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Cloning and characterization of the β-xylosidase from *Dictyoglomus turgidum* for high efficient biotransformation of 10-deacetyl-7-xylosltaxol

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ABSTRACT

With the aim of finding an extracellular biocatalyst that can efficiently remove the C-7 xylose group from 10-deacetyl-7-xylosltaxol, a *Dictyoglomus turgidum* β-xylosidase was cloned and expressed in *Escherichia coli* BL21 (DE3). The molecular mass of purified Dt-Xyl3 was approximately 84 kDa. The recombinant Dt-Xyl3 was most active at pH 5.0 and 75 °C, retaining 88% activity at 65 °C for 1 h, and displaying excellent stability over pH 4.0–7.5 for 24 h. In terms of kinetic parameters, the K_m and V_{max} values for *p*NPX were 0.8316 mM and 5.0178 μmol/mL·min, respectively. Moreover, Dt-Xyl3 was activated by Mn^{2+} and Ba^{2+} and inhibited by Cu^{2+}, Ni^{+} and Al^{3+}. In particular, it displayed high tolerance to salts with 60.8% activity in 20% (*w/v*) NaCl. Ethanol and methanol at 5–15% showed little effect on the enzymatic activity. Dt-Xyl3 demonstrated multifunctional activities followed by *p*NPX, *p*NPAraf and *p*NPG and had a high selectivity for cleaving the outer xylose moieties of 10-deacetyl-7-xylosltaxol with K_{cat}/K_m 110.87 s^{-1}/mM, which produced 10-deacetyl-taxol to semi-synthesize paclitaxel. Under the optimized conditions (60 °C, pH 4.5, enzyme dosage of 0.5 U/mL), 1 g of 10-deacetyl-7-xylosltaxol was transformed to its corresponding aglycone 10-deacetyl-taxol within 30 min, with a molar conversion of 98%. This is the first report that *Dictyoglomus turgidum* can produce extracellular GH3 β-xylosidase with highly specific activity for 10-deacetyl-7-xylosltaxol biotransformation, thus leading to the application of β-xylosidase Dt-Xyl3 as a biocatalyst in biopharmaceutics.

1. Introduction

β-D-Xylosidase is one of the key enzymes for xylan hydrolysis [1]. It can hydrolyze lignocellulose and hemicellulose resources (especially xylans, which are complex heteropolysaccharides consisting of a backbone of β-1,4-linked xylose residues and side chains depending on the source) combined with β-D-xylanase to produce xylooligosaccharides or xylose, which are widely used in the biomass energy, food, feed and medicine industries [2–4]. However, the roles of β-xylosidase include but are not limited to the degradation of xylooligosaccharides. In recent years, more attention has been paid by researchers to the use of β-xylosidase as a biocatalyst for the bioconversion of xylose-containing natural active substances from forest sources [5,6]. Compared with traditional chemical catalysts, biocatalysts such as β-xylosidase provide better selectivity, higher yield and mild reaction conditions. Shin et al. [7] characterized a GH39 β-xylosidase from *Thermoanaerobacterium thermosaccharolyticum* and used it to produce ginsenosides Rg1 and Rh1 from notoginsenosides R1 and R2 with a molar conversion yield of 100% in 4 and 18 h, respectively. Zhang et al. [8] cloned, expressed and characterized a thermostable GH3 β-xylosidase from *Thermotoga petrophila* and used it to transform 20(S)-Rg3 from ginsenoside extract in cooperation with a β-glucosidase with a corresponding molar conversion of 95.0%. Moreover, Li et al. [6] characterized a highly xylose-tolerant GH39 β-xylosidase from *Dictyoglomus thermophilum* and used it to completely biotransform astragaloside IV to produce cycloastragenol with stronger anti-aging activity in 3 h. Therefore, it is particularly important to screen, clone and express β-xylosidase with high specificity for the biotransformation of bioactive substances.

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According to different GH families and sources, β-xylosidases display different enzymatic properties [9]. To date, many β-xylosidases have been found in bacteria, archaea, fungi and plant, and most belong to GH families 1, 3, 39, 43 and 120 based on the GH classification system CAZy (www.cazy.org) [10–12]. Generally, β-xylosidases with high temperature resistance, high sugar resistance and greater specificity have better application prospects in industrial processes, since high temperatures not only increase the solubility of transformed substrates but also reduce the risk of contamination, and high sugar tolerance can resist the inhibition of substrate feedback [13,14]. However, β-xylosidases from fungal sources have optimum temperatures between 40 and 60 °C and an optimum pH range of 3–5, which are not suitable for biocatalytic transformation under high temperatures [15,16]. Therefore, more attention has been paid to the discovery of β-xylosidases from thermophilic bacteria in recent years [17,18]. *Dictyoglomus turgidum*, a hypertermophilic bacterium, is regarded as a treasury of thermostable and sugar-tolerant glycosidase genes. To date, several β-glucosidases have been cloned, characterized and purified from *Dictyoglomus turgidum* [19,20]. However, β-xylosidases from *Dictyoglomus turgidum* have rarely been reported. Therefore, the discovery of thermostable β-xylosidases with high substrate specificity from *Dictyoglomus turgidum* has become the focus of our current work, with important research significance and value.

Paclitaxel (Taxol), isolated from the bark of *Taxus brevifolia*, is a kind of diterpenoid that plays an essential role in the treatment of some cancers, such as breast cancer, non-small cell lung cancer, advanced or recurrent cervical cancer, and others [21–23]. Paclitaxel is distributed in many *Taxus* species; however, paclitaxel in yew plants is present in very small quantities, at only 0.01%-0.02% by weight from stem bark [24]. In addition, the chemical synthesis route of paclitaxel is complicated, the reaction conditions are difficult to control and the synthesis yield is very low [25]. All of the above reasons have hindered the extensive application of paclitaxel in the clinic. Fortunately, the contents of other toxoids such as 7-β-xylosyl-10-deacetylataxol (7-XDT) and its analogues are much higher than paclitaxel, up to 0.5% by weight, which is 20–50 times that of paclitaxel [26]. By removing the C-7 xylose, the product 10-deacetylataxol (DT) can be used for the semi-synthesis of paclitaxel [27]. Compared with chemical methods to remove the C-7 xylose from 7 to XDT, biological methods (enzymatic catalysis processes) are more efficient and environmentally friendly. Cheng et al. [28] isolated the glycoside hydrolase *Lxyl-p1-2* from *Lentinula edodes* and used it to convert 7-β-xylosyltaxanes with a bioconversion rate of approximately 80%. Dou et al. [29] reported a novel cellulosome-like multienzyme complex produced by *Cellulosimicrobium cellulans* that undertook efficient glycoside hydrolysis of water-insoluble 7-xylosy-10-deacetylpaclitaxel.

In this study, we cloned, expressed and characterized a novel thermostable GH3 multifunctional enzyme β-xylosidase/glucosidase/arabinosidase from *Dictyoglomus turgidum*. This enzyme displayed highly selective hydrolysis performance in removing the outer C-7 xylose of 7-xylosy-10-deacetylpaclitaxel. These extraordinary properties make Dt-Xyl3 more suitable for preparation of 10-deacetylpaclitaxel and its analogues, which are widely used in the pharmaceutical and commercial industries.

2. Materials and methods

2.1. Bacterial strains and plasmids

Dictyoglomus turgidum DSM 6724 was purchased from Deutsche Sammlung von Mikroorganismen und Zelkulturen (www.dsmz.de). *Escherichia coli* BL21 and Top10F' cells were grown in Luria-Bertani (LB) broth at 37 °C. BL21-pET-20b-Dt-xyl3 was constructed in our lab by transforming the host strain *E. coli* BL21 with the recombinant plasmid pET-20b-Dt-xyl3 (restriction enzyme cutting sites up- and downstream were *NdeI* and *XhoI*, respectively) encoding the Dt-Xly3 sequence.

β-Xylosidases Xln-DT from *Dictyoglomus thermophilum* [6], Dth3 from *Dictyoglomus thermophilum* [30], Tth XyB3 from *Thermotoga Thermarum* [17], Tth Xyl from *Thermoanaerobacterium thermosaccharolyticum* [31] and Tpe Xln3 from *Thermotoga petrophila* [8] were preserved and prepared by the Microbial Technology Research Laboratory, Nanjing Forestry University.

2.2. Media and materials

LB agar plates (1% peptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) containing 100 μg/mL ampicillin were used to cultivate the strain. LB broth was used for seed culture in a shake flask.

The substrates *p*NP-β-D-xylopyranoside (*p*NPX), *p*NP-β-D-glucopyranoside (*p*NPG), *o*NP-β-D-glucopyranoside (*o*NPG), *p*NP-β-D-galactopyranoside (*p*NPGal), *p*NP-α-L-rhamnopranoside (*p*NPR), *p*NP-a-L-arabinofuranoside (*p*NPArf) and *p*NP-a-L-arabinopyranoside (*p*NPArp) were purchased from Sigma-Aldrich (USA). 7-β-Xylosyl-10-deacetylataxol (> 98% Purity, HPLC) and 10-deacetylataxol (> 98% Purity, HPLC) were purchased from Chendu Institute of Biology, CAS (www.cdmust.com).

2.3. Plasmid constructions and DNA manipulation

The β-xylosidase-encoding gene *Dt-xyl3* (Accession No. ACK42995.1) with a size of 2268 bp was amplified from *Dictyoglomus turgidum* DSM 6724 genomic DNA by PCR with the primers *Dt-xyl3-F* (CGCCATATGGAGAAAGAAAAGATTGAGGAG) and *Dt-xyl3-R* (CCGCTCGAGTTTTACTATGAAAAATCCCTT). The restriction enzyme sites are the underlined sequences. The PCR products were digested with *NdeI* and *Xho*I and subcloned into the pET-20b vector, finally yielding the expression plasmid pET-20b-*Dt-xyl3*.

DNA manipulation followed standard operating procedures. A Gel Extraction Kit and a Plasmid Kit (Axygen, USA) were used for purifying the PCR products and plasmids. DNA restriction enzymes (*NdeI* and *Xho*I), T4 DNA ligase and Ex-taq polymerase were purchased from TaKaRa (Dalian, China). DNA transformation was manipulated by steps for heat shock transformation at 42 °C for 2 min.

2.4. Nucleotide sequence and structural analyses of Dt-xyl3

The nucleotide sequences of *Dt-xyl3* and other known β-xylosidase genes were analyzed by the multiple sequence alignment tool Clustal X1.9. Databases were searched by using BLAST (www.blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI and against CAZy.

The protein sequences were submitted to Swiss Model (www.swissmodel.expasy.org) and I-TASSER (www.zhanglab.ccmb.med.umich.edu/I-TASSER) for protein structure homology modeling. Tertiary structures and predicted active catalytic centers of GH3 β-xylosidase Dt-xyl3 were visualized using Swiss-Pdb Viewer and analyzed by PyMOL.

2.5. Expression and enzyme purification

E. coli BL21 (DE3) with the recombinant plasmid pET-20b-*Dt-xyl3* was grown in LB ampicillin medium at 37 °C (180 r/min in an orbital shaker). The cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM with an optical density at OD_{600} of approximately 0.8, and the bacteria were further incubated at 25 °C for approximately 12 h.

The bacteria (200 mL) were harvested by centrifugation at 5, 000 × *g* (4 °C) for 10 min, washed several times with distilled water and resuspended in 20 mL of 1 × binding buffer (5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl buffer, pH 7.9). The soluble proteins containing recombinant Dt-xyl3 were obtained by ultrasonication, heat-treated at 75 °C for 30 min and then cooled in an ice bath and

centrifuged at 8000 × g (4 °C) for 30 min. Finally, the supernatants were purified with a Ni-nitrilotriacetic acid (NTA) affinity column (Novagen, USA), and the enzyme protein was collected by eluting with 1 × binding buffer (pH 7.9). The target protein was examined by 12% SDS-PAGE gel and stained with Coomassie brilliant R-250, and the protein bands were analyzed by density scanning with an image analysis system (Bio-Rad, USA) [32]. The purified protein concentration was measured by a Bradford protein Assay Kit (Sangon Biotech, China) using albumin from bovine serum as a standard.

2.6. β-Xylosidase assay and biochemical characterization

*p*NP method. The reaction mixture contained the following: 10 μL of 20 mM substrate *p*NPX dissolved in sodium phosphate buffer (50 mM, pH 6.0), 180 μL of sodium phosphate buffer (50 mM, pH 6.0) and 10 μL of purified enzyme. The reaction was carried out at 75 °C for 10 min, and the reaction was quenched by adding 600 μL of Na_2CO_3 (1 M) [6]. The enzymatic activity was immediately measured by detecting the liberated *p*NP at 405 nm. For every sample, the activity was measured three separate times. One unit of β-xylosidase activity (1 U) was defined as the amount of enzyme required to liberate 1 μmol of *p*NP per minute under the above assay conditions, which is consistent with the literature reference [17].

The purified Dt-xyl3 was biochemically characterized using *p*NPX as the substrate. The activity of purified Dt-xyl3 was measured at 65–90 °C per 5 °C at pH 5.0 (50 mM citric acid/sodium citrate buffer). To estimate enzyme thermostability, Dt-xyl3 was preincubated without substrate at 65, 75, and 85 °C for 2 h (every 30 min), and then the residual β-xylosidase activity was determined at 75 °C and pH 5.0 (50 mM citric acid/sodium citrate buffer). The activity of the enzyme without preincubation was defined as 100%. The optimum pH was evaluated by incubation at 75 °C for 10 min in citric acid/sodium citrate buffer and sodium phosphate buffer (50 mM) with various pH values (pH 4.0–7.5). To estimate enzyme stability at pH 4.0–7.5 in citric acid/sodium citrate buffer and sodium phosphate buffer, the purified Dt-xyl3 was preincubated in different buffers without substrate at 4 °C overnight. After 24 h, the residual β-xylosidase activity was determined at 75 °C and pH 5.0 (50 mM citric acid/sodium citrate buffer).

Enzymatic activity was detected in the presence of various xylose, glucose and arabinose concentrations (10, 20, 30, 50, 80, 100, 200, 300 and 500 mM) and different concentrations of NaCl (3.0–30.0%, w/v). The effects of adding different ions or chemical reagents on the β-xylosidase activity of purified Dt-xyl3 were evaluated. Fe^{3+}, Ni^{2+}, Na^{+}, Sr^{2+}, Ca^{2+}, Cu^{2+}, Li^{+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, K^{+}, Al^{3+}, NH_4^{+} and the chemical agent EDTA were assayed at final concentrations of 1 mM and 5 mM in the reaction mixture. The activity of purified Dt-xyl3 was measured with the addition of organic solvents (methanol, ethanol or DMSO) at final concentrations of 5%, 10%, 15%, 20%, 25%, 30%, 40% and 50% in the mixture. The purified Dt-xyl3 was preincubated with each reagent for 10 min at 75 °C before adding into *p*NPX. The activity of the enzyme without the metal ions, chemical reagent and organic solvents was defined as 100%.

The substrate specificity of the purified enzyme was tested by using *p*NPX, *p*NPG, *o*NPG, *p*NPGal, *p*NPR, *p*NPArf and *p*NPArp. The kinetic parameters of purified Dt-xyl3 were determined at 75 °C using *p*NPX as the substrate at various final concentrations ranging from 0.2 to 8 mM prepared in pH 5.0 citric acid/sodium citrate buffer under standard reaction conditions [17]. The data were analyzed by nonlinear regression using the Michaelis-Menten equation.

2.7. Enzymatic transformation of 7-β-Xylosyl-10-deacetylataxol

For 7-β-Xylosyl-10-deacetylataxol as the substrate, six purified β-xylosidases (Dt-xyl3, Xln-DT, Dth3, Tth XyB3, Tth Xyl and Tpe Xln3) were compared to hydrolyze 7-XDT with the enzymatic transformation reaction mixture. The volume of the transformation reaction mixture was 200 μL and contained sodium phosphate buffer (50 mM, pH 5.0), 1 g/L 7-XDT (DMSO as a solvent) and 0.5 U/mL (relative to final enzyme dosage) β-xylosidase. The reaction system was incubated at 75 °C for 30 min, then suspended in an ice bath and stopped by adding 400 μL of methanol. After centrifugation at a speed of 8 000 r/min, the methanol extract of this material was assayed by HPLC. The control was created using only substrate without enzyme.

For comparing the conversion efficiency of different β-xylosidases to 7-XDT, the kinetic constants of Dt-xyl3, Xln-DT, Dth3, Tth XyB3, Tth Xyl and Tpe Xln3 were determined with 7-XDT as the substrate at concentrations of 0.1–3 g/L.

To select suitable transformation conditions, the effects of the main factors (temperature, pH, enzyme dosage, 7-β-xylosyl-10-deacetylataxol concentration and enzymatic transformation time) were investigated. The variation ranges were as follows: temperature ranging from 50 to 90 °C, pH value ranging from 4.0 to 7.0, enzyme dosage of 0.1–4 U/mL, 7-β-xylosyl-10-deacetylataxol concentration of 0.5–5 g/L and enzymatic transformation time (5 min, 10 min, 15 min, 20 min, 30 min, 60 min and 120 min). The molar bioconversion rate of 7-XDT to DT, expressed as molar conversion (%), was calculated by the following formula: Molar conversion (%) = $[C_i/M_i]/[C_t/M_t] \times 100$, where C_i and M_i are the initial concentration and molar mass of 7-XDT, respectively, and C_t and M_t are the DT concentration and the molar mass of DT after time t, respectively. The concentrations of 7-XDT and DT were calculated according to standard equations ($y = 8189.3X + 342.63$, $R^2 = 0.9991$ for 7-XDT and $y = 5933.1X + 132.29$, $R^2 = 0.9995$ for DT).

2.8. Assay of 7-XDT and DT by HPLC

7-XDT and DT were analyzed using an Agilent HPLC 1260 system (USA) and a C18 column (4.6 × 250 mm; i.d., 5 μm; S.No. USNH017518, USA) with distilled water (A) and acetonitrile (B) as the mobile phase. A 25-min binary gradient elution was performed. An isocratic elution of 44% solvent B lasted for the initial 10 min; a linear gradient elution of 44–48% solvent B was performed from 10 to 13 min, followed by a linear gradient elution of 48–100% solvent B from 13 to 18 min; and finally, the column was returned to its starting condition in 7 min. The injection volume was 20 μL for each sample, the flow rate was 1 mL/min, and absorbance at 230 nm was monitored.

3. Results and discussion

3.1. Cloning, sequence, and structural analysis of the β-xylosidase gene Dt-xyl3

According to the complete genome sequence analysis of *Dictyoglomus turgidum* DSM 6724, a putative protein with possible β-xylosidase/glucosidase/arabinosidase activity (GenBank accession No. ACK42995.1) was found. The gene fragment of *Dt-xyl3* was obtained with primers *Dt-xyl3*-f and *Dt-xyl3*-r using cDNA as the template. The full-length gene of *Dt-xyl3* was shown to be 2268 bp and encoded 756 amino acids with a predicted MW of 83.9 kDa. To date, more than 130 families have been classified as glycoside hydrolases [9]. Homologous amino acid sequences of the deduced Dt-xyl3 and other foregone β-xylosidase proteins were searched in GenBank. To determine the evolutionary relationships among the β-xylosidases, phylogenetic trees were constructed by using the Neighbor-Joining (NJ) method. The NJ trees showed that there were seven clades, and each clade was composed of a separated monophyletic group (Fig. 1). Among them, Clade I was the β-xylosidases from bacteria, archaea and fungi, which belong to GH3. GH3 β-xylosidase and β-glucosidase enzymes are pivotal for the degradation of hemicellulosic biomass and other natural active substrates, such as the GH3 β-xylosidase/α-arabinosidase from *Thermotoga thermarum* [17], which exhibited high hydrolytic activity on xylooligosaccharides, and the β-glucosidase from *Thermotoga petrophila* [8]

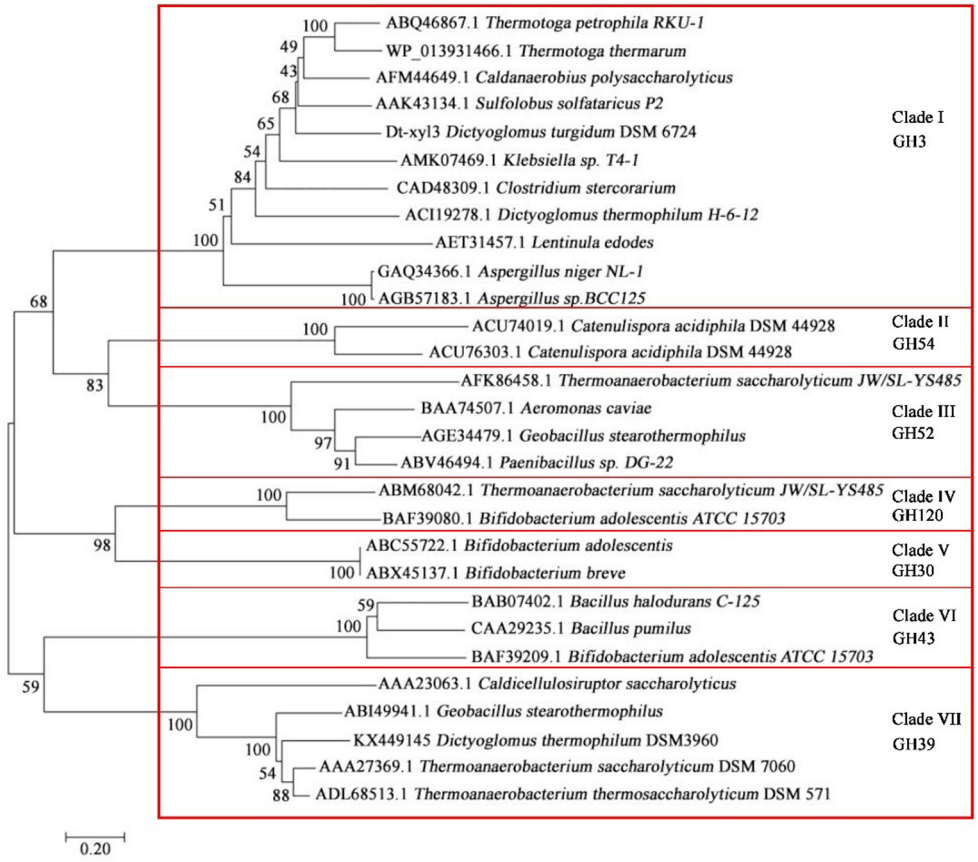

Fig. 1. Neighbor-joining (NJ) tree results from the analysis of the β-xylosidase Dt-xyl3 with 29 amino acid sequences. (Numbers on nodes correspond to percentage bootstrap values for 1000 replicates).

with high ginsenoside Rb1 to ginsenoside 20(S)-Rg3 bioconversion productivity. In the NJ trees, the β-xylosidase Dt-xyl3 had a distant relationship with other β-xylosidases that belonged to the GH3 family. Dt-xyl3 had the highest sequence similarity of 88.58% with the β-glucosidase from *Dictyoglomus thermophilum* (GenBank accession No. WP_012548714.1). Although these enzymes belong to the GH3 family, the sequence similarity differs greatly. The multiple amino acid sequence alignment indicated that Dt-xyl3 had a sequence similarity of 42% with the β-xylosidases from bacteria such as Thermotoga petrophila RKU-1 (GenBank accession No. ABQ46867.1) and *Thermotoga thermarum* (GenBank accession No. WP_01393146.1), 44% with the β-xylosidases from archaea such as *Caldanaerobius polysaccharolyticus* (GenBank accession No. AFM44649.1) and Sulfolobus solfataricus *P*2 (GenBank accession No. AAK43134.1), and 24% with the β-xylosidases from fungi such as *Aspergillus* sp.BCC125 (GenBank accession No. AGB57183.1) and Lentinula edodes (GenBank accession No. AET31457.1). The detailed multiple amino acid sequence alignment of Dt-xyl3 with the previously reported enzymes described above is shown in Fig. S1. Clade II to Clade VII were β-xylosidases belonging to 30, 39, 43, 52, 54 and 120, of which most were from thermophilic bacteria. The members of the thermophilic genus *Dictyoglomus turgidum* had a 59–68% distant relationship with the genera of Clade II, Clade III, Clade VI and VII, which suggested that its enzymatic properties might be distinct. Based on the analysis of Dt-xyl3 with Blast and NJ trees, this protein could be a member of the GH3 family.

Dt-xyl3 of I-TASSER modeling started from the structure templates identified by LOMETS from the PDB library. The homology model of Dt-xyl3 was built using templates of the highest significance in the threading alignments, which were measured by the Z-score. The templates in this section were the 2 best templates (PDB 5YOTA and 5Z87A) selected from the LOMETS threading programs with Z-scores of 4.46 and 8.64; a Z-score > 1 implies good alignment and vice versa. The confidence of each model was quantitatively measured based on the C-score calculated by the significance of threading template alignments and convergence parameters of the structure assembly simulations. The C-score is generally within the [-5–2] range, where a C-score of a higher value indicates a model with a higher confidence and vice versa. In this paper, the C-scores based on PDB 5YOTA and 5Z87A were 0.77 and −0.60, respectively, which indicated that the final model predicted by I-TASSER was realistic. TM-score was estimated by C-score and protein length following the correlation observed between these qualities. Since the 2 proteins (5YOTA and 5Z87A) from the PDB were ranked by cluster size, the TM-scores followed by 5Z87A and 5YOTA were 0.932 and 0.920, respectively. The ligand binding sites according to the template of PDB 3WLKX were 138GLU, 186LEU, 200ARG, 233LYS, 234HIS, 278MET, 281TYR, 313ASP, 314TYR, 346LEU and 517GLU (Fig. 2).

The full sequence of the β-xylosidase gene *Dt-xyl3* was ligated into the expression vector pET-20b at *NdeI* and *XhoI* to generate the plasmid pET-20b-*Dt-xyl3*. Then, a positive *E. coli* colony with recombinant plasmid pET-20b-*Dt-xyl3* was finally introduced to express recombinant Dt-xyl3 with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) for approximately 12 h at 25 °C. Among the expressed proteins, soluble expression accounted for approximately 14.3%, while the remaining 86.7% was inclusion bodies (data were not shown). During 12 h of incubation, cultures grown on IPTG reached a maximum β-xylosidase activity of 2.4 U/mL in LB medium.

3.2. Purification and characterization of recombinant β-xylosidase Dt-xyl3

To study the biochemical properties and characteristics of any enzyme, protein purification is an essential step. The crude β-xylosidase

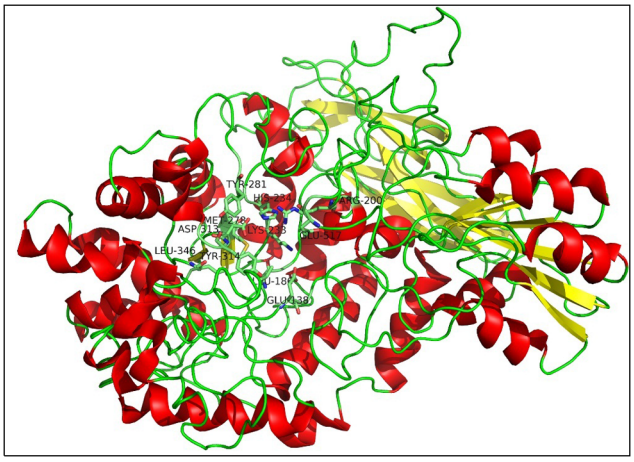

Fig. 2. Tertiary structures of the GH3 β-xylosidase Dt-xyl3 from *Dictyoglomus turgidum* and predicted active center sites. (138GLU, 186LEU, 200ARG, 233LYS, 234HIS, 278MET, 281TYR, 313ASP, 314TYR, 346LEU, 517GLU).

Dt-xyl3 was purified by heat treatment and Ni^{2+}-affinity column chromatography with a final purification of 3.07-fold and a yield of 57.7% (detailed purification results from a 200-mL culture are shown in Table 1). After all purification preparations, the expressed protein was shown as a single band on a 12% SDS-PAGE gel with a molecular mass of more than 75 kDa without other bands (Fig. 3), which corresponded to the predicted molecular mass of the monomer (83,916 Da).

The specific activity of the purified Dt-xyl3 was measured by using substrates at 1.0 mM (*p*NPX, *p*NPG, *o*NPG, *p*NPGal, *p*NPR, *p*NPArf and *p*NPArp) at 75 °C and pH 6.0 (Table 2). The K_m and V_{max} of the enzyme using *p*NPX as the substrate were 0.8316 mM and 5.0178 μmol/mL·min, respectively, followed by *p*NPArf, *p*NPG and *p*NPArp. The purified Dt-xyl3 was not active with *o*NPG, *p*NPGal and *p*NPR. The kinetic parameters were measured from Lineweaver-Burk double reciprocal plots, which suggested that Dt-xyl3 has highly specific activity on residual xylose. The enzyme can hydrolyze xylobiose and cellobiose to produce xylose and glucose (Fig. S2).

The relative enzymatic properties of the purified β-xylosidase Dt-xyl3 were determined by using *p*NPX as the substrate. The optimal temperature for Dt-xyl3 was revealed to be 75 °C (Fig. 4a), which was similar to the GH39 β-xylosidase Xln-DT from *Dictyoglomus thermophilum* but significantly higher than the GH3 β-xylosidase Lxyl-p1-2 from *Lentinula edodes* and the β-xylosidase from *Aspergillus niger* USP-67 [28,33]. In industrial applications, enzyme heat resistance has been a bottleneck for economically viable enzyme use. Thus, enzyme thermostability has become an appreciable property in practical processes. The thermal stability of Dt-xyl3 was evaluated at 65, 75 and 85 °C (Fig. 4b). The Dt-xyl3 was highly stable at 65 °C for 2 h, keeping almost 90% of its initial activity when incubated below 65 °C for 60 min. However, the thermostability of Dt-xyl3 significantly decreased above 75 °C over 30 min and was completely lost at 85 °C after 30 min. The purified β-xylosidase Dt-xyl3 had the highest enzyme activity at pH 5.0 after testing a pH range of 4.0–7.5 (Fig. 4c), which was similar to the GH3 β-xylosidase from *Neurospore crassa* [18] and GH43 β-xylosidase from *Phanerochaete chrysosporium* [34] but significantly lower than the GH43 β-xylosidases from *Thernonyces lanuginosus* and *Humicola insolens* Y1 [35,36]. Moreover, the pH stability of Dt-xyl3 was excellent at 2, 6, 12 and 24 h, respectively. Thus, the enzyme can maintain excellent pH

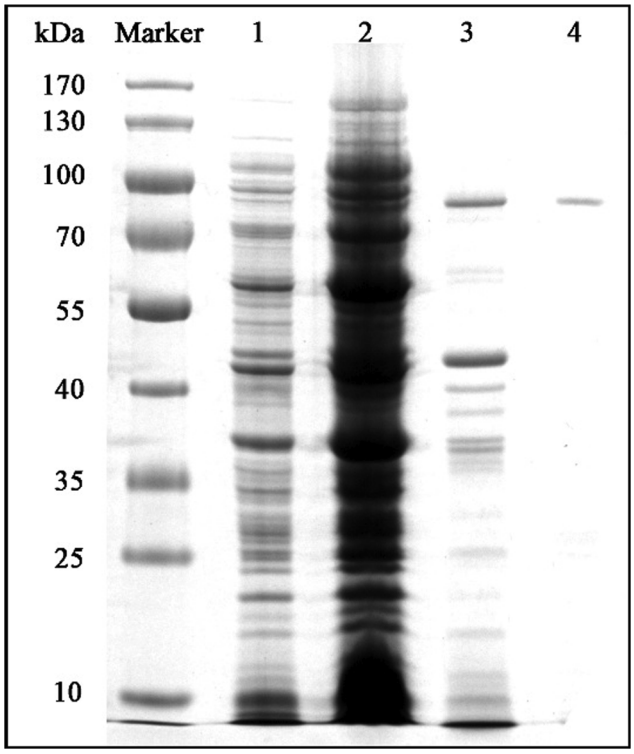

Fig. 3. SDS-PAGE analysis of recombinant β-xylosidase Dt-xyl3 expressed in *E. coli* BL21 (DE3). Lane Marker: protein marker; Lane 1: the crude extract of *E. coli* BL21 (DE3) harboring pET-20b; Lane 2: the crude extracts of β-xylosidase Dt-xyl3; Lane 3: the cell extracts after sonication were heat-treated at 75 °C for 30 min; Lane 4: purified β-xylosidase Dt-xyl3 by Ni-NTA resin affinity chromatography.

Table 2
Kinetic parameters of recombinant Dt-xyl3 towards various chromogenic substrates as measured by *p*NP release at 75 °C.

Substrate [a]	Kinetic parameters	
	K_m (mM)	V_{max} (μmol/mL·min)
p-Nitrophenyl-β-D-xylopyranoside	0.8316	5.0178
p-Nitrophenyl-β-D-galactopyranoside	ND [b]	
p-Nitrophenyl-α-L-arabinofuranoside	2.014	7.542
p-Nitrophenyl-α-L-arabinopyranoside	2.4319	13.595
p-Nitrophenyl-α-L-rhamnopyranoside	ND	
p-Nitrophenyl-β-D-glucopyranoside	2.394	2.8672

[a] Final concentration of each was 1.0 mM.
[b] Not determined, specific activity is not determined by the analytical methods used in this study.

Table 1
Purification of recombinant protein Dt-xyl3.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Culture extract	575.3	260.3	2.21	100	1
Heat treatment [a]	389.6	68.59	5.68	67.7	2.57
Ni affinity chromatography	332.2	48.82	6.79	57.7	3.07

Substrate for Dt-xyl3 was *p*-Nitrophenyl-β-D-xylopyranoside.
[a] The cell extracts after sonication were heat treated at 75 °C for 30 min, and then cooled in an ice bath, centrifuged at 8,000 g for 10 min at 4 °C and the supernatant was kept.

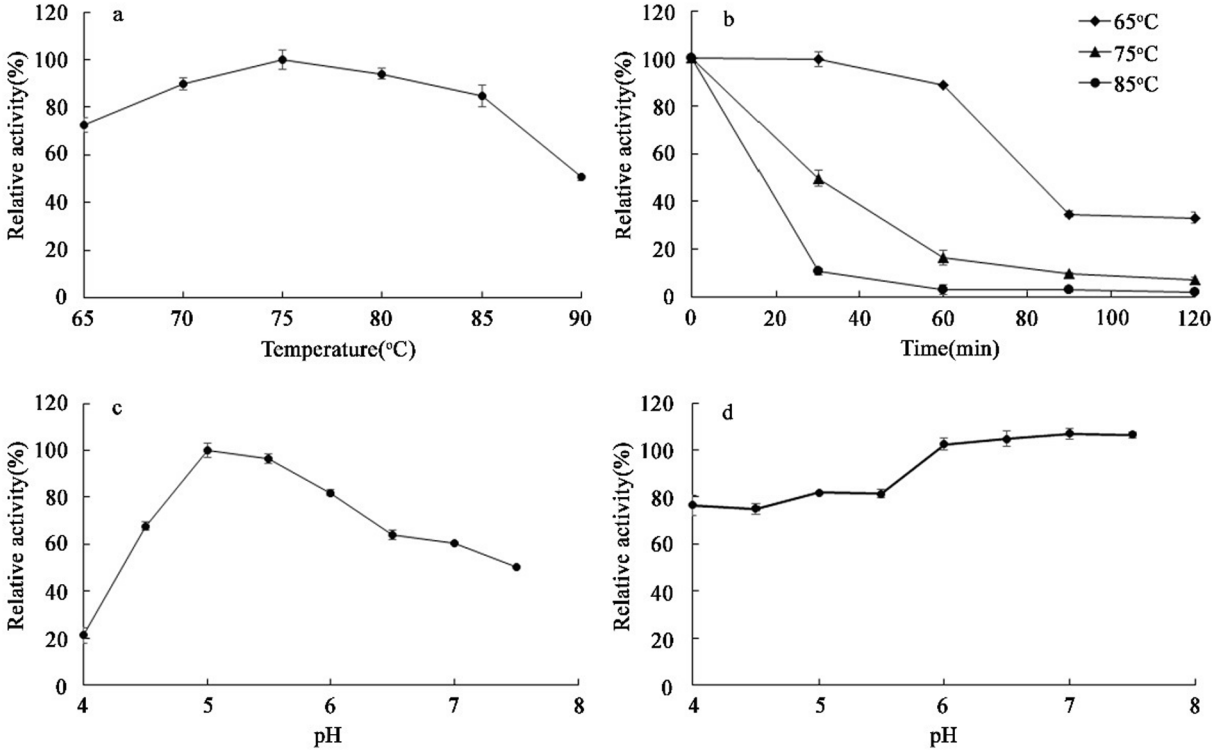

Fig. 4. Characterization of the recombinant β-xylosidase Dt-xyl3. (a) Effect of optimum temperature on Dt-xyl3 activity; (b) The thermostability of the enzyme Dt-xyl3 (the residual activity was monitored while the enzyme was incubated at 65 °C (*filled diamond*), 75 °C (*filled triangle*), and 85 °C (*flled roundness)*; the maximum activity was defined as 100%); (c) Effect of optimum pH on Dt-xyl3 activity; (d) pH stability of the enzyme Dt-xyl3.

stability over 12 h and even over 24 h. The activity of Dt-xyl3 was gradually reduced to 80% at pH 4.0–5.5 after 24 h and was over 100% at pH 6.0–7.5, respectively (Fig. 4d). This remarkable pH stability is highly favorable in many industrial applications.

K_i is the sugar inhibition coefficient, which is defined as the concentration of sugar when the residual enzyme activity is 50%. The higher the value of K_i, the stronger the sugar tolerance of the enzyme [6,17]. Xylose-tolerant β-xylosidase is thought to be useful for biochemical application fields because xylose is a strong inhibitor of β-xylosidase during enzymatic hydrolysis. Unfortunately, the majority of β-xylosidases from fungi are sensitive to xylose, such as those from *Neurospora crassa* [15], *Trichoderma reesei* [37] and *Aspergillus oryzae* [38], which display K_i values for xylose of 1.72, 2.3 and 2.72 mM, respectively. The bacterial β-xylosidase Dt-xyl3 from *Dictyoglomus turgidum* was insensitive to inhibition with K_i values of 20 mM xylose, 50 mM glucose and 500 mM arabinose, which were 7.35–11.63 times higher than those of the above β-xylosidases (Fig. 5a). Agricultural wastes, such as bagasse and corncob, are abundant and cheap lignocellulose materials that are used to solve problems in the sugar industry and biofuel ethanol industry. These agricultural wastes are formed by cellulose, hemicelluloses and lignin, of which approximately 25–35% is hemicelluloses. In these materials, the main hemicellulosic polymers are arabinoxylans, with a high content of xylose and lower content of arabinose. Therefore, β-xylosidase with high xylose, arabinose and glucose tolerance can quickly alleviate the feedback inhibition of sugar to xylooligosaccharides, thus accelerating the hydrolysis of xylooligosaccharides [39]. Previously, many NaCl-tolerant (18–20% NaCl concentrations) *endo*-β-xylanases have been reported with potential applications in soy sauce production, which can cut the total cost and optimize sterilization processes [40]. So far, however, there have been few reports of halophilic β-xylosidases [41,42]. In this study, when the NaCl concentration was under 10% (w/v), the purified Dt-xyl3 was activated by 132.4%-156.3%. In the presence of 15% NaCl, the β-xylosidase activity of Dt-xyl3 decreased by 88.9% (Fig. 5b). With the continuous increase of NaCl concentration, the β-xylosidase activity of Dt-xyl3 was severely inhibited, which was similar to the GH39 β-xylosidase from *Sphingomonas* [41]. The excessive tolerance of β-xylosidase to organic solvents (methanol, ethanol and DMSO) is one of the key factors for the use of β-xylosidase as a natural and efficient catalyst in the production of biomass and the bioconversion of other active substances. Enzymes with high organic solvent tolerance are affected at higher concentrations of organic solvents, which indicates prominent

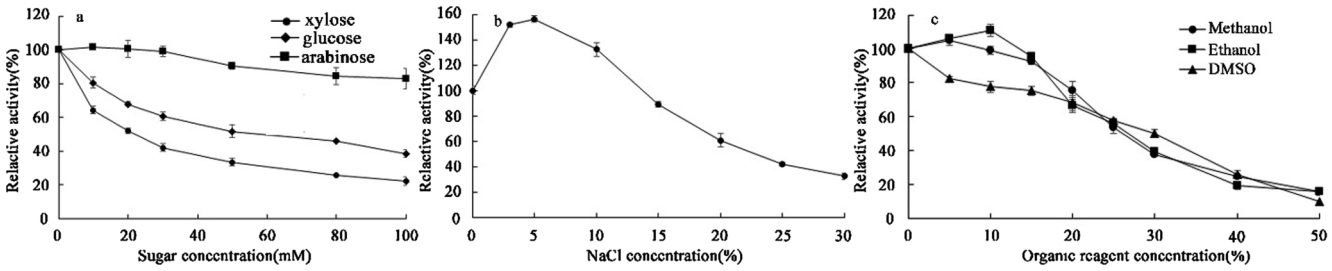

Fig. 5. Activity of purified β-xylosidase Dt-xyl3. (a) Effect of xylose (*flled roundness*), glucose (*filled diamond*) and arabinose (*filled squared*) on the activity of Dt-xyl3; (b) Effect of NaCl on the activity of Dt-xyl3; (c) Effect of methanol (*filled roundness*), ethanol (*filled squared*) and DMSO (*filled triangle*) on the activity of Dt-xyl3.

Table 3
Effects of metal cations and reagents on the recombinant Dt-xyl3 activity.

Cation of reagent	Relative enzyme activity (%)	
	1 mM	5 mM
Contrast	100	100
Fe^{3+}	106.9	90.7
Ni^{+}	90.6	22.7
Na^{+}	112.8	113.4
Sr^{2+}	112.6	116
Ca^{2+}	107.4	97.3
Cu^{2+}	66.3	9.9
Li^{+}	98.2	95.8
Co^{2+}	117.4	74.2
Zn^{2+}	110.3	60.3
Mn^{2+}	159.2	141
Mg^{2+}	109.6	41.3
Ba^{2+}	114.2	103.4
K^{+}	107.8	109.8
EDTA	89.9	71.7
Al^{3+}	35.3	9.5
NH_4^{+}	93.4	94

Values shown were the mean of duplicate experiments, and the variation about the mean was below 5%.

application advantages in the field of non-aqueous phase biocatalysis. For example, saccharification and fermentation need to be synchronized in the preparation of bioethanol, and some organic reagents are often used to aid the dissolution of natural active substances in enzymatic conversion systems. In this study, organic solvents (methanol, ethanol and DMSO) affected the activity of the β-xylosidase Dt-xyl3. The residual enzyme activity was over 100% with low concentrations of methanol and ethanol (10%, w/v), which was similar to the β-xylosidase Xln-DT from *Dictyoglomus thermophilum* and the β-glucosidase from *Thermotoga petrophila* [6,8]. With increasing concentrations of organic solvent, enzyme activity was inhibited slowly (Fig. 5c). When the concentrations of methanol and ethanol were over 30%, the residual enzyme activity decreased to 50%. The inhibitory effects of three organic reagents on β-xylosidase Dt-xyl3 were as follows: DMSO > methanol > ethanol. The results suggested that β-xylosidase Dt-xyl3 could be more suitably used in some industrial applications.

Furthermore, as shown in Table 3, the effects of metal ions and chemical reagents on the β-xylosidase enzyme activities of Dt-xyl3 were determined using *p*NPX as the substrate with final concentrations of 1 mM and 5 mM, and enzyme solution without metal ions and chemical reagents was used as the control. At 1 mM, most metal ions did not inhibit the activity of β-xylosidase Dt-xyl3 except Cu^{2+} and Al^{3+}, which was similar to the β-xylosidases from *Aspergillus niger* ADH-11 [11], *Cellulosimicrobium cellulans* sp.21 [43] and *Dictyoglomus thermophilum* [6]. β-Xylosidase Dt-xyl3 activities were strongly inhibited up to 66.3% and 9.9% in the presence of 1 mM and 5 mM Cu^{2+}, respectively. The cause of this phenomenon may be because Cu^{2+} is known to catalyze the auto-oxidation of cysteine molecules, which leads to the formation of intra- and inter-molecular di-sulfide bonds or sulfenic acid [44]. In addition, β-xylosidase enzyme activities were inhibited to varying degrees at a metal ion concentration of 5 mM and almost completely inhibited by 5 mM Al^{3+}. The addition of Mn^{2+} enhanced the β-xylosidase enzyme activity by 59.2% and 41.0% at 1 mM and 5 mM, respectively, which was similar to the results obtained for α-mannosidase from hen oviduct and β-xylosidase from *Dictyoglomus thermophilum*, possibly because Mn^{2+} may activate and protect the active center of β-xylosidases [6]. At different concentrations of EDTA (1 or 5 mM), there were no significant inhibition effects on enzyme activity, which indicated that the chelating agent EDTA did not notably affect β-xylosidase activity.

3.3. Screening of β-xylosidases for the biotransformation of 7-XDT

Paclitaxel is known as a "blockbuster drug", showing outstanding activity against various cancers including breast, lung, cervical cancers and AIDS-related Kaposi sarcoma [45–47]. However, its content is extremely low when isolated from the bark of the tree yew. A hundred-year-old *Taxus* can provide only a 300-mg dose of paclitaxel. Furthermore, the chemical synthesis of paclitaxel is complicated, and it is difficult to improve the purity due to its numerous byproducts. The content of 7-β-xylosyl-10-deacetylataxol, the by-product simultaneously extracted with paclitaxel, can be as high as 0.5%, which is 25–50 times that of paclitaxel. 7-β-xylosyl-10-deacetylataxol can be hydrolyzed via biological or chemical methods to obtain 10-deacetyltaxol for the semi-synthesis of paclitaxel. Compared with organic reagents such as periodate or other strong acids applied in chemical methods, the bioconversion principle uses enzymes as biocatalysts to remove xylose groups, a process that is therefore considered to be mild and environmentally friendly (the transformation diagram is shown in Fig. S3). Up to now, only a few of the GH3 family β-xylosidases, such as Lxyl-p1-1 and Lxyl-p1-2 from *Lentinula edodes*, have been reported to have the ability to hydrolyze 7-XDT to form DT [28].

It is universally known that the catalytic activities of β-xylosidases from different sources and families vary greatly due to their different structures. For instance, Tth XyB3 from *Thermotoga Thermarum* can cut xylooligosaccharide but not remove the C-6 xylose moiety on notoginsenoside R1 [17], and Tpe Xln3 from *Thermotoga petrophila* can hydrolyze ginsenoside Rb2 and Rc to ginsenoside Rd but not cut the xylose moiety of astragaloside IV at the C-3 position [8]. Therefore, in a study that screened for the best β-xylosidases that can specifically cleave the outer xylose moiety at position C-7 of 7-XDT, six β-xylosidases (Dt-xyl3 from *Dictyoglomus turgidum*, Xln-DT from *Dictyoglomus thermophilum*, Dth3 from *Dictyoglomus thermophilum*, Tth XyB3 from *Thermotoga Thermarum*, Tth Xyl from *Thermoanaerobacterium saccharolyticum* and Tpe Xln3 from *Thermotoga petrophila*, the detailed information is shown in Table 4) were used to biotransform 7-XDT. As shown in Table 4, only 4 GH3 family β-xylosidases could biotranform 7-XDT (the molar conversion rates of 7-XDT hydrolyzed by Dt-xyl3, Dth3, Tth xyB3 and Tpe Xln3 β-xylosidases were 98.0%, 86.1%, 42.3% and 36.8%, respectively, in 30 min), and GH39 β-xylosidase Xln-DT from

Table 4
Kinetic parameters of β-xylosidase activity of Dt-xyl3 and other β-xylosidases.

Name	Source	GH family	Sequence homology (%)	Kinetic parameters				Refs.
				K_m (mM)	V_{max} (μM/min)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1}/mM)	
Dt-xyl3	*Dictyoglomus turgidum*	3	100[a]	0.031	16.667	3.437	110.87	This study
Dth3	*Dictyoglomus thermophilum*	3	29.99	4.021	20.00	0.609	0.151	[30]
Tth XyB3	*Thermotoga Thermarum*	3	41.99	0.334	1.667	0.029	0.087	[17]
Tpe Xln3	*Thermotoga petrophila*	3	9.63	0.521	0.0125	0.002	0.004	[8]
Tth Xyl	*Thermoanaerobacterium saccharolyticum*	120	12.30	ND				This study
Xln-DT	*Dictyoglomus thermophilum*	39	9.13	ND				[6]
Lxyl-p1-1	*Lentinula edodes*	3	21.25	2.9	1.51	0.87	0.30	[28]
Lxyl-p1-2	*Lentinula edodes*	3	21.84	1.79	3.34	1.92	1.07	[28]

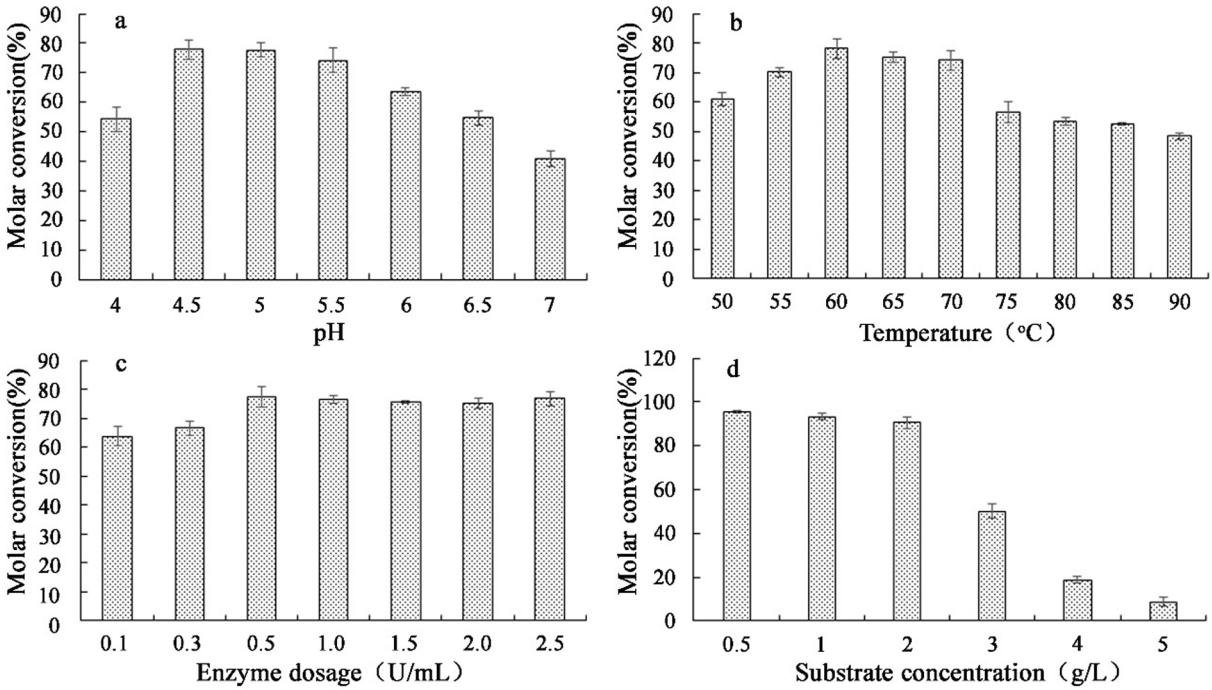

Fig. 6. Optimal conditions for the hydrolysis of the C-7 xylosidic bond of 7-XDT. (a) Effect of pH; (b) Effect of temperature; (c) Effect of enzyme dosage; (d) Effect of 7-XDT concentration.

Dictyoglomus thermophilum and GH120 β-xylosidase Tth Xyl from *Thermoanaerobacterium saccharolyticum* had no enzymatic activity for 7-XDT. The k_{cat}/K_m ratio of Dt-xyl3 for 7-XDT was much higher than those of Dth3, Tth XyB3, Tpe Xln3, Lxyl-p1-1 and Lxyl-p1-2 (110.87 s^{-1}/mM versus 0.151, 0.087, 0.004, 0.30 and 1.07 s^{-1}/mM, respectively), representing the fastest conversion efficiency of a β-xylosidase that can specifically remove xylose at the C-7 position from 7 to XDT. The k_{cat}/K_m ratio, which is called catalytic efficiency, reflects the affinity and catalytic ability of enzymes for substrates at the same time. Hence, the k_{cat}/K_m ratio is often used to compare different enzymes with the same substrate. The larger the k_{cat}, the faster the rate of enzymatic conversion, and the smaller K_m, the greater the affinity between enzyme and the substrate. As shown in Table 4, the k_{cat}/K_m ratio of Dt-xyl3 for 7-XDT was 103 to 370-fold higher than those of Lxyl-p1-1 and Lxyl-p1-2, which indicated that Dt-xyl3 had the strongest catalytic activity for 7-XDT among the reported GH3 β-xylosidases.

3.4. Optimal conditions for the biotransformation of 7-XDT by Dt-xyl3

To determine the optimal conditions for the bioconversion of 7-XDT to DT, single factors (temperature, pH, enzyme dosage and substrate concentration) were initially investigated. Considering the effects on the β-xylosidase Dt-xyl3, 5% DMSO as the cosolvent for the above hydrolysis reaction did not affect enzyme activity. Within a 30-min reaction time and with DMSO as the cosolvent, the optimal temperature and pH for Dt-xyl3 against 7-XDT were 4.5 and 60 °C (Fig. 6a-b). With higher enzyme dosages, higher completion rates for the biotransformation process were achieved. As shown in Fig. 6c, the optimal enzyme dosage of Dt-xyl3 was 0.5 U/mL, and with a continuous increase in enzyme dosage, the bioconversion rate did not increase significantly. In addition, the substrate concentration was a critical factor affecting the hydrolysis efficiency. Different concentrations of 7-XDT (from 0.5 g/L to 5 g/L) were incubated with Dt-xyl3 at a dosage of 0.5 U/mL at 60 °C and pH 4.5 for 30 min (Fig. 6d). For Dt-xyl3, the molar conversion rate was inhibited slightly at the high concentration of 3 g/L 7-XDT with a 45.4% molar conversion rate. When the concentration of 7-XDT was below 3 g/L, the bioconversion rate was approximately 100%. With a concentration of 7-XDT up to 5 g/L, the bioconversion rate dropped below 10%, which showed that a high concentration of substrate could inhibit the activity of β-xylosidase Dt-xyl3. Finally, the time-course biotransformation analysis of the enzymatic reaction by recombinant Dt-xyl3 is shown in Fig. 7a. A total of 1 g/L 7-XDT was transformed to its corresponding aglycone 10-deacetyl-taxol within 30 min, with a molar conversion of 98%, which was faster than the Lxyl-p1-1 and Lxyl-p1-2 (24 h) from *Lentinula edodes* [28]. As shown in Fig. 7b, approximately all 7-XDT was converted into DT without any byproducts that could be identified by HPLC. Thus, the total production cost from 7 to XDT to paclitaxel can be greatly reduced. Taken together, our work provides a rapid and highly efficient biocatalytic pathway to obtain DT for the semi-synthesis of taxol. All of these results suggest that this recombinant Dt-xyl3 has great potential for biotransformation to produce natural medicine.

4. Conclusions

In this paper, a novel thermostable and halophilic multifunctional β-xylosidase/β-glucosidase/α-arabinosidase, Dt-xyl3 from *Dictyoglomus turgidum*, was cloned and expressed in *E. coli* BL21 (DE3) to specifically release xylose from 7 to XDT. Phylogenetic analysis showed that Xln-DT had distant relationships with other β-xylosidases belonging to the GH 3 family. Enzymatic characterization indicated that Xln-DT had a high optimal temperature and optimal pH of 5.0. Most importantly, there was 50% inhibition at 20 mM xylose, 50 mM glucose and 500 mM arabinose. Compared with the other GH3 β-xylosidases, Dt-xyl3 possessed higher efficiency in 7-β-xylosyl-10-deacetylataxol hydrolysis, removing the xylose moiety at the C-7 carbon. This study indicates that recombinant Dt-xyl3 would be suitable for producing natural medicine, such as 7-β-xylosyl-10-deacetylataxol, and greatly improve the utilization of 7-β-xylosyl-10-deacetylataxol for the semi-synthesis of taxol.

Declaration of Competing Interest

The authors declare that they have no competing interests.

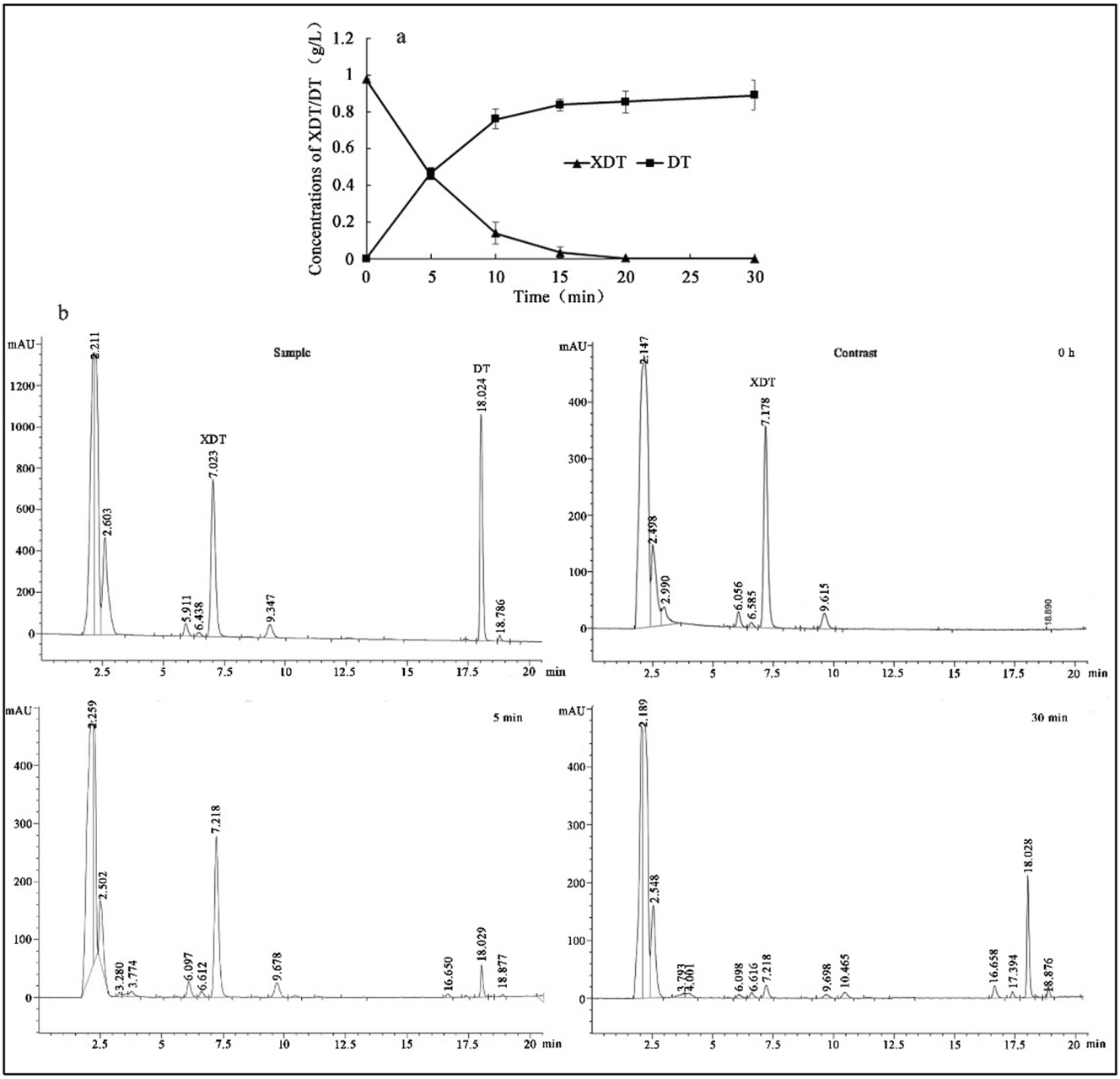

Fig. 7. HPLC analysis of 7-XDT hydrolysis by Dt-xyl3. (a) A time-course of bioconversion of 7-XDT by Dt-xyl3; (b) 7-XDT (1 g/L) incubated with 0.5 U/mL Dt-xyl3 for 0, 10 and 30 min.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103357.

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