Appl Biochem Biotechnol
DOI 10.1007/s12010-017-2598-x

Enzymatic Hydrolysis of Black Liquor Xylan by a Novel Xylose-Tolerant, Thermostable β-Xylosidase from a Tropical Strain of *Aureobasidium pullulans* CBS 135684

Wichanee Bankeeree[1] · **Rinji Akada**[2] · **Pongtharin Lotrakul**[1] · **Hunsa Punnapayak**[1] · **Sehanat Prasongsuk**[1]

Received: 30 May 2017 / Accepted: 6 September 2017

Abstract From three cell-associated β-xylosidases produced by *Aureobasidium pullulans* CBS 135684, the principal enzyme was enriched to apparent homogeneity and found to be active at high temperatures (60–70 °C) over a pH range of 5–9 with a specific activity of 163.3 units (U) mg^{-1}. The enzyme was thermostable, retaining over 80% of its initial activity after a 12-h incubation at 60 °C, with half-lives of 38, 22, and 10 h at 60, 65, and 70 °C, respectively. Moreover, it was tolerant to xylose inhibition with a K_i value of 18 mM. The K_m and V_{max} values against *p*-nitrophenyl-β-d-xylopyranoside were 5.57 ± 0.27 mM and 137.0 ± 4.8 μmol min^{-1} mg^{-1} protein, respectively. When combining this β-xylosidase with xylanase from the same *A. pullulans* strain, the rate of black liquor xylan hydrolysis was significantly improved by up to 1.6-fold. The maximum xylose yield (0.812 ± 0.015 g g^{-1} dry weight) was obtained from a reaction mixture containing 10% (*w*/*v*) black liquor xylan, 6 U g^{-1} β-xylosidase and 16 U g^{-1} xylanase after incubation for 4 h at 70 °C and pH 6.0.

Keywords Black yeast · Thermoactive enzyme · Xylose tolerant · Xylan hydrolysis

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12010-017-2598-x) contains supplementary material, which is available to authorized users.

✉ Sehanat Prasongsuk
sehanat.p@chula.ac.th

[1] Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

[2] Department of Applied Chemistry, Yamaguchi University Graduate School of Science and Technology for Innovation, Ube 755-8611, Japan

Published online: 16 September 2017

Introduction

Hemicellulose is the most abundant heteropolysaccharide in plant biomass and is comprised of xylan, β-(1,4)-linked xylopyranose residues, as the backbone polymer with various substituents, including acetyl, arabinosyl, and glucuronyl residues. The complete hydrolysis of hemicellulose yields a mixture of monosaccharides, including xylose and arabinose, which can be used as substrates in the food, fuel, and chemical industries [1]. Hemicellulose can be extracted from any plant biomass, but recently, those prepared from agricultural residues have gained more attention due to their cheap cost and abundance. Most studies have focused on the extraction of xylan from corn cobs, rice hulls, cereal straw, and other feedstock [2]. On the contrary, xylan recovery from waste water of biomass-related industries such as pulp and paper mills, especially the black liquor, has rarely been reported in spite of its high potential [3]. Approximately 8600 L of black liquor are generated every day from *Eucalyptus grandis* kraft pulping process by a commercial pulp and paper company in Thailand. The amount of xylan extracted from this black liquor was about 13 g L^{-1} or 95% of xylan recovery from the pulp feedstock [4]. The degradation of hemicellulose requires the synergistic action of several enzymes. Among these, xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.37) are the most well studied. Most xylanases randomly hydrolyze glycosidic linkages along the xylan chain to release xylooligosaccharides (XOs) and xylobioses that are subsequently hydrolyzed by β-xylosidase to xylose monomers. The key enzyme in the complete degradation is β-xylosidase, since xylosyl residues and the reducing end product can inhibit xylanase. From a biotechnological viewpoint, the combination of β-xylosidase with other hemicellulose-degrading enzymes has opened new possibilities for a number of applications, including in the pulp and paper, food, beverage, feed, textile, and pharmaceutical industries [5]. For such applications, a large quantity of the enzyme possessing specific properties, including thermostability and tolerance to elevated concentration of substrate, product, and other additives, is required [6].

Among several microorganisms producing xylan-degrading enzymes, *Aureobasidium pullulans* has been recognized as one of the best producers with xylanase and β-xylosidase activity as high as 373.0 U mL^{-1} and 10.0 U mg^{-1} cell dry weight, respectively [7–9], compared to the extracellular enzymes from other fungi including *Penicillium janczewskii* (151.1 and 1.9 U mL^{-1}, respectively) [10] and *Aspergillus niger* (91.5 and 2.1 U mL^{-1}, respectively) [11]. In 2013, *A. pullulans* CBS 135684 isolated from Thailand was found to produce a cellulase-free xylanase that was active at relatively high temperatures (70–80 °C) over a wide optimal pH range from 4 to 10 [9]. These properties are relatively uncommon in the xylanases from previously reported *A. pullulans* strains and other typical industrial yeast species. Since this strain also exhibited β-xylosidase activity, it was of interest to investigate whether β-xylosidase also possessed such remarkable properties similar to the xylanase, and whether it could be used in any xylose-based applications.

Therefore, the goals of this study were to enrich and characterize the β-xylosidase from *A. pullulans* CBS 135684 and to explore the potential of using this enzyme in the saccharification of xylan extracted from the black liquor effluent of the pulp and paper industry.

Materials and Methods

Microorganism

Aureobasidium pullulans CBS 135684 was obtained from the fungal culture collection of the Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The yeast was grown in yeast malt (YM) agar medium at room temperature (28 ± 2 °C) for 2 days and short-term stock cultures were stored at 4 °C. For long-term storage, the strain was stored at − 20 °C in YM broth containing 20% (*v*/*v*) glycerol.

Production of β-Xylosidase

The seed culture was prepared by growing *A. pullulans* in YM broth at room temperature with agitation at 150 rpm for 72 h. The inoculum was adjusted to 2.5×10^6 cells mL^{-1} and then 100 μl was transferred into a 250-mL Erlenmeyer flask containing 100 mL of production medium [12]. The incubation was continued under the same condition for 5 days, when *A. pullulans* cells were then harvested by centrifugation at 9000×*g* for 10 min and freeze-dried at − 60 °C for 6 h. The lyophilized cells (1 g) were ground, suspended in 100 mL of sterile distilled water and centrifuged at 5000×*g* for 10 min for debris removal with harvesting of the supernatant as the crude enzyme.

Enzyme and Protein Assays

For determination of the β-xylosidase activity, the reaction mixture consisting of 0.1 mL of appropriately diluted crude enzyme solution (see "Production of β-Xylosidase") or purified enzyme solution (1 mg protein per mL), 0.2 mL of 5 mM *p*-nitrophenyl-β-D-xylopyranoside (pNPX; Sigma, USA) in deionized water, and 0.7 mL of 50 mM acetate buffer (pH 6.0) was incubated at 70 °C for 10 min. The reaction was terminated by adding 1.0 mL of 0.5 M Na_2CO_3, and the absorbance at 410 nm was measured. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol (pNP) from pNPX per min. Protein concentrations were measured by the method of Lowry et al. [13] using bovine serum albumin (Sigma, USA) as the standard.

Enzyme Enrichment and SDS-PAGE Analysis

Enrichment of the assumed principal β-xylosidase was performed at 4 °C. The crude enzyme preparation (see "Production of β-Xylosidase") was concentrated tenfold by ultrafiltration (10 kDa MW membrane cut-off, Amicon, Beverly, MA, USA) prior to precipitation using ammonium sulfate (40–80% saturation). After recovery by centrifugation at 10,000×*g* for 20 min, the pellet was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and dialyzed for 16 h against the same buffer. The concentrated enzyme (40 mg protein mL^{-1}) was applied to a pre-equilibrated DEAE Sepharose Fast Flow column (7 mm × 25 mm, GE Healthcare, Buckinghamshire, UK) at a flow rate of 1.0 mL min^{-1}. The adsorbed proteins were eluted with a linear gradient of 0–1.0 M NaCl in 50 mL of

the same buffer. Five-milliliter fractions were collected and analyzed for β-xylosidase activity and protein content. Fractions that represented the major portion of β-xylosidase activity were pooled and dialyzed against 50 mM acetate buffer (pH 4.0). The concentrated enzyme solution (18 mg protein mL^{-1}) was then purified through a pre-equilibrated SP Sepharose Fast Flow column (7 mm × 25 mm, GE Healthcare) at a flow rate of 1.0 mL min^{-1}. Elution was performed with a linear gradient of 0–1.0 M NaCl in 50 mL of the same buffer and collected in 5-mL fractions. Fractions with β-xylosidase activity were pooled, dialyzed against 50 mM acetate buffer (pH 6.0), and applied to a pre-equilibrated Sephacryl-S100 gel filtration column (30 cm × 5 cm Sephacryl S-100 HR, Sigma, USA). Elution was performed at a flow rate of 0.5 mL min^{-1} collecting 3-mL fractions that were assayed for β-xylosidase activity and protein content as described above. Protein profiles of the collected fractions were determined by resolution through SDS-PAGE (12.5% (*w*/*v*) resolving gel in 1.5 M Tris-HCl, pH 8.8), run at 20 mA for 2 h, and stained with Coomassie Brilliant Blue R-250 [14].

Determination of β-Xylosidase Properties

Effects of pH and Temperature on the Enzyme Activity

Effects of temperature and pH on the enriched β-xylosidase activity were determined by incubating the reaction mixture (1 U mL^{-1} enzyme and 5 mM pNPX, total volume 1 mL) at various temperatures (40–80 °C) in 50 mM sodium acetate (pH 3.0–6.0), sodium phosphate (pH 6.0–9.0), or glycine (pH 9.0–11.0) reaction buffer for 10 min. The relative activity was calculated as the percentage of the maximal activity.

Effect of Temperature on the Enzyme Stability

Enzyme thermostability was assessed by measuring the residual β-xylosidase activity after incubation at different temperatures from 50 to 70 °C in the absence of pNPX for a 6–4-h period. The initial activity of the enriched β-xylosidase at 1 U mL^{-1} was set as 100% relative activity.

Effects of Different Ions and Organic Solvents on the Enzyme Activity

To investigate the effects of different cations and a divalent cation chelator on the enzyme activity, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4{\cdot}7H_2O$, KCl, $MgCl_2$, $MnSO_4$, NaCl, $ZnCl_2$, and EDTA were separately added to the reaction mixture at 1 mM prior to the enzyme assay under the optimal conditions (see "Effects of pH and Temperature on the Enzyme Activity"). The hydrolytic activity of β-xylosidase was also determined in the presence of some organic solvents including, primary alcohols ($C_{1–6}$), secondary alcohols (2-propanol and 2-butanol), tertiary alcohol (2-methyl-2-propanol), acetone, and chloroform at final concentrations of 10–30% (*v*/*v*) under the optimal condition (see "Effects of pH and Temperature on the Enzyme Activity"). The relative activity was calculated as a percentage of the enzyme activity without the aforementioned chemicals added.

Substrate Specificity

Substrate specificity was determined by incubating the enriched enzyme (1 μg protein per reaction) in the presence of various individual substrates, including pNPX, cellobiose, carboxymethyl cellulose (CMC), beechwood xylan, oat spelt xylan, and commercial xylooligosaccharides ($X_{2–4}$), at different final concentrations from 0 to 1 mM. The assay was performed under the optimal condition and the activity was measured by quantification of the released pNP (for pNPX), xylose (for xylooligosaccharides and xylan), and glucose (for cellobiose and CMC). The amount of released reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [15]. One U was defined as the amount of enzyme that liberated 1 μmol of each product per min. A 100% relative activity was set as that when xylobiose was used as the substrate. The hydrolytic products were qualitatively analyzed by thin-layer chromatography (TLC) on silica gel plates (60F 254, 0.2 mm, Merck, Germany) [16]. The unit activity was also used to calculate the kinetic values of the enzyme, in terms of the Michaelis constant (K_m) and maximum velocity (V_{max}), by fitting the initial hydrolysis rates to the Lineweaver-Burk equation.

Monosaccharide Inhibition

The effects of various monosaccharides on the enzyme activity were studied using 1 mM pNPX as the substrate in the presence of xylose, glucose, arabinose, mannose, or xylitol at different final concentrations (1–70 mM). The K_i value of inhibition was defined as the amount of a monosaccharide required to inhibit 50% of the β-xylosidase activity, and was calculated from the Dixon plot. Kinetic constants were determined from the reactions that were performed with 5 mM or 10 mM monosaccharide at varying pNPX concentrations from 0 to 1 mM under optimal conditions to further confirm the type of inhibition.

Synergy of Xylanase and β-Xylosidase on Black Liquor Xylan Hydrolysis

The xylan used in this study was extracted from the black liquor of a commercial pulp and paper plant (Double A (1991) Public Co., Ltd., Thailand) as described by Bankeeree et al. [4]. In order to enhance the xylan degradation, response surface methodology (RSM) was used to determine the optimum condition, in terms of the incubation time and amount of β-xylosidase and extracellular xylanase from *A. pullulans* CBS 135684. A Box-Behnken design matrix with three factors at three different levels and three replicates at the center point was used for fitting the second-order response surface [17]. The reaction was performed with 10% (*v*/*v*) black liquor xylan (Fluka, USA) in 50 mM acetate buffer (pH 6.0) at 70 °C. The amount of xylose from each reaction was taken as the response and analyzed by HPLC using a RP-C18 column (Nucleodur 100-5 C18 ec, 250 × 4, Macherey Nagel). The column was eluted with 70:30 (*v*/*v*) acetonitrile: water at 1.0 mL min^{-1} and detection was performed using a differential refractometer R-401. Statistical analysis of the data was performed using the design package Design-Expert software (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the analysis of variance (ANOVA) and to determine the significance of each term in the equations fit. In order to verify the accuracy of the predicted model, a repeated experiment was performed using the optimized amount of each enzyme.

Other Data Analysis

Statistical differences among the means of data ($n = 3$) were calculated when appropriate using one-way analysis of variance (ANOVA) and either Duncan's multiple range test (DMRT) or Student's t test (2 tailed) with the SPSS 17.0 software package (SPSS Inc., Chicago, U.S.A.). Differences at $P \leq 0.05$ were considered significant.

Results and Discussion

β-Xylosidase Production

Although *A. pullulans* is well known as an excellent xylanase producer, there have been only a few reports on its β-xylosidase, where most of them have shown that the enzymes were cell associated [18, 19]. Similar to those reports, the β-xylosidase activity of *A. pullulans* CBS 135684 was only detected only in the cell extract. After cultivation for 5 days in production medium with corncob as the sole carbon source, the maximum activity of this enzyme was 2.36 ± 0.01 U mg^{-1} cell dry weight (DW), some 1.5-fold higher than that when commercial beechwood xylan was used (1.58 ± 0.02 U mg^{-1} cell DW). The maximum activity of β-xylosidase from *A. pullulans* CBS 135684 was higher than those previously reported from *A. pullulans* CBS 58475 (0.9 U mg^{-1}) [20] and *Penicillium janczewskii* (1.9 U mg^{-1}) [21].

β-Xylosidase Enrichment and SDS-PAGE Analysis

The likely principal cell-associated β-xylosidase was enriched to apparent homogeneity, with the summary of the enrichment steps shown in Table 1 and Fig. 1. The DEAE-Sepharose column chromatography yielded two separate peaks of β-xylosidase activity (Fig. 1a). The unbound enzyme peak, which represented the major portion (~ 70%) of β-xylosidase activity, was further fractionated using SP Sepharose Fast Flow column chromatography, where the bound fraction yielded two separate peaks of enzyme activity upon subsequent elution with NaCl (Fig. 1b). The enzyme from the major peak (~ 50% total activity) was subsequently fractionated over Sephacryl S-100 gel filtration chromatography to yield a single peak of β-

Table 1 Enrichment of the (principal) cell-associated β-xylosidase from *A. pullulans* CBS 135684

Enrichment step	Total protein (mg)	Total activity (U)[a]	Specific activity (U mg^{-1} protein)	Purification (fold)	Yield (%)
Cell extract	1054 ± 13.79	5258 ± 24.87	4.97 ± 0.05	1.00	100.00
Ultrafiltration	885 ± 11.35	4815 ± 20.14	5.42 ± 0.06	1.09	91.58
$(NH_4)_2SO_4$ precipitation (50–80% saturation)	583 ± 9.64	3548 ± 13.59	6.05 ± 0.08	1.22	67.48
DEAE-Sepharose	84 ± 2.94	2052 ± 18.46	24.18 ± 0.70	4.87	39.03
SP Sepharose Fast Flow	18 ± 0.34	1785 ± 9.84	98.59 ± 1.55	19.84	33.95
Sephacryl S-100	6 ± 0.12	985 ± 2.63	163.32 ± 2.70	32.86	18.73

[a] 1 U is the amount of enzyme required to release 1 μmol *p*-nitrophenol equivalent per min under the assay conditions. Experiments were performed in triplicate. Data are shown as the mean ± SD (error bars), derived from triplicate experiments

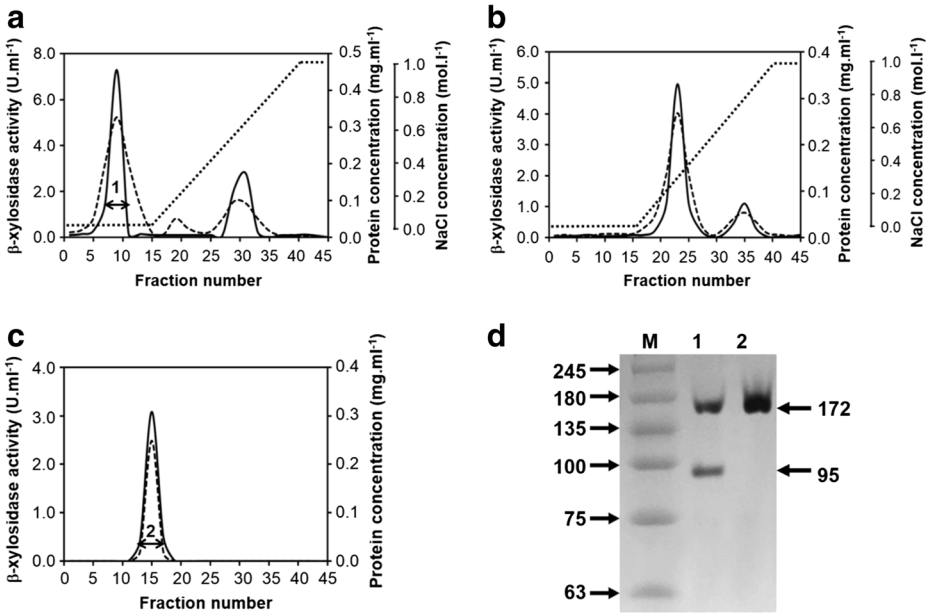

Fig. 1 Sequential purification steps of β-xylosidase from *A. pullulans* CBS 135684. The amounts of protein (- - -) and β-xylosidase activity (—) in fractions eluted from **a** DEAE Sepharose, **b** SP Sepharose Fast Flow, and **c** Sephacryl S-100 gel filtration column chromatography were profiled. The proteins that adsorbed to DEAE Sepharose or SP Sepharose Fast Flow column chromatography were eluted with a linear gradient of 0–1.0 mol L^{-1} NaCl (·····) in the corresponding buffer. SDS-PAGE analysis **d** of the enriched β-xylosidase from *A. pullulans* CBS 135684 was performed on a 10% (*w*/*v*) gel in 1.5 M Tris-HCl (pH 8.8) and stained with Coomassie Brilliant Blue R-250. Lane M: Blueye prestained protein ladder (GeneDireX, Taiwan). Lane 1: the enriched β-xylosidase from DEAE Sepharose column (~ 30 μg protein). Lane 2: the enriched β-xylosidase from SP Sepharose Fast Flow column (~ 30 μg protein). Lane 3: the enriched β-xylosidase from Sephacryl S-100 gel filtration column chromatography (~ 30 μg protein)

xylosidase activity (Fig. 1c). These results indicated that *A. pullulans* CBS 135684 potentially produced at least three cell-associated β-xylosidases, which, if so, is the first report of multiple forms of β-xylosidase in *A. pullulans*. However, their genetic and post-translational relationship to each other remains unknown. The overall percent recovery of this (potentially principal) enzyme was approximately 18.7% with a 32.9-fold enrichment and a specific activity of 163.3 U mg^{-1}. The final enriched enzyme showed a single band of ~ 172 kDa on one-dimensional SDS-PAGE resolution with Coomassie Brilliant Blue staining (Fig. 1d), which was similar to the size estimated from the Sephacryl S-100 gel filtration chromatography (data not shown). Differences in size between this enriched β-xylosidase from *A. pullulans* CBS 135684 and those previously reported from other strains of *A. pullulans* (88.5 kDa) [18–20] implied that it might be a distinct enzyme.

Effects of the pH and Temperature on the β-Xylosidase Activity

The effects of pH and temperature on activity of the enriched β-xylosidase are summarized in Fig. 2a. The maximum β-xylosidase activity was found at 70 °C and pH 6.0, while it was also active over a relatively wide pH range (5.0–9.0) with more than 80% of the maximum activity at the same temperature. This preference for a mildly acidic to mildly basic condition makes the enzyme strikingly different from the previously reported *Aureobasidium* β-xylosidases that

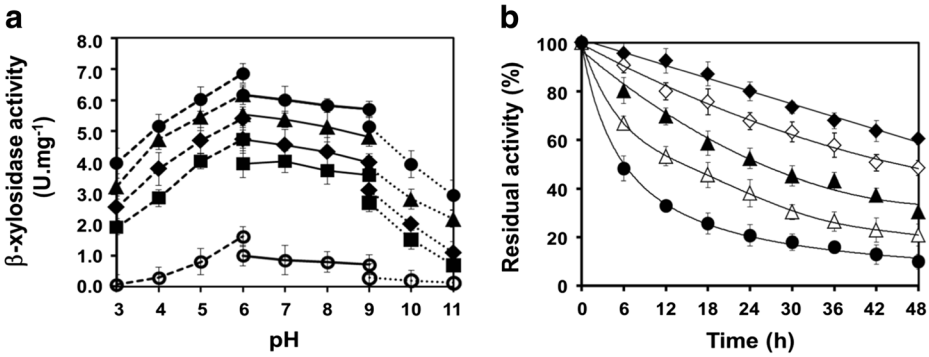

Fig. 2 Effect of the **a** pH and temperature on the enzyme activity and **b** the thermostability profile of the enriched β-xylosidase from *A. pullulans* CBS 135684. **a** The β-xylosidase activity was assayed by incubating the purified enzyme with 5 mM pNPX in 50 mM sodium acetate (pH 3.0–6.0; - - -), phosphate (pH 6.0–10.0; —), or glycine (pH 10.0–11.0; ·····) buffer at 40 °C (■), 50 °C (♦), 60 °C (▲), 70 °C (●), or 80 °C (○) for 10 mins. Relative activity was calculated as a percentage of the maximum activity (50 mM sodium acetate buffer (pH 6.0), 70 °C). **b** The thermostability profile of the enriched β-xylosidase was investigated by separate preincubation in 50 mM sodium acetate buffer (pH 6.0) at 50 °C (♦), 55 °C (◊), 60 °C (▲), 65 °C (Δ), or 70 °C (●) without the substrate for 48 h. The residual β-xylosidase activity was then calculated as a percentage of the initial activity. Data are shown as the mean ± SD (error bars), derived from triplicate experiments

were active at a moderately acidic pH range (3.5–4.5) [19, 20]. The *A. pullulans* CBS 135684 β-xylosidase activity at each pH was significantly increased when the incubation temperature was increased from 40 °C to the optimal at 70 °C. However, a significantly lower enzyme activity was observed at 80 °C (e.g., 12.08 ± 0.42% at pH 6.0), which might be caused by protein denaturation. A fungal β-xylosidase that is active in an alkaline environment and a high temperature are uncommon (Table 2). With its broad optimum pH and thermophilic properties, this enzyme is likely to be applicable in several industrial processes, and especially in the bioconversion of lignocellulosic materials as this is optimally performed at a high pH and temperature.

Effect of Temperature on the β-Xylosidase Stability

The enriched β-xylosidase was found to be relatively stable at 50–60 °C with more than 80% of its original activity remaining after 18 h incubation at these temperatures (Fig. 2b). The half-life of the purified enzyme at 70 °C, the optimum temperature of enzyme activity, was approximately 10 h. In comparison, the previously reported β-xylosidases from the thermophilic bacterium *Geobacillus thermodenitrificans* [21] and the thermotolerant fungi *Aspergillus fumigatus* [31] were clearly less stable, with a half-life at 70 °C of less than 3 h in both cases.

Effects of Different Ions and Organic Solvents on the β-Xylosidase Activity

The enriched enzyme activity was not significantly affected by the presence of any of the tested ions at 1 mM, except for Cu^{2+} that reduced the residual enzyme activity to 80.8 ± 1.4% (Table 3). The sensitivity of β-xylosidase to Cu^{2+} is relatively common for this enzyme from fungi, including those from *Fusarium proliferatum* [25] and *Paecilomyces thermophila* [32]. The presence of EDTA did not significantly affect the β-xylosidase activity, suggesting that divalent cations were not essential for the catalytic activity of this enzyme.

Table 2 Properties of the enriched (principal) β-xylosidase from *A. pullulans* CBS 135684 and those reported from other fungal strains

Species	MW (kDa)	Optimum activity		Specific activity (U mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_i (mM)	Reference
		pH	Temperature (°C)					
Aureobasidium pullulans CBS 135684	95.0	6.0	70	163.3	5.6	137.0	24.0	This study
Aureobasidium pullulans ATCC 20524	88.5	3.5	70	288.0	3.5	263.0	–	[19]
Aspergillus japonicas	120.0	4.0	70	42.6	0.3	98.0	2.9	[22]
Aspergillus niger GS1	111.0	5.0	60	386.7	6.1	1364.0	–	[23]
Aspergillus oryzae[a]	84.7	4.5	55	150.0	1.0	250.0	2.7	[24]
Fusarium proliferatum	91.2	4.5	60	53.0	0.8	75.0	5.0	[25]
Humicola grisea var. *thermoide*[a]	87.0	6.5	55	19.6	1.4	13.0	–	[26]
Humicola insolens Y1[a]	83.2	6.0	60	11.6	2.5	37.3	29.0	[27]
Penicillium janczewskii	100.0	6.0	65	1.9	3.4	33.2	6.0	[10]
Penicillium sclerotiorum	144.0	2.5	60	31.1	0.5	0.8	32.1	[28]
Paecilomyces thermophila J18[a]	52.3	7.0	55	37.9	4.5	90.2	–	[29]
Trichoderma reesei	80.0	3.5	60	16.0	0.5	12.0	1.4	[22]
Talaromyces amestolkiae	200.0	3.0	70	47.1	0.2	52.0	1.7	[30]

[a] Recombinant enzyme

Since organic solvents have been used for solubilizing hydrophobic substrates in enzymatic reactions, their effects on the activity of the enriched β-xylosidase were also determined in this study. The addition of alcohols significantly decreased the β-xylosidase activity in a concentration-dependent manner ($P \leq 0.05$) and also with the increasing alkyl chain length of the alcohol (Table 4), the latter of which might be due to the increasing hydrophobicity with increasing alkyl chain length [33]. However, this enzyme seemed to be particularly tolerant to methanol and ethanol, since it was not significantly inhibited by both alcohols at 10% (*v*/*v*) and retained more than 80% of its initial activity in the presence of 30% (*v*/*v*) of either alcohol. This level of tolerance was higher than that previously reported for the β-xylosidase from the thermophilic fungus *Sporotrichum thermophile* [34] and the thermophilic bacterium *Geobacillus thermodenitrificans* [21], where the presence of 10% (*v*/*v*) methanol or ethanol caused a 30% decrease in their activity. On the other hand, the CBS 135684 β-xylosidase was highly sensitive to 2-methyl-2-propanol, with a more than 50% reduction of its initial activity at a concentration as low as 10% (*v*/*v*). Similar to most enzymes, the β-xylosidase was sensitive to the denaturation caused by acetone and chloroform, with up to 12 and 24% of the enzyme activity being lost in the presence of 10% (*v*/*v*) acetone and chloroform, respectively.

Table 3 Effects of different cations (at 1 mM) on the activity of the (principal) β-xylosidase from *A. pullulans* CBS 135684

Additive	Relative activity (%)*
None	100.0 ± 1.4^{b}
EDTA	104.5 ± 2.7^{b}
KCl	101.2 ± 7.1^{b}
NaCl	100.7 ± 2.3^{b}
$CaCl_2$	107.2 ± 8.7^{b}
$CoCl_2$	100.2 ± 8.7^{b}
$CuSO_4$	80.8 ± 1.4^{a}
$FeSO_4$	105.9 ± 5.9^{b}
$LiCl_2$	102.1 ± 2.5^{b}
$MgCl_2$	104.5 ± 3.7^{b}
$MnSO_4$	103.3 ± 4.9^{b}
$ZnCl_2$	103.4 ± 2.2^{b}

Means with different superscript letters are significantly different (ANOVA and DMRT, $P \leq 0.05$)

*100% relative activity corresponded to activity of the β-xylosidase under the standard assay condition without ion/EDTA added. Data are shown as the mean ± SD (error bars), derived from triplicate experiments

β-Xylosidase Substrate Specificity

Substrate specificity of the enriched β-xylosidase was tested with synthetic substrates, natural polymers, and XOs, with the results summarized in Table 5. Compared to xylobiose, the enzyme exhibited a significantly higher activity towards pNPX (114%) but noticeably less towards xylotriose (85%) and xylotetraose (71%). The enzyme was also able to hydrolyze cellobiose, although at a much lower amount (21%). No hydrolytic activity was detected when polymeric glucans, both natural beechwood xylan and synthetic CMC, were used as its

Table 4 Effect of organic solvents on the activity of the (principal) β-xylosidase from *A. pullulans* CBS 135684

Additive	Relative activity (%)*		
	10% (*v*/*v*)	20% (*v*/*v*)	30% (*v*/*v*)
None	100.0 ± 1.4^{ef}	100.0 ± 1.4^{H}	100.0 ± 1.4^{z}
Methanol	103.0 ± 3.3^{f}	99.4 ± 4.2^{H}	95.0 ± 2.9^{y}
Ethanol	101.5 ± 4.3^{ef}	92.3 ± 2.9^{G}	83.5 ± 1.0^{x}
Propanol	94.8 ± 3.3^{de}	60.0 ± 2.0^{E}	41.7 ± 2.0^{u}
Butanol	76.4 ± 1.3^{c}	26.1 ± 1.3^{C}	19.7 ± 1.2^{r}
Pentanol	73.7 ± 5.9^{bc}	24.9 ± 1.0^{C}	15.8 ± 1.2^{q}
Hexanol	68.5 ± 5.9^{b}	20.0 ± 1.9^{B}	13.7 ± 2.0^{q}
2-Propanol	99.9 ± 3.5^{ef}	72.0 ± 2.3^{F}	50.3 ± 1.8^{v}
2-Butanol	88.5 ± 5.9^{d}	56.2 ± 1.0^{DE}	29.3 ± 0.9^{s}
2-Methyl-2-propanol	30.2 ± 3.2^{a}	9.4 ± 0.4^{A}	ND
Acetone	88.1 ± 2.2^{d}	70.2 ± 2.4^{F}	57.3 ± 2.2^{w}
Chloroform	76.7 ± 2.3^{c}	53.7 ± 5.9^{D}	38.6 ± 1.1^{t}

Means with different superscript letters (a–f, A–H, and q–z for data in the first to the third column, respectively) in the same column are significantly different (ANOVA and DMRT, $P \leq 0.05$)

ND not detectable

*100% relative activity corresponded to activity of the β-xylosidase under standard assay conditions without any solvent added. Data are shown as the mean ± SD (error bars), derived from triplicate experiments

Table 5 Substrate specificity and kinetic analysis of the (principal) β-xylosidase from *A. pullulans* CBS 135684 against synthetic and natural substrates

Substrates	Relative activity (%) at 1 mM of substrate	Products	K_m* (mM)	V_{max}* (μmol min^{-1} mg^{-1})
p-Nitrophenyl xylopyranoside	114.4 ± 2.9[e]	*p*-Nitrophenol	5.67 ± 0.27	136.99 ± 4.81
Xylobiose (X_2)	100.0 ± 4.1[d]	X_1, X_2	4.60 ± 0.11	95.50 ± 0.96
Xylotriose (X_3)	85.2 ± 3.4[c]	X_1, X_2, X_3	6.97 ± 0.13	41.30 ± 0.74
Xylotetraose (X_4)	70.8 ± 2.3[b]	X_1, X_2, X_3, X_4	8.26 ± 0.28	21.30 ± 0.25
Beech wood xylan	ND	–	–	–
Cellobiose (C_2)	20.94 ± 2.5[a]	Glucose (C_1)	12.64 ± 0.34	6.97 ± 0.14
Carboxymethyl cellulose	ND	–	–	–

ND not detectable

Means with different superscript letters are significantly different (ANOVA and DMRT, $P \leq 0.05$)

*Kinetic values were calculated from reactions performed with different concentrations of substrate (0–1 mM) in 50 mM sodium acetate buffer (pH 6.0) at 70 °C for 10 min. Data are shown as the mean ± SD (error bars), derived from triplicate experiments

substrate. A similar β-D-glucosidase activity of β-xylosidase has been reported previously for the β-xylosidase from *Penicillium janczewskii* (1.3% of β-xylosidase activity) [35] and *Aspergillus japonicus* (31.6% of β-xylosidase activity) [22].

The deduced Michaelis-Menten kinetic constants for K_m and V_{max} against xylobiose for the enriched *A. pullulans* CBS 135684 β-xylosidase were 4.60 ± 0.11 mM and 95.5 ± 0.96 μmol xylose min^{-1} mg^{-1} protein, respectively. Thus, the enzyme bound natural xylobiose more tightly but hydrolyzed it less efficiently than the synthetic pNPX (5.57 ± 0.27 mM and 136.99 ± 4.81 μmol pNP min^{-1} mg^{-1} proteins, respectively). The V_{max} value against pNPX of *A. pullulans* CBS 135684 β-xylosidase was much lower than that reported previously for another *A. pullulans* strain, but higher than those of several filamentous fungi (Table 2). The K_m value of the enzyme increased almost 1.8-fold as the chain length of the substrate increased from X_2 to X_4, suggesting a reduced binding affinity and so a decreased catalytic rate with longer chain substrates. Xylose was detected from the TLC analysis as an initial product of the enzymatic hydrolysis in all the XOs in this study (Supplementary data 1), which indicates that the enriched β-xylosidase from *A. pullulans* CBS 135684 is an exo-cutting enzyme.

Monosaccharide Inhibition of β-Xylosidase

Xylose as the final product is a well-known inhibitor of many microbial β-xylosidases [10]. The presence of the monosaccharides used in this study including glucose, arabinose, mannose, and xylitol at different concentrations from 1 to 70 mM did not significantly inhibit the β-xylosidase activity (Supplementary data 2). In contrast, the enzyme activity gradually decreased with increasing xylose concentrations and was completely inhibited at 55 mM xylose. The plot of the reciprocal of the reaction rate ($1/V$) against the xylose concentration was used to estimate the inhibition constant (K_i) between xylose and the enzyme (Fig. 3a). The calculated value of K_i from this Dixon plot was 18.20 ± 0.16 mM, which was 1.8- to 9.1-fold higher than those previously reported for other β-

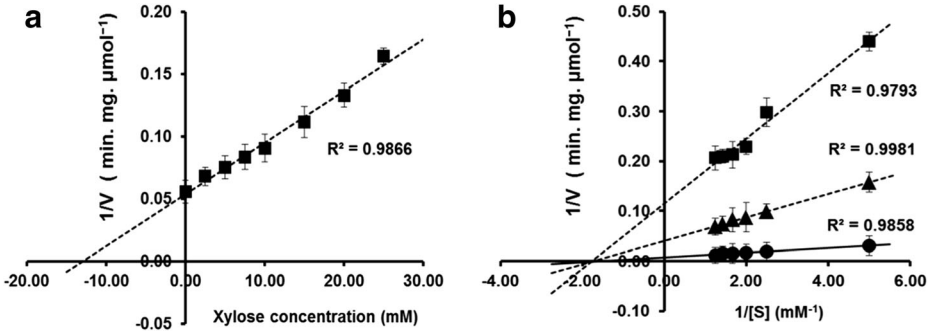

Fig. 3 Effect of the xylose concentration on the activity of the enriched β-xylosidase from *A. pullulans* CBS 135684, as shown by **a** Dixon and **b** Lineweaver-Burk plots. **a** The Dixon plot was constructed by plotting xylose concentrations (0–25 mM) against the reciprocal of the initial rate when reactions were conducted for 10 min with 5 mM p-NPX in 50 mM of sodium acetate buffer (pH 6.0). **b** The Lineweaver-Burk plot was constructed by plotting the reciprocal of the xylose concentrations at 10 mM (▲), 20 mM (■), and 0 mM (●) against the initial rate when reactions were conducted for 10 mins with 0–0.8 mM p-NPX in 50 mM of sodium acetate buffer (pH 6.0). Data are shown as the mean ± SD (error bars), derived from triplicate experiments

xylosidases of 2–10 mM [10, 22]. Therefore, with a lower sensitivity to xylose inhibition, this enzyme has a great potential in the conversion of hemicellulose in many fields. At 5 and 10 mM, xylose altered the V_{max} (24.39 ± 0.85 and 8.70 ± 0.76 μmol min^{-1} mg^{-1}, respectively) but did not significantly modify the K_m (5.70 ± 0.35 and 5.82 ± 0.10 mM, respectively) when PNX was used as the substrate (Fig. 3b). This suggests non-competitive inhibition, where xylose might bind with the enzyme at sites other than the active site used in catalysis and interferes with the catalytic hydrolysis without affecting the substrate binding [36].

Synergy of Xylanase and β-Xylosidase on Xylan Hydrolysis

The xylan used in this study was extracted from black liquor effluent released during the kraft pulping process of *Eucalyptus grandis* wood from a commercial company in Thailand. Hemicelluloses in wood chips are partially depolymerized during chemical pulping resulting in dissolved short-chain xylans in the effluent [37]. Therefore, black liquor xylan is an attractive substrate for xylose and XOs production compared with commercial long-chained xylans due to its low cost, suitable structure for degradation, and environmental advantage of waste treatment. In preliminary experiments, the effect of the crude β-xylosidase preparation from *A. pullulans* CBS 135684 (Production of β-Xylosidase) on xylan hydrolysis was investigated using 10% (*w*/*v*) black liquor xylan, due to its similar properties to those of the purified enzyme (Supplementary data 3). Since the β-xylosidase preferably hydrolyzed $X_{2–4}$, the crude xylanase from *A. pullulans* CBS 135684 was used in combination with the crude β-xylosidase to hydrolyze oligoxylans longer than X_4 and enhance the xylose yield. At 5 U g^{-1} xylanase, the amount of reducing sugar released from the reactions with and without 5 U g^{-1} β-xylosidase was 0.22 ± 0.03 and 0.36 ± 0.02 g g^{-1} DW of xylan, respectively. Thus, a 1.6-fold increase in xylan production yield was obtained by the co-digestion compared to that with the xylanase alone. In an attempt to optimize the condition for xylan hydrolysis, the effect of the xylanase and β-xylosidase concentrations on the xylose yield at different incubation times were investigated using a sequential univariate approach to select the operational ranges of variables (data not shown). Subsequently, a RSM using a Box-Behnken design was applied within these ranges of parameters to obtain the maximum response. The maximum amount of xylose produced, 0.812 ± 0.015 g g^{-1} DW of xylan (trial number 14 in Table 6), was very close

Table 6 Box-Behnken design matrix of black liquor xylan hydrolysis by xylanase and β-xylosidase from *A. pullulans* CBS 135684

Trial	Variable factor			Xylose yield (mg g^{-1} DW xylan)*	Predicted value (m g^{-1})
	Xylanase (U g^{-1})	β-Xylosidase (U g^{-1})	Time (h)		
1	10 (− 1)	3 (− 1)	4 (0)	0.57 ± 0.03	0.55 ± 0.01
2	22 (+ 1)	3 (− 1)	4 (0)	0.57 ± 0.02	0.56 ± 0.01
3	10 (− 1)	9 (+ 1)	4 (0)	0.56 ± 0.03	0.55 ± 0.01
4	22 (+ 1)	9 (+ 1)	4 (0)	0.58 ± 0.01	0.58 ± 0.01
5	10 (− 1)	6 (0)	2 (− 1)	0.54 ± 0.01	0.54 ± 0.01
6	22 (+ 1)	6 (0)	2 (− 1)	0.56 ± 0.03	0.56 ± 0.01
7	10 (− 1)	6 (0)	6 (+ 1)	0.69 ± 0.02	0.69 ± 0.01
8	22 (+ 1)	6 (0)	6 (+ 1)	0.70 ± 0.01	0.70 ± 0.01
9	16 (0)	3 (− 1)	2 (− 1)	0.49 ± 0.02	0.47 ± 0.01
10	16 (0)	9 (+ 1)	2 (− 1)	0.43 ± 0.01	0.41 ± 0.01
11	16 (0)	3 (− 1)	6 (+ 1)	0.57 ± 0.02	0.55 ± 0.01
12	16 (0)	9 (+ 1)	6 (+ 1)	0.64 ± 0.03	0.63 ± 0.01
13	16 (0)	6 (0)	4 (0)	0.80 ± 0.02	0.81 ± 0.01
14	16 (0)	6 (0)	4 (0)	0.81 ± 0.02	0.81 ± 0.01
15	16 (0)	6 (0)	4 (0)	0.81 ± 0.02	0.81 ± 0.01

Numbers in parenthesis (− 1, 0, or + 1) are the coded value (low, normal or high). The second-order regression equation, providing the xylose yield as a function of the variables. This model was significant, based on the *F* value (328.63) and the model terms value of Prob > *F* (0.001)

* Data are shown as the mean ± SD (error bars), derived from triplicate experiments

to the predicted value of 0.808 ± 0.009 g g^{-1} DW (Table 6). Comparison of the predicted and experimental values revealed a good correspondence between them, with a coefficient of determination (R^2) of 0.95. Three-dimensional response plots and their corresponding contour plots (Fig. 4) were drawn on the basis of the model equation to investigate the interaction among the variables and to determine the optimum concentration of each compound for xylan hydrolysis. Canonical analysis revealed the maximum xylose yield of 0.812 ± 0.015 g g^{-1} DW with 16 U g^{-1} xylanase and 6 U g^{-1} β-xylosidase 6 U g^{-1} after incubation at 70 °C for 4.5 h. The validity of the predicted results by the regression model was confirmed by a repeated experiment under the same (predicted to be optimal) concentrations, where three replications gave an average yield of xylose (0.809 ± 0.018 g g^{-1} DW) that was close to the predicted value (0.808 ± 0.009 g g^{-1} DW). The synergistic effect of selected variables corresponded to a 2.25-fold increase in the production yield compared to the preliminary experiment. In addition, the production yield of xylose in this study was higher than those previous

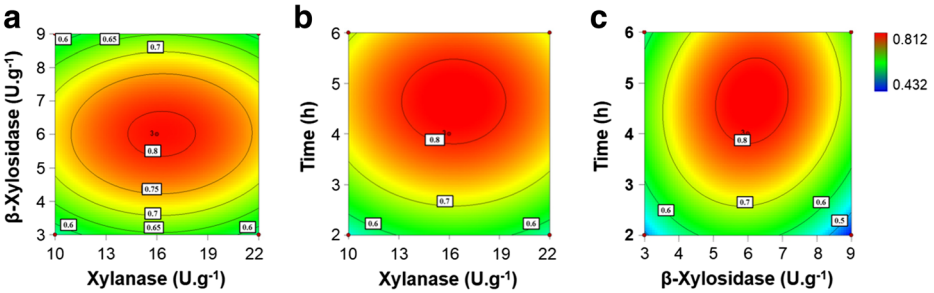

Fig. 4 The response surface and contour plots showing the interactions between the different concentrations of xylanase and β-xylosidase and reaction time on the xylose concentration. The experiment was performed according to the central composite design

reports of xylan hydrolysis by the co-incubation with xylanase and β-xylosidase from *Penicillium janczewskii* (0.22 g g^{-1} after 24 h) [10], and the co-action of the xylanase from *Anoxybacillus flavithermus* and β-xylosidase from *Sulfolobus solfataricus* (0.63 g g^{-1} after 4 h) [38].

Conclusion

The major cell-associated β-xylosidase from the Thai *A. pullulans* strain CBS 135684 was enriched to apparent homogeneity, characterized and found to exhibit several interesting properties, including being active and stable over a high temperature (60–70 °C), catalyzed well at mild alkali pHs and was tolerant to xylose and alcohols. The maximum xylose yield from black liquor xylan hydrolysis with crude xylanase (16 U g^{-1}) and β-xylosidase (6 U g^{-1}) was over 80% of the theoretical yield under a high concentration of xylan (10%, *w*/*v*) at an elevated temperature (70 °C) after incubation for 4.5 h. In conclusion, the β-xylosidase from *A. pullulans* CBS 135684 is a prospective enzyme for a wide range of applications, especially for the bioconversion of hemicellulose.

Acknowledgements This research was performed under the Core-to-Core Program, which was financially supported by the Japan Society for the Promotion of Science (JSPS), National Research Council of Thailand (NRCT), Vietnam Ministry of Science and Technology (MOST), the National University of Laos, Beuth University of Applied Sciences, and Brawijaya University. In addition, financial support from the Rachadapisek Somphot Endowment under Outstanding Research Performance Program and the Rachadapisek Sompote Fund for Postdoctoral Fellowship, Chulalongkorn University, are acknowledged.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Hu, J., Davies, J., Mok, Y. K., Gene, B., Lee, Q. F., Arato, C., & Saddler, J. N. (2016). Enzymatic hydrolysis of industrial derived xylo-oligomers to monomeric sugars for potential chemical/biofuel production. *ACS Sustainable Chemistry & Engineering, 4*, 7130–7136.
2. Quiñones, T. S., Retter, A., Hobbs, P. J., Budde, J., Heiermann, M., Plöchl, M., & Ravella, S. R. (2015). Production of xylooligosaccharides from renewable agricultural lignocellulose biomass. *Biofuels, 6*, 147–155.
3. Lisboa, S. A., Evtuguin, D. V., Neto, C. P., & Goodfellow, B. J. (2005). Isolation and structural characterization of polysaccharides dissolved in Eucalyptus globulus kraft black liquors. *Carbohydrate Polymers, 60*, 77–85.
4. Bankeeree, W., Prasongsuk, S., Imai, T., Lotrakul, P., & Punnapayak, H. (2016). A novel xylan-polyvinyl alcohol hydrogel bead with laccase entrapment for decolorization of reactive black 5. *BioResources, 11*, 6984–7000.
5. Kumar, K. S., Arumugam, N., Permaul, K., & Singh, S. (2016). Thermostable enzymes and their industrial applications. *Microbial Biotechnology: An Interdisciplinary Approach*, Taylor and Francis, CRC Press, Boca Raton, Florida. Chapter 5, pp. 115–162.
6. Shao, W., Xue, Y., Wu, A., Kataeva, I., Pei, J., Wu, H., & Wiegel, J. (2011). Characterization of a novel β-Xylosidase, XylC, from Thermoanaerobacterium saccharolyticum JW/SL-YS485. *Applied and Environmental Microbiology, 77*, 719–726.
7. Leathers, T. D. (1986). Color variants of Aureobasidium pullulans overproduce xylanase with extremely high specific activity. *Applied and Environmental Microbiology, 52*, 1026–1030.

8. Manitchotpisit, P., Leathers, T. D., Peterson, S. W., Kurtzman, C. P., Li, X. L., Eveleigh, D. E., Lotrakul, P., Prasongsuk, S., Dunlap, C. A., Vermillion, K. E., & Punnapayak, H. (2009). Multilocus phylogenetic analyses, pullulan production and xylanase activity of tropical isolates of Aureobasidium pullulans. *Mycological Research, 113*, 1107–1120.
9. Bankeeree, W., Lotrakul, P., Prasongsuk, S., Chaiareekij, S., Eveleigh, D. E., Kim, S. W., & Punnapayak, H. (2014). Effect of polyols on thermostability of xylanase from a tropical isolate of Aureobasidium pullulans and its application in prebleaching of rice straw pulp. *SpringerPlus, 3*, 37–48.
10. Terrasan, C. R. F., Guisan, J. M., & Carmona, E. C. (2016). Xylanase and β-xylosidase from Penicillium janczewskii: purification, characterization and hydrolysis of substrates. *Electronic Journal of Biotechnology, 23*, 54–62.
11. Li, H., Liu, J., Wu, J., Xue, Y., Gan, L., & Long, M. (2014). Comparative analysis of enzymatic hydrolysis of miscanthus xylan using Aspergillus niger, Hypocrea orientalis, and Trichoderma reesei xylan-degrading enzymes. *BioResources, 9*, 2191–2202.
12. Bankeeree, W., Lotrakul, P., Prasongsuk, S., Kim, S. W., & Punnapayak, H. (2016). Enhanced production of cellulase-free thermoactive xylanase using corncob by a black yeast, Aureobasidium pullulans CBS 135684. *Korean Chemical Engineering Research, 54*, 822–829.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.
14. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.
15. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–428.
16. Lemmnitzer, K., Süß, R., & Schiller, J. (2015). *TLC/MALDI MS of carbohydrates planar chromatography—mass spectrometry* (pp. 327–344). Boca Raton, Florida: CRC Press.
17. Box, G. E., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*, 455–475.
18. Iembo, T., Da Silva, R., Pagnocca, F. C., & Gomes, E. (2002). Production, characterization, and properties of β-glucosidase and β-xylosidase from a strain of Aureobasidium sp. *Applied Biochemistry and Microbiology, 38*, 549–552.
19. Ohta, K., Fujimoto, H., Fujii, S., & Wakiyama, M. (2010). Cell-associated β-xylosidase from Aureobasidium pullulans ATCC 20524: Purification, properties, and characterization of the encoding gene. *Journal of Bioscience and Bioengineering, 110*, 152–157.
20. Dobberstein, J., & Emeis, C. C. (1991). Purification and characterization of β-xylosidase from Aureobasidium pullulans. *Applied Microbiology and Biotechnology, 35*, 210–215.
21. Jain, I., Kumar, V., & Satyanarayana, T. (2014). Applicability of recombinant β-xylosidase from the extremely thermophilic bacterium Geobacillus thermodenitrificans in synthesizing alkylxylosides. *Bioresource Technology, 170*, 462–469.
22. Semenova, M. V., Drachevskaya, M. I., Sinitsyna, O. A., Gusakov, A. V., & Sinitsyn, A. P. (2009). Isolation and properties of extracellular β-xylosidases from fungi Aspergillus japonicus and Trichoderma reesei. *Biochemistry (Moscow), 74*, 1002–1008.
23. Díaz-Malváez, F. I., García-Almendárez, B. E., Hernández-Arana, A., Amaro-Reyes, A., & Regalado-González, C. (2013). Isolation and properties of β-xylosidase from Aspergillus niger GS1 using corn pericarp upon solid state fermentation. *Process Biochemistry, 48*, 1018–1024.
24. Kirikyali, N., Wood, J., & Connerton, I. F. (2014). Characterisation of a recombinant β-xylosidase (xylA) from Aspergillus oryzae expressed in Pichia pastoris. *AMB Express, 4*, 68–74.
25. Saha, B. C. (2003). Purification and properties of an extracellular β-xylosidase from a newly isolated Fusarium proliferatum. *Bioresource Technology, 90*, 33–38.
26. Iembo, T., Azevedo, M. O., Bloch Jr., C., & Filho, E. X. F. (2006). Purification and partial characterization of a new β-xylosidase from Humicola grisea var. thermoidea. *World Journal of Microbiology and Biotechnology, 22*, 475–479.
27. Xia, W., Shi, P., Xu, X., Qian, L., Cui, Y., Xia, M., & Yao, B. (2015). High level expression of a novel family 3 neutral β-xylosidase from Humicola insolens Y1 with high tolerance to d-xylose. *PloS One, 10*, e0117578.
28. Knob, A., & Carmona, E. C. (2012). Purification and properties of an acid β-xylosidase from Penicillium sclerotiorum. *Annals of Microbiology, 62*, 501–508.
29. Teng, C., Jia, H., Yan, Q., Zhou, P., & Jiang, Z. (2011). High-level expression of extracellular secretion of a β-xylosidase gene from Paecilomyces thermophila in Escherichia coli. *Bioresource Technology, 102*, 1822–1830.
30. Nieto-Domínguez, M., de Eugenio, L. I., Barriuso, J., Prieto, A., de Toro, B. F., Canales-Mayordomo, Á., & Martínez, M. J. (2015). Characterization of a novel pH-stable GH3 β-xylosidase from Talaromyces

amestolkiae: an enzyme displaying regioselective transxylosylation. *Applied and Environmental Microbiology, 81*, 6380–6392.
31. Lenartovicz, V., de Souza, C. G. M., Moreira, F. G., & Peralta, R. M. (2003). Temperature and carbon source affect the production and secretion of a thermostable β-xylosidase by Aspergillus fumigatus. *Process Biochemistry, 38*, 1775–1780.
32. Yan, Q. J., Wang, L., Jiang, Z. Q., Yang, S. Q., Zhu, H. F., & Li, L. T. (2008). A xylose-tolerant β-xylosidase from Paecilomyces thermophila: characterization and its co-action with the endogenous xylanase. *Bioresource Technology, 99*, 5402–5410.
33. Singh, S. M., Cabello-Villegas, J., Hutchings, R. L., & Mallela, K. M. G. (2010). Role of partial protein unfolding in alcohol-induced protein aggregation. *Proteins: Structure, Function, and Bioinformatics, 78*, 2625–2637.
34. Katapodis, P., Nerinckx, W., Claeyssens, M., & Christakopoulos, P. (2006). Purification and characterization of a thermostable intracellular β-xylosidase from the thermophilic fungus Sporotrichum thermophile. *Process Biochemistry, 41*, 2402–2409.
35. Terrasan, C. R., Temer, B., Sarto, C., Silva, J. F. G., & Carmona, E. C. (2013). Xylanase and β-xylosidase from Penicillium janczewskii: production, physico-chemical properties, and application of the crude extract to pulp biobleaching. *BioResources, 8*, 1292–1305.
36. Jordan, D. B., & Braker, J. D. (2007). Inhibition of the two-subsite β-d-xylosidase from Selenomonas ruminantium by sugars: competitive, noncompetitive, double binding, and slow binding modes. *Archives of Biochemistry and Biophysics, 465*, 231–246.
37. Jönsson, A.-S., Nordin, A.-K., & Wallberg, O. (2008). Concentration and purification of lignin in hardwood kraft pulping liquor by ultrafiltration and nanofiltration. *Chemical Engineering Research and Design, 86*, 1271–1280.
38. Kambourova, M., Mandeva, R., Fiume, I., Maurelli, L., Rossi, M., & Morana, A. (2007). Hydrolysis of xylan at high temperature by co-action of the xylanase from Anoxybacillus flavithermus BC and the β-xylosidase/α-arabinosidase from Sulfolobus solfataricus Oα. *Journal of Applied Microbiology, 102*, 1586–1593.