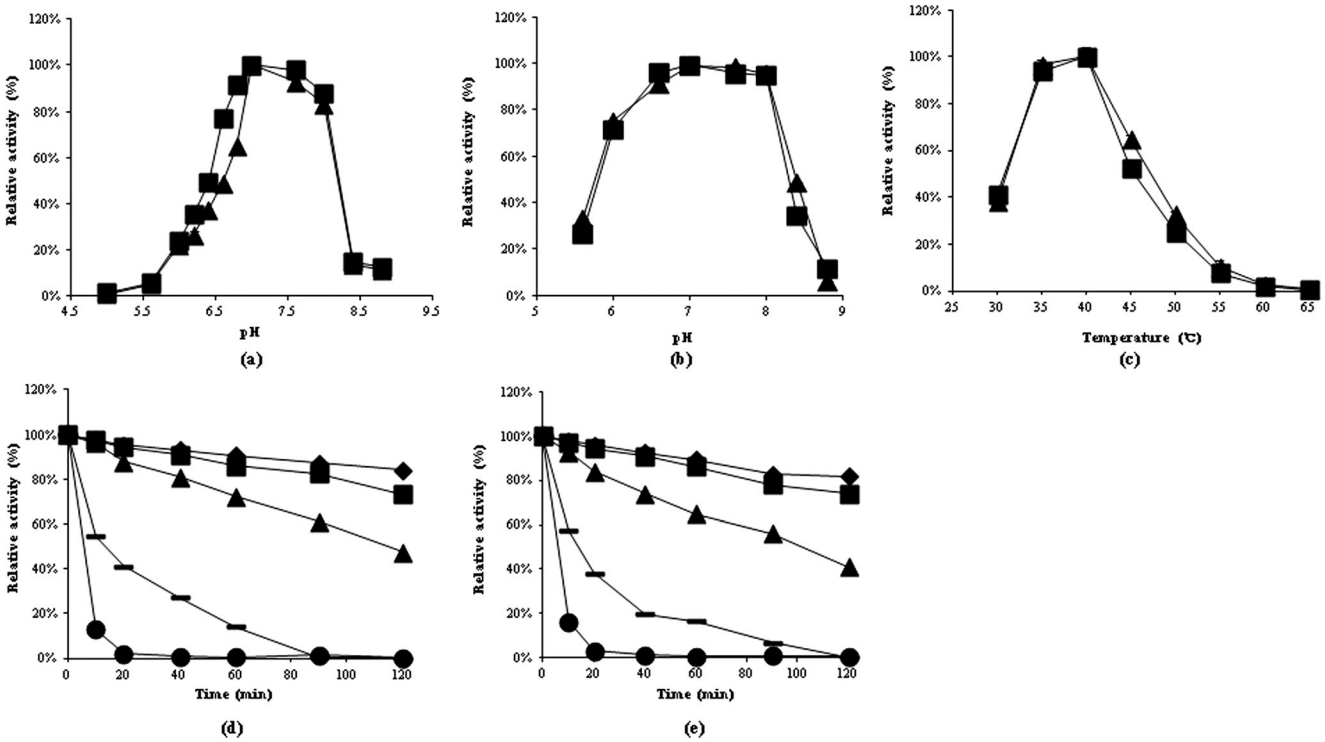

Fig. 6 Characterization of the His-Xyl62 and Xyl62-His enzyme activities. **a** The effect of different pH values on activity. **b** pH stability of His-Xyl62 and Xyl62-His. **c** The effect of different temperatures on activity. **d** Temperature stability of His-Xyl62. **e** Temperature stability of Xyl62-His. In **a**, **b**, and **c**, the black triangle represents His-Xyl62, and the black square represents Xyl62-His. In **d, e**, the black diamond represents 35 °C, the black square represents 40 °C, the black triangle represents 45 °C, the black transverse line represents 50 °C, and the black circle represents 55 °C

high activity levels at 35–45 °C. His-Xyl62 and Xyl62-His should be suitable for the commercial applications on neutral and mesophilic conditions. Interestingly, some β-xylosidases from thermophilic bacteria and archaea have been reported. The optimal reaction conditions are pH 6.0 and 65 °C for β-xylosidases XylC from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, as well as the recombinant $XylC^{rec}$ using pNPX as the substrate (Weilan et al. 2011). The GH43 β-xylosidase XynB3 from *Geobacillus stearothermophilus* T-6 has the highest activity at 65 °C and pH 6.5, with a clear preference for xylose-based substrates (Shallom et al. 2005). However, the β-xylosidases from

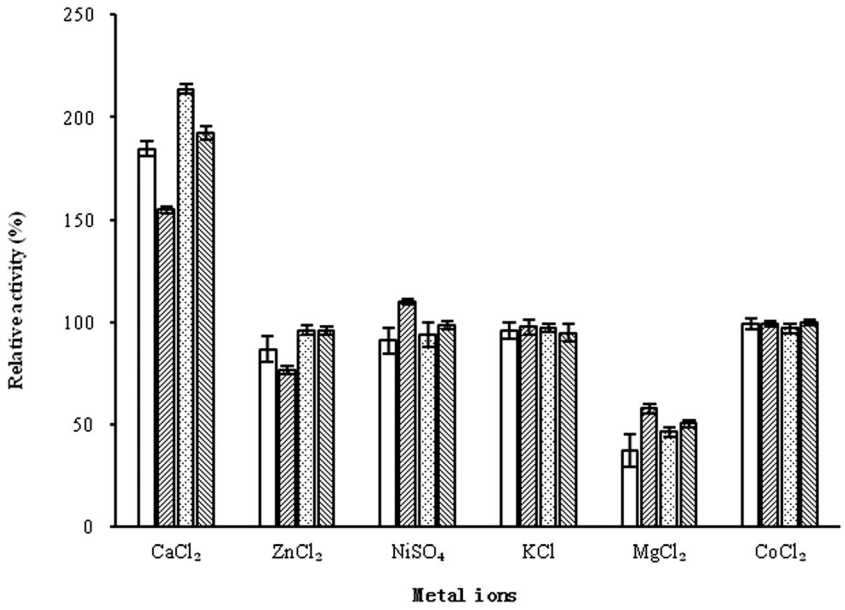

Fig. 7 Effects of different metal ions on enzyme activity. White: 2 mmol/L (His-Xyl62), light upward diagonal line: 10 mmol/L (His-Xyl62), dotted diamond:2 mmol/L (Xyl62-His), light downward diagonal line: 10 mmol/L (Xyl62-His). The figure demonstrated that six metal ions represented divergent effects to the enzymatic activity, and Ca^{2+} improves the enzymatic activity while Mg^{2+} inhibits the enzymatic activity

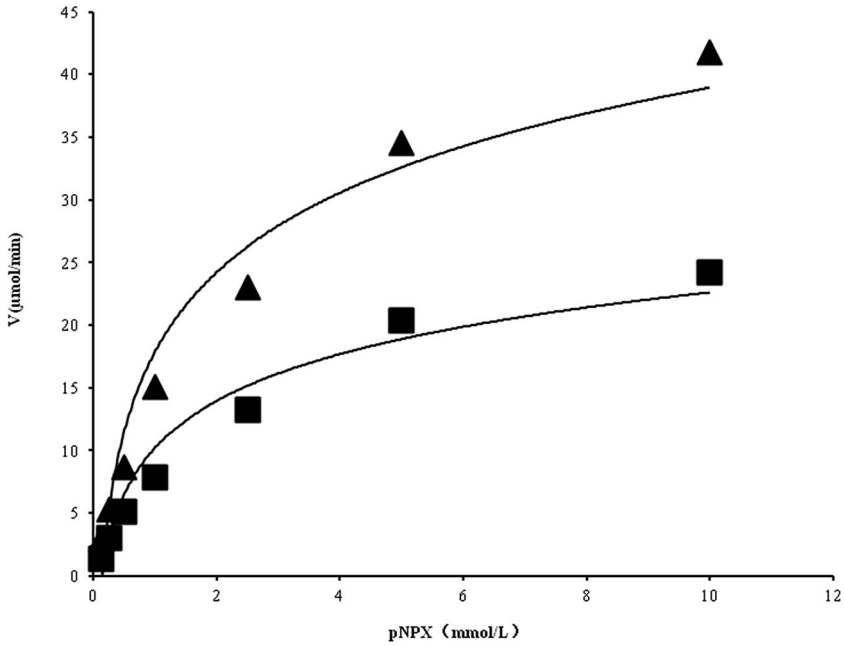

Fig. 8 Nonlinear regression fitting to the Michaelis–Menten equation for hydrolysis rates versus pNPX concentrations. Black triangle: His-Xyl62; black square: Xyl62-His. The figure exhibited the kinetic studies of His-Xyl62 and Xyl62-His using pNPX as substrate

Bacillus thermantarcticus (Lama et al. 2004) and *Bifidobacterium adolescentis* display optimal activity at pH 5.0–7.0 and 50–70 °C (Lagaert et al. 2011). The increasing focus on thermostable xylosidases indicates that Xyl62 may be subjected to molecular modifications to improve thermostability.

The activities of some β-xylosidases, such as Xyl62, are significantly enhanced by Ca^{2+}, including enzymes from *Neocallimastix frontalis* (Studier 2005), *Scytalidium thermophilum* (Zanoelo et al. 2004), and *Humicola insolens* (Xinzhou et al. 2014). Ca^{2+} slightly enhances the activity of β-xylosidases from *S. thermophilum* (Zanoelo et al. 2004), *Aureobasidium* (Hayashi et al. 2001), *Paecilomyces thermophila* (Yan et al. 2008) and *Aspergillus phoenicis* (Rizzatti et al. 2001). According to other studies, Ca^{2+} does not enhance the activity of the β-xylosidase from

Table 3 Catalytic efficiency of GH 43 β-xylosidases from different bacteria on pNPX

Species	K_M (mM)	*k*cat (s^{-1})	Catalytic efficiency (*k*cat /K_M, $s^{-1} \times mM^{-1}$)	Reference
Enterobacter ludwigii LY-62	1.55[a] 2.80[b]	8.51[a] 6.94[b]	5.49[a] 2.48[b]	This report
Lactobacillus brevis ATCC 367	6.93	73.40	10.60	(Jordan et al. 2012)
Thermobifida fusca YX	0.55	6.72	12	(Moraïs et al. 2012)
Bacillus halodurans C-125	4.40	12.10	2.75	(Sinnott 1990)
Geobacillus thermoleovorans IT-08	0.066	0.00032	0.0048	(Ratnadewi et al. 2013)
Geobacillus stearothermophillus T-6	17	57	3.35	(Shallom et al. 2005)[48]
Selenomonas ruminantium	0.39	11.80	30.26	(Jordan and Braker 2011)
Bacillus pumilus IPO	3.90	76	19.49	(Xu et al. 1991)
Bacillus pumilus 12	1.50	7.60	5.07	(Van Doorslaer et al. 1985)
Geobacillus thermoleovorans IT-08	0.55	0.18	0.33	(Wagschal et al. 2009)
Enterobacter sp.	2.92	1.32	0.45	(Campos et al. 2013)
Bifidobacterium animalis subsp. *lactis* BB-12	15.60	60.60	3.88	(Viborg et al. 2013)
Bacteroides ovatus	4.57	7.82	1.71	(Jordan et al. 2017)
Weissella sp. strain 92	7.40	258	34.90	(Falck et al. 2015)
Lactobacillus brevis DSM 20054	11	46	4.18	(Michlmayr et al. 2013)

[a] His-Xyl62

[b] Xyl62-His

B. thermantarcticus (Lama et al. 2004), and it slightly inhibited the activity of the β-xylosidase from *Phanerochaete chrysosporium* (Huy et al. 2013).

The determined kinetic parameters and relative parameters of GH43 β-xylosidases from different bacterial sources are listed in Table 3. For comparison, the k_{cat}/K_M value of the β-xylosidase from *Enterobacter* sp. is 0.45 $s^{-1} \times mM^{-1}$ (Campos et al. 2013). The catalytic efficiency value of the *E. ludwigii* LY-62 β-xylosidase is lower than β-xylosidases from *Thermobifida fusca* YX (Moraïs et al. 2012), *Selenomonas ruminantium* (Jordan and Braker 2011), *Bacillus pumilus* IPO (Xu et al. 1991), *Weissella* spp. strain 92 (Falck et al. 2015), and *Lactobacillus brevis* ATCC 367 (Jordan et al. 2012). However, the study of β-xylosidase enzymatic characteristics should focused on the differences for the diversified applications.

E. ludwigii has rarely been studied as a bacterium containing a xylanolytic enzyme system. In the present study, *E. ludwigii* LY-62 with high β-xylosidase activity was isolated and identified. The β-xylosidase Xyl62 from *E. ludwigii* LY-62 was studied and successfully expressed as fusion proteins. Both the N-terminal and C-terminal His-tagged fusion proteins were confirmed as functional β-xylosidases with distinct characteristics. This report is the first to describe a β-xylosidase from *E. ludwigii.*

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals Not applicable.

Informed consent Not applicable.

Ethics approval Not applicable.

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