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ARTICLE

Biochemical characterization of a noble xylanase from *Paenibacillus* sp. EC116

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Abstract In our present study, a new Endo-1,4-β-xylanase (EC 3.2.1.8) was isolated, which belongs to glycosyl hydrolase family 10 member. The new enzyme designated as *xyn*A was expressed for biochemical characterization. The *xyn*A xylanase was identified in *Paenibacillus* sp. EC116. The novel gene had the size of 2673 base pairs, which correspond to 891 amino acid residues. The expressed enzyme has a size of ~100 kDa. The amino acid sequence was similar to that of endo-1,4-beta-xylanase from *Paenibacillus sp*. FSL R5-192 (ETT36211.1) (96 % identical). The xylanase has an optimal temperature of 40 °C and the pH optimum of 6.0. The specific activity of the xylanase toward birchwood xylan was about 0.69 μmol min^{-1} mg^{-1}, the V_{max} value of 1.639 μmol mg^{-1} min^{-1}, and the K_m value of 35.1 mg ml^{-1}. The EC116 xylanase was relatively stable up to 60 °C. Xylanase enzymes have numerous industrial applications, which need to meet the specific requirements for each application. We hope that our EC116 xylanase is one of the candidates for commercial applications.

Keywords B-glucosidase · Glycosyl hydrolase family 10 · *Paenibacillus sp.* · Saccharification · Xylanase

Introduction

Xylan is a non-crystalline complex polysaccharide with a backbone of -D-1,4-linked xylopyranoside units whose hydroxyl groups are substituted with arabinosyl, acetyl, and glucuronosyl side chains (La Grange et al. 2001). Xylans are the main carbohydrates in the hemicellulosic fraction of vegetable tissues and form an interface between lignin and other polysaccharides such as cellulose (Goulart et al. 2005). Hydrolysis of xylan is one of the essential steps toward the efficient utilization of lignocellulosic materials in nature.

Endo-β-1,4-D-xylanase (EC 3.2.1.8) (i.e., xylanase) randomly catalyzes the hydrolysis of internal β-1,4-D-xylosidic bonds of the xylan or heteroxylan backbone to produce xylooligosaccharides (Berrin and Juge 2008). Based on their remarkable diversity in amino acid sequences and hydrophobic clusters, enzymes of endo-β-1,4-D-xylanase with distinct catalytic domains have been observed in glycoside hydrolase (GH) families 5, 7, 8, 10, 11, and 43 (Collins et al. 2005). More recently, Luo et al. (2010) reported xylanases from GH family 30.

Although xylanases have been biochemically characterized from various microorganisms such as bacteria (Deesukon et al. 2011), fungi (Watanabe et al. 2014), and actinomycetes (Ninawe et al. 2008), the search for new xylanases with different or better biochemical properties for industrial applications still continues. In our current

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study, a xylanase gene from *Paenibacillus* sp. was cloned and expressed, and the product was purified. The biochemical properties, the amino acid sequence, and the secondary structure were studied. The efficiency of the purified xylanase and saccharification with pretreated rice straw was also investigated.

Materials and methods

Isolation and identification of a xylanase-producing microbe

A loam soil was sampled in the ImSil-Gun region of Korea. From soil, *Paenibacillus* sp. EC116 was isolated using xylan agar plates having 2 g L^{-1} CBR-xylan, 10 g L^{-1} Bacto Tryptone, 5 g L^{-1} Bacto-yeast extract, 10 g L^{-1} NaCl, and 15 g L^{-1} Bacto agar at 30 °C for 48 h. Based on the formation of clear zones on the plates, each colony was selected for further study. Using 16S rRNA sequencing study, the xylanase-producing bacterial strain was identified. The dye-tagged xylan substrate, which is xylan-linked with Cibacron brilliant red 3B-A (CBR-xylan), was prepared using a previously described chemical cross-linking method (Fülöp and Ponyi 1997).

Screening of a genomic library of xylanase-encoding genes

From the novel isolate, genomic DNA was purified (Dubnau and Davidoff-Abelson 1971) to be subjected to the partial digestion with *Sau*3AI. DNA fragments (4–5 kb) in size were obtained from 0.8 % agarose gel. After digestion of pHSG298 vector (Takara Bio, Japan) with *Bam*HI, the resulting vector was treated with bacterial alkaline phosphatase to prevent self-ligation. The genomic DNA fragments obtained from above were ligated with the treated vector. A genomic DNA library was made by transforming the recombinant plasmids into *E. coli* DH5α. Bacterial colonies that have xylanase activity were screened on xylan agar plates containing 10 g L^{-1} oat spelt xylan, 5 g L^{-1} Bacto-yeast extract, 10 g L^{-1} Bacto Tryptone, 10 g L^{-1} NaCl, 100 mg L^{-1} Trypan blue, 15 g L^{-1} Bacto agar, and 100 μg mL^{-1} kanamycin. When colonies were surrounded by clear zones, the colonies were isolated and regrown on xylan agar plates. A choice plasmid (pHSG-xyl) was sequenced from positive clones.

Cloning, expression, and purification of xylanase

Using primers, which were designed based on the gene DNA sequence, the whole xylanase gene was amplified by PCR from pHSG-xyl. The forward primer was 5′-TCCATGGCCAAGGCAGTTACGAAAGTG-3′ (the *Nco*I site is underlined) and the reverse primer was 5′-ACAAGCTTATGCCCGAGTTTGCTCTTTC-3′ (the *Hind*III site is underlined). PCR was performed in vial size of 20 μL containing 100 ng of pHSG-eng as the template, 2 μL of 10 × PCR buffer, 0.5 μL of 10 mM dNTP, 1 μL of 10 μM each primer, and 1 U EF *Taq* DNA polymerase (Solgent, Korea). For PCR reactions, an initial denaturation at 96 °C (5 min) was followed by 35 cycles of denaturation at 96 °C (30 s), annealing at 56 °C (30 s), and extension at 72 °C (1 min). PCR products were cloned into pGEM-T easy vector (Promega, USA). The obtained clones were subject to double-digestion with *Nco*I/*Hind*III. The resulting DNA fragments were inserted between *Nco*I and *Hind*III of pET22b(+) vector (EMD Millipore, Germany).

Plasmids that contain the xylanase gene were transformed into *E. coli* BL21 (DE3). The resulting transformed *E. coli* was cultured in Luria–Bertani medium at 37 °C with agitation. The overnight culture was grown further by adding to Luria–Bertani medium (100 mL) having 100 μg mL^{-1} ampicillin and grown at 37 °C overnight with agitation. Expression of the recombinant enzyme was induced for 12 h at 30 °C by adding 0.01 mM isopropyl-β-D-thiogalactopyranoside when the OD_{600} value reached 0.5. Cells were harvested by centrifugation to be suspended in lysis buffer (5 mL; 100 mM sodium phosphate, pH 7.0, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitor cocktail). The harvested cells were sonicated for disruption. After centrifugation (10,000×*g*, 20 min, 4 °C), for removal of cell debris, the crude extract was mixed with 2 mL of Ni-nitrilotriacetic acid agarose (Qiagen, USA) for 1 h at 4 °C. The incubated solution was loaded onto a chromatography column pre-washed with wash buffer (20 mM Tris, 20 mM imidazole, 300 mM NaCl, pH 8.0). Then, the bound protein was treated for elution with the wash buffer having 250 mM imidazole. After concentration of the eluted solution using Amicon ultra centrifugal filter units (EMD Millipore), the concentrate was dialyzed with buffer (100 mM sodium phosphate buffer, pH 7.0, 20 % glycerol). The dialyzed protein solution was analyzed by SDS-polyacrylamide gel electrophoresis on polyacrylamide gel (12 %) and was visualized by Coomassie Brilliant Blue R-250 (CBB R250; Sigma-Aldrich, USA) staining. Bradford reagent (Bradford 1976) was used to quantify protein with the standard of bovine serum albumin.

Enzyme activity, kinetic studies, and protein assays

Using the method of Deesukon et al. (2011), enzyme activity of xylanase was assayed with appropriate modification. The enzyme amount that liberates 1 μmol of reducing sugar equivalent per minute was considered 1 unit

of xylanase activity. The activity of β-glucosidase was determined with the same method used for xylanase activity except that carboxymethyl cellulose (CMC; Sigma-Aldrich) was employed in place of xylan. The effect of different chemicals on the enzyme activity was estimated by inclusion of the chemicals (1 mM) to the reaction solution. The kinetic parameters, K_m and V_{max}, of the xylanase were assessed from a Lineweaver–Burk plot under optimal assay conditions (10 min of incubation, 70 °C, pH 5.5). The substrate concentrations of carboxymethyl cellulose ranged from 1.0 to 10.0 mg mL^{-1}. SDS-polyacrylamide gel electrophoresis was carried out on 12.5 % gel. The isolated proteins were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) for visualization.

The enzyme activities against various substrates, including CMC (Sigma-Aldrich), Avicel (Sigma-Aldrich), birch wood xylan (Sigma-Aldrich), and beech wood xylan (Sigma-Aldrich) were analyzed. The reaction mixtures were composed of 1.0 % substrate in 50 mM sodium phosphate buffer (pH 6.0) and enzyme solution. After incubation at 40 °C for 30 min, dinitrosalicylic acid reagent was added, and the mixture was heated at 100 °C for 5 min. The concentration of reducing sugars was measured at 575 nm (Miller et al. 1960).

Temperature and pH optima of xylanase

For endoglucanase activity, the optimal pH was estimated by incubating the purified enzyme for 30 min at 30 °C in various buffers having different pH ranges. For determination of the optimal temperature for activity, the purified enzyme was incubated in sodium acetate buffer (50 mM, pH 5.5) for 30 min at different temperatures ranging from 10 to 90 °C. To explore the thermostability, the enzyme was incubated at various temperatures in the absence of substrate, and residual endoglucanase activity was determined as mentioned above.

Enzymatic hydrolysis

Rice straw was collected in Jeongeup-si region of Korea. Air dried rice straw without classification was cut to a length of 3–5 cm. Prior to pretreatment, the biomass was ground using a blender, and the particles between the sizes of 50 and 100 mesh were collected. The rice straw was pretreated at 121 °C for 30 min with 2.5 % H_2SO_4, 15 % NH_4OH, 0.5 M Na_2CO_3, or 0.5 M NaOH. The pretreated solid particles were washed using deionized water until the washed water pH reached about 7.0. Then, the sample was dried in a dry oven (50 °C). Enzymatic hydrolysis was carried out in a 150 mL Erlenmeyer flask at 40 °C at 180 rpm. The substrate consistency was adjusted to 3 % (w/v) using 100 mM sodium phosphate buffer (pH 6.0).

Results and discussion

Screening and isolation of microorganisms

Each individual strain was isolated from soil and was confirmed by the observation of a clear zone. The ability to produce reducing sugars from xylan was used to screen the strains. Strains having active xylanase were confirmed by comparing the 16S rRNA sequence data with those in GenBank. The selected strain was positioned in a phylogenetic tree (Fig. 1). Based on the 16S rRNA sequence data, the selected strain had the highest identity of 99 % toward that of *Paenibacillus pabuli* (Gene Bank Accession No. NR_040853.1). The strain was designated as *Paenibacillus* sp. EC116.

Cloning of xylanase gene from *Paenibacillus* sp. EC116

After digestion with *Sau*3A1 of the genomic DNA of strain EC116, DNA fragments were inserted into the pHSG298 plasmid. The resulting mixture was transformed into *E. coli*. The insert DNAs were sequenced with colonies having xylanase activities obtained. The predicted protein sequence was analyzed with BLASTP to identify the *xynA* having open reading frame (ORF) of 2,673 bp. The ORF had a size of 100.5 kDa that consisted of 891 amino acids. The full xylanase gene did not contain a linker sequence but rather contained three carbohydrate-binding module (CBM) sequences (Fig. 2). The SignalP 3.0 program indicated that *xyn*A includes a signal peptide of 29 amino acids and a probable mature xylanase of 861 amino acids, yielding a mature protein of 97.3 kDa. Comparison of the deduced amino acid sequence was performed in the NCBI protein databases. The new *xynA* xylanase belonged to glycosyl hydrolase (GH) family 10. The xylanase had the identity of 96, 38, and 37 % toward endo-1,4-beta-xylanases from *Paenibacillus sp*. FSL R5-192 (ETT362 11.1), *Paenibacillus mucilaginosus* KNP414 (YP_0046 42748.1), and *Thermoanaerobacterium thermosulfurigenes* (AAB08046.1), respectively (Fig. 3). Both the nucleotide and amino acid sequences of the EC116 xylanase were deposited to GenBank (accession number, KT877348).

The genus *Paenibacillus* contains 143 species with published names found in http://www.bacterio.cict.fr/p/paenibacillus.html. The general characteristics of the genus *Paenibacillus* include facultative anaerobic, spore-forming, and rod-shaped bacteria, with *anteiso*-$C_{15:0}$ as the dominant

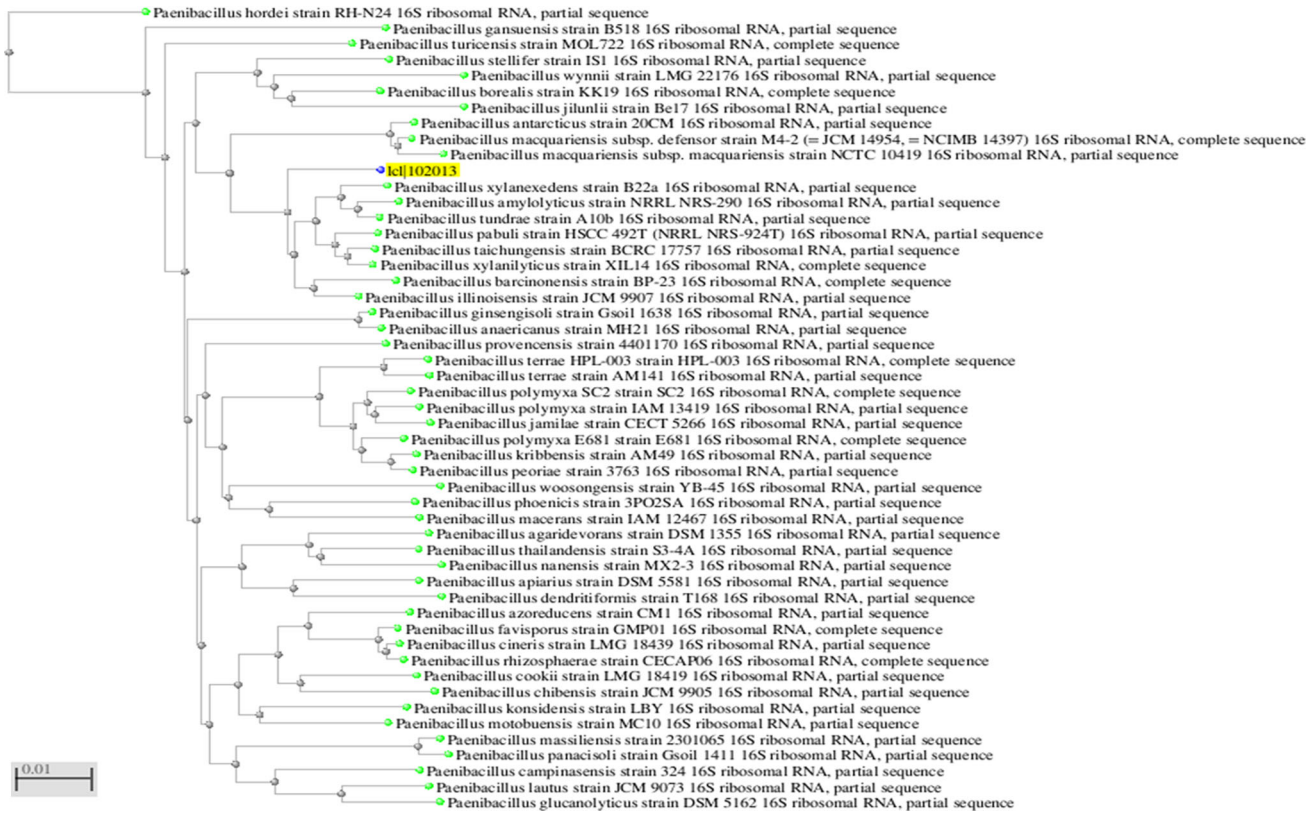

Fig. 1 A neighbor-joining phylogenetic tree based on 16S rRNA sequences showing the position of *Paenibacillus* sp. EC116 among related bacterial taxa

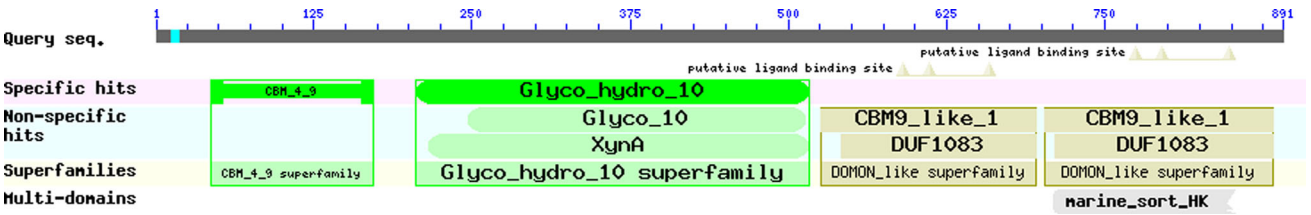

Fig. 2 Conserved domain of xylanase protein from *Paenibacillus* sp. EC116 (NCBI). CBM, carbohydrate-binding domain (44–171 bp); Glyco_hydro_10, glycosyl hydrolase family 10 (205–516 bp); Glyco_10, glycosyl hydrolase family 10 (246–514 bp); XynA, Beta-1,4-xylanase (216–516 bp); CBM9_like_1, DOMOM-like type 9 carbohydrate-binding module of xylanases (526–695 bp); CBM9_-like_1, DOMOM-like type 9 carbohydrate-binding module of xylanases (702–883 bp)

fatty acid, as well as a G+C content of 45–57 mol% (Ash et al. 1993). When compared with their GH family 11 counterparts, GH family 10 xylanases have higher molecular weights (>30 kDa), lower substrate specificities, and acidic isoelectric points (pIs). In addition, the three-dimensional (3D) structures of GH family 10 xylanases are very similar to those of GH family 5 enzymes, all consisting primarily of the $(\beta/\alpha)_8$ barrel fold, which has a shape of salad bowl (Larson et al. 2003).

Expression of the recombinant xylanase and its purification

While excluding the signal sequence, the DNA sequence including the beginning of catalytic domain to stop codon was amplified for the expression of *xyn*A gene from *Paenibacillus* sp. EC116. Thus, *pel*B sequence replaced the natural signal sequence (Lei et al. 1987). The resulting DNA fragment containing the *xynA* gene was inserted into

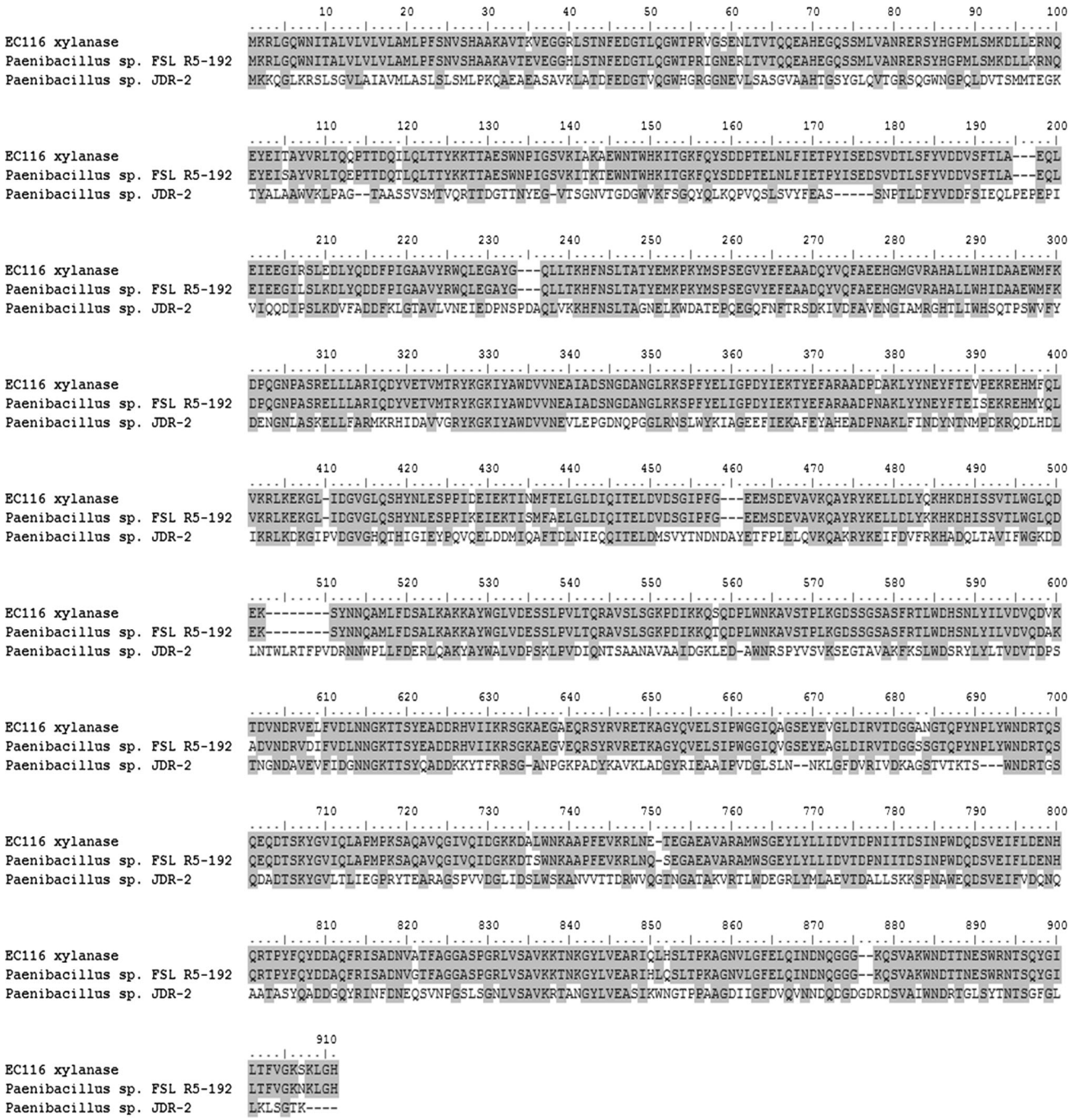

Fig. 3 Comparison of the deduced amino acid sequence of xylanase from *Paenibacillus* sp. EC116 with those of some other xylanases

the pET22b(+) expression vector and transformed into *E. coli* BL21 (DE3) for induction. Although half of the protein was found in inclusion bodies, the expressed protein was still produced in bulk (Fig. 4). As described in Table 1, the recombinant enzyme was purified. The purified protein has a purification of 2.5-fold (18.8 % yield) and specific activity of 0.69 unit/mg. When the purified enzyme was visualized with SDS-polyacrylamide gel electrophoresis and staining, a protein of ~100 kDa was evident (Fig. 4).

Biochemical characterization of recombinant *xyn*A

Enzyme activity of the recombinant xylanase was assessed, while varying the temperature and pH. The purified xylanase has shown to have enzyme activity from 10 to 80 °C.

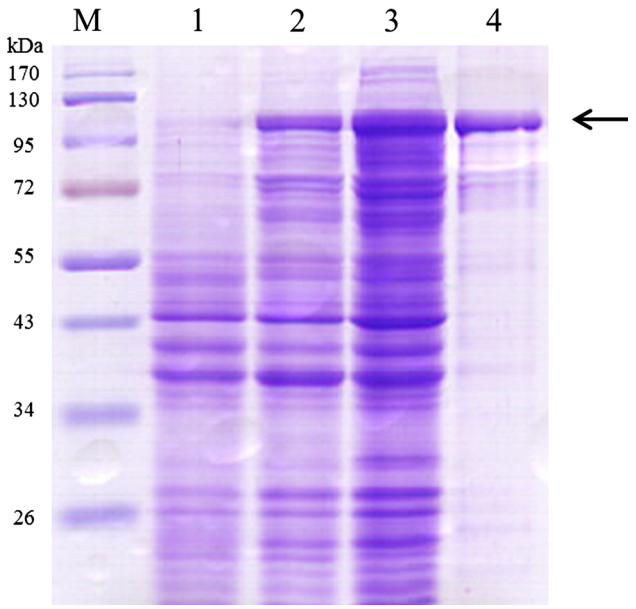

Fig. 4 SDS-polyacrylamide gel electrophoresis analysis of purified xylanase from *Paenibacillus* sp. EC116. Lane M, molecular weight marker; lane 1, whole cells (no induction); lane 2, whole cells (after induction with isopropyl-β-D-thiogalactopyranoside); lane 3, crude extract; and lane 4, purified xylanase. The arrow indicates the protein band corresponding to the xylanase. 12 % acrylamide gel was used for SDS-polyacrylamide gel electrophoresis

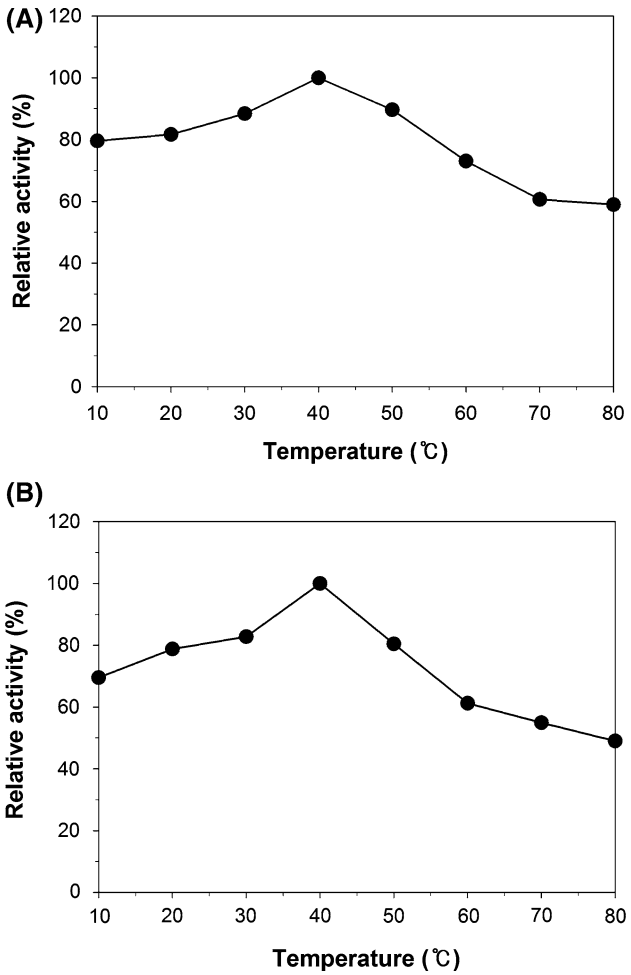

Fig. 5 Optimal temperature for activity of the xylanase from *Paenibacillus* sp. EC116. Enzyme activity was assessed after incubation of reactions (30 min) at various temperatures, with 2 % xylan (**A**) and carboxymethyl cellulose (**B**) as the substrate

Maximal activity was found at 40 °C. The enzyme activity declined above 40 °C; for example, at 80 °C with 60 % relative activity against xylanase activity (Fig. 5A) and 45 % relative activity against β-glucosidase activity (Fig. 5B). Below 40 °C, activity declined gradually, that is, at 10 °C with 80 % relative activity against xylanase activity (Fig. 5A) and 70 % relative activity against β-glucosidase activity (Fig. 5B).

The optimal pH against xylanase activity was 6.0. The activity decreased sharply at pH values lower than pH 4.5 but declined gradually over pH 7.0 (Fig. 6A). Xylanases from various microorganisms often have optimal activity at pH 6.0–7.0 (Gowdhaman et al. 2014). The optimal pH against β-glucosidase activity was 7.0. However, when pH values are <6.0 and >7.5, activity decreased rapidly (Fig. 6B). Many β-glucosidases from various microorganisms often have optimal activity around pH 7.0 (Rajasree et al. 2013).

Table 1 Summary of xylanase purification from *Paenibacillus* sp. EC116

Steps	Total volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
(a)						
Crude extract	9.0	23.022	6.407	0.278	100	1.0
Ni–NTA affinity chromatography	1.5	1.743	1.203	0.690	18.77	2.5
(b)						
Crude extract	9.0	23.022	7.815	0.329	100	1.0
Ni–NTA affinity chromatography	1.5	1.743	0.921	0.529	11.79	1.6

The substrate was (a) xylan and (b) carboxymethyl cellulose

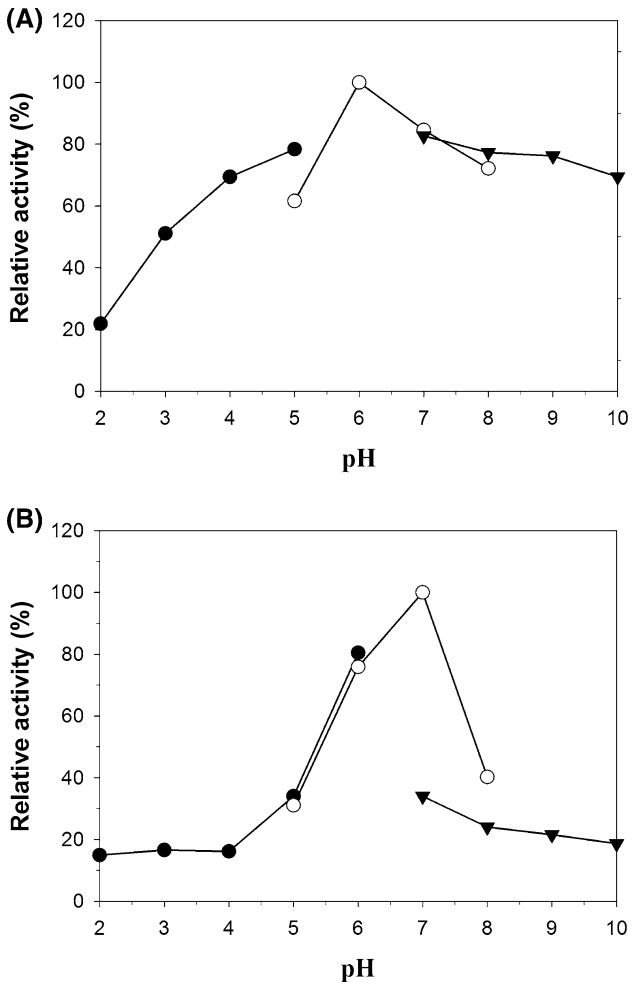

Fig. 6 Optimal pH for activity of the xylanase from *Paenibacillus* sp. EC116. Enzyme assays were performed at various pH with 30 min incubations (30 °C) using 2 % xylan (**A**) and carboxymethyl cellulose (**B**) as the substrate. Buffer systems (all at 50 mM) employed are Tris–HCl buffer (pH 7–10; *filled triangle*), Na phosphate buffer (pH 5–8; *open circle*), and Na citrate buffer (pH 2–6; *filled circle*)

After incubating the recombinant enzyme at varying temperatures, the thermal stability of the purified enzyme was measured (Fig. 7). The xylanase was stable enough to retain 80 % residual activity after incubating at 60 °C for 1 h. By making a Lineweaver–Burk plot, the kinetic parameters (values of V_{max} and K_m) were assessed (Fig. 8). The assay was performed (40 °C, pH 6.0), while employing various concentrations of xylan from 1.0 to 10.0 mg mL^{-1}. The purified xylanase had the values of V_{max} (1.639 μmol mg^{-1} min^{-1}) and K_m (35.10 mg mL^{-1}). The effects of different metal ions toward the endoglucanase activity were tested. No significant influence was observed from Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} ions on enzyme activity, the activity ranging from 80 to 120 %. On the other hand, enzyme activity increased with Co^{2+} and Mn^{2+} by 180 and 175 %, respectively, when compared to the control value (Supplementary

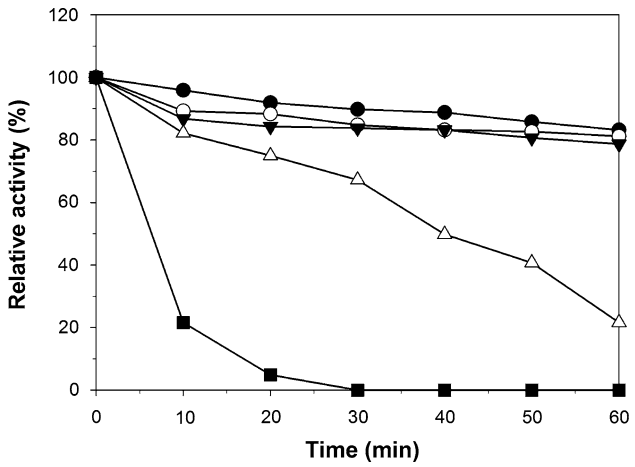

Fig. 7 The thermostability of the xylanase from *Paenibacillus* sp. EC116. Temperature stability was assessed after incubation of the recombinant enzyme at 40 °C (*filled circle*), 50 °C (*open circle*), 60 °C (*filled triangle*), 70 °C (*open triangle*), and 80 °C (*filled square*) for 30 min. The residual activity was assessed by incubating reaction for 30 min at 40 °C in Na phosphate buffer (50 mM, pH 6.0), employing 2 % xylan as the substrate

Table 1). This phenomenon of activity stimulation has also been observed for the xylanase of *Streptomyces coelicolor* Ac-738 (Lisov et al. 2014).

The substrate specificity was compared to the EC116 xylanase. Each carbohydrates of 1 % including CMC), Avicel, beech wood xylan, and birch wood xylan was employed as substrates. The results showed that birch wood xylan has the highest activity (100 %), followed by beech wood xylan (86 %). In addition, the EC116 xylanase exhibits a relatively low activity of 43 % with CMC (Supplementary Table 2).

Saccharification of the pretreated rice straw

The saccharification ability of *Paenibacillus* EC116 xylanase in pretreated rice straw was compared in acid (2.5 % H_2SO_4), alkaline (15 % NH_4OH, 0.5 M Na_2CO_3 or 0.5 M NaOH), and non-pretreated rice straws. *Paenibacillus* EC116 xylanase produced the highest reducing sugar in rice straw pretreated with 0.5 M Na_2CO_3 (Supplementary Fig. 1).

It has been known that the enzyme xylanases have many industrial applications; for example, in the clarification of wine and juice, in the hydrolysis of biomass for biorefinery applications (Collins et al. 2005; Paës et al. 2012), as an animal feed additive for the digestibility improvement (Cowieson et al. 2006), and helping dough rise for breadmaking (Ahmad et al. 2014). It seems like that the application of xylanases as described above may be more economic and more environmental friendly than that of chemical alternatives (Subramaniyan and Prema 2002). However, among the over 250 known xylanases, not many possesses physical and catalytic properties which can suit the specific requirements tailored to any of these

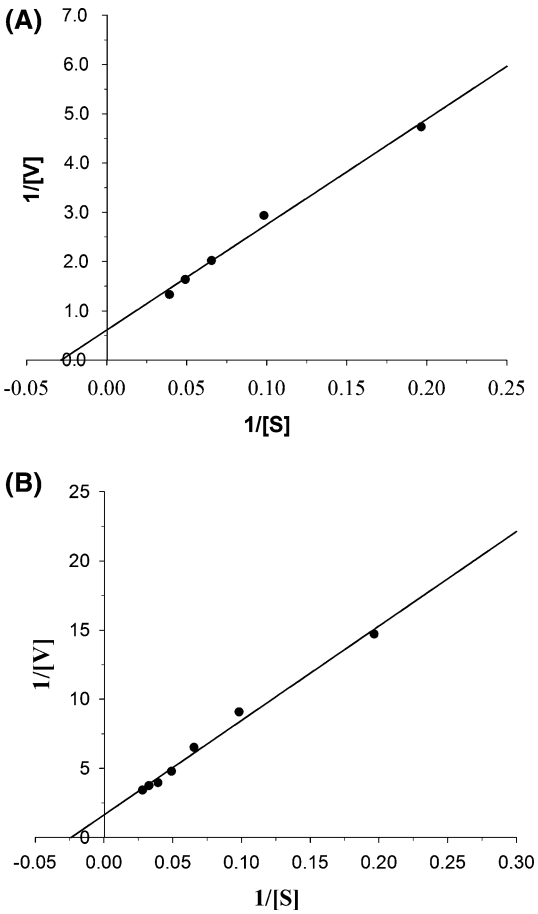

Fig. 8 Lineweaver–Burk plot of xylanase from *Paenibacillus* sp. EC116. The Lineweaver-Burk plot was created from the results of the xylanase assay under optimal assay conditions as described in "Methods and materials" section using xylan (**A**) or carboxymethyl cellulose (**B**) as the substrate

commercial applications (Collins et al. 2005; Paës et al. 2012). Our continued effort to explore the natural diversity of xylanases might provide these industries with candidates for commercial applications.

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