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Original Article

Overexpression and characterization of alkaliphilic *Bacillus firmus* strain K-1 xylanase

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ABSTRACT

The alkaliphilic *Bacillus firmus* strain K-1 produces an alkaliphilic xylanase (Xyn11A) which can be stable across a wide pH range and active at high temperatures. However, the enzyme suffers from low activity when isolated directly from the culture broth using corn husk. A method was developed which employed recombinant DNA technology to produce recombinant Xyn11A (rXyn11A) in an *Escherichia coli* (*E. coli*) expression system. The thioredoxin-fused Xyn11A (Thio-rXyn11A) could be expressed as a soluble form in BL21 (DE3). The expressed protein was tobacco etch virus (TEV) cleavaged to remove the thioredoxin tag and subjected to further purification using Ni^{2+}-NTA affinity chromatography followed by gel-filtration chromatography. Activity of rXyn11A was shown to be under the same conditions as the native enzyme isolated directly from the K-1, having a broad range of active pH (5.0–12.0), with the maximum activity obtained from pH 5.0 at 60 °C. Interestingly, the obtained rXyn11A exhibited a very large increase in specific activity (3034 U/mg), which was 84-times higher than that reported in the native enzyme when observed under the same conditions. Also seen in the xylan hydrozation assay, the rXyn11A hydrolyzed insoluble xylans around 100-times more effectively than the native enzyme. The results from this study demonstrated a successful method for generating the enzyme rXyn11A with much improved activity, making it feasible for industrial applications.

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Introduction

Xylan is a major component of hemicellulose found abundantly in the cell walls of plants (Joseleau et al., 1992). It has a backbone of β-1,4-linked xylose units which are substituted with arabinose and acetate residues (Millward-Sadler et al., 1994; Tomme et al., 1996). Complete degradation of xylan requires the action of several types of enzymes such as endo-β-1,4-xylanase, β-xylosidase, α-arabino-furanosidase, α-glucuronidase and acetyl-xylan esterase (Harris and Ramalingam, 2010). Endo-β-1,4-xylanase (EC 3.2.1.8) has a most important role in the degradation of xylan, as it hydrolyzes β-1,4-glycosidic linkages within the xylan backbone to yield short-chain xylooligosaccharides of different lengths (Biely et al., 1985). Xylan-degrading enzymes are attractive because of their useful applications in various industrial processes (Wong et al., 1988), including improving the digestibility of animal feedstocks (Yin et al., 2001), the modification of cereal-based foodstuffs (O'Shea et al., 2014), the de-lignification of paper pulp (Gübitz et al., 1997) and for use in biorefinery industries (Subramaniyan and Prema, 2002).

Xylan-degrading enzymes have been classified into the glycoside hydrolase families GH5, GH8, GH10, GH11, GH30, and GH43 based on amino acid sequence similarities and hydrophobic cluster analysis (Paës et al., 2012). Of note, only the glycoside hydrolase family11 (GH11) displays exclusively "true endoxylanase" activity through endohydrolysis of (1 → 4)-β-D-xylosidic linkages in xylans and (1 → 3)-β-D-glycosidic linkages in (1 → 3)-β-D-xylans (Carbohydrate-Active Enzymes database; Cantarel et al., 2009). GH11 xylanases are often subdivided into acidic and alkaline xylanases based on their optimal pH (Sapag et al., 2002). Generally, the structure of GH11 members contains two domains: a catalytic (CD) and a carbohydrate-binding one (CBM) (Paës et al., 2012). However, other reports (Cuyvers et al., 2011, 2012) showed GH11 xylanases isolated from *Bacillus subtilis* or *Aspergillus niger* containing only a catalytic domain. Such protein entities display a secondary binding

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site (SBS) that functions similarly to a CBM site, for the binding of insoluble xylans (Vandermarliere et al., 2008).

An alkaliphilic bacterium, *Bacillus firmus* strain K-1, produces two xylanases, Xyn10A and Xyn11A, belonging to families 10 and 11 of the glycosyl hydrolases, respectively, when grown in xylan medium (Chang et al., 2004). Only Xyn11A, a single-domain xylanase, appears to be able to bind and hydrolyze insoluble xylans effectively (Ratanakhanokchai et al., 1999). The Xyn11A from K-1 is an alkaliphilic xylanase which is active in a wide pH range from 5.0 up to 12.0 at an optimal temperature of 60 °C (Ratanakhanokchai et al., 1999), making it attractive for application in the pulp and paper industry as well as for the biotransformation of biomass into biofuel (Viikari et al., 1994; Beg et al., 2001; Sandrim et al., 2005). The current study described the expression, purification and characterization of the recombinant xylanase from *B. firmus* K-1, and the ability to hydrolyze a variety xylan type and agricultural wastes type was determined.

Materials and method

Microorganisms, plasmids and oligonucleotide primers

E. coli XL1blue (Stratagene, La Jolla, CA, USA) was used as a competent cell for cloning. Plasmid pET15b (Novagen, Madison, WI, USA) carrying ampicillin resistance was used for cloning the polymerase chain reaction (PCR) fragments. Oligonucleotide primers were synthesized by First BASE Laboratories Sdn Bhd (Selangor, Malaysia). In this study, NXyn11f and NXyn11r primers which have sequences 5′AAGGATCCAGTGCCAATACCTATTGGCAA3′ and 5′TTCTCGAGTTACCAGACCGTTACATTCGATC3′, respectively, were used.

Construction of the Xyn11A expression vector

The *Xyn11A* gene (GenBank accession no. AF317713) encoding Xy11A from residue 27 to 210 was amplified using PCR. PCR amplification of 30 cycles with the *KOD hot start* DNA polymerase (Novagen; Madison, WI, USA) was carried out in a 50 μL reaction containing 2 mM dNTPs 2 μL, 10× buffers 2 μL, 25 mM $MgSO_4$ 1.5 μL, 10 mM of each primer 1 μL and DNA template 1 μL. Each cycle consisted of heating at 95 °C for 3 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 3 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen; Germantown, MD, USA). The xylanase (*xyn11A*) gene was cloned into *Bam*HI and *Xho*I sites of pET15b vector (Novagen; Madison, WI, USA) which was modified to contain a hexahistidine tag, bacterial thioredoxin and a tobacco etch virus (TEV) cleavage site encoding the sequence at the N-terminus (Suwannaboon et al., 2013). The ligation mixture was transformed into *E. coli* XL1blue. *E. coli* cells were cultivated in Luria-Bertani (LB) medium supplemented with 100 μg ampicillin/mL at 37 °C, overnight. Plasmid DNA was extracted and purified using a QIAprep Spin Miniprep Kit (Qiagen; Germantown, MD, USA). The resulting plasmids were verified using DNA sequencing (First BASE Laboratories Sdn Bhd; Selangor, Malaysia).

Protein purification of rXyn11A and hexahistidine tag removal

The rXyn11A expressing constructs were transformed into *E. coli* BL21 (DE3). The transformants were cultured in LB medium containing ampicillin (100 μg/mL). After growth at 37 °C to mid-log phase (optical density measured at a wavelength of 600 nm (OD_{600}) = 0.6–0.8), the bacterial culture was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 M at 20 °C for 16 h. Cells were harvested using centrifugation at 7000 rpm for 3 min, and resuspended in 20 mL of lysis buffer containing 15 mM imidazole, 300 mM NaCl and 20 mM Tris-base buffer (pH 8.0). Cells were disrupted using sonication. The supernatant was collected using centrifugation at 10,000 × g for 20 min. The rXyn11A was purified using Ni^{2+}-NTA His-Bind affinity chromatography (Qiagen; Germantown, MD, USA) by washing five times with a single column volume of washing buffer that contained 15 mM imidazole, 500 mM NaCl and 20 mM Tris-base buffer (pH 8.0). The fusion proteins were recovered in the elution buffer containing 250 mM imidazole, 150 mM NaCl and 20 mM Tris–base buffer (pH 8.0).

In order to remove the hexahistidine tag and thioredoxin, the fusion protein was transferred to TEV cleavage buffer containing 3 mM reduced glutathione, 0.3 mM oxidized glutathione, 15 mM imidazole, 300 mM NaCl, and 20 mM Tris–base buffer (pH 8.0) using disposable PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) and incubated with TEV protease at a 5:1 mass ratio and incubated at 4 °C overnight (Suwannaboon et al., 2013). The TEV reaction was subjected to Ni^{2+}-NTA His-Bind affinity chromatography (Qiagen; Germantown, MD, USA) followed by gel-filtration chromatography, with 500 μL of approximately 23 mg/mL protein being injected into a Superdex 200 XK-16 column (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) and the cleavage products were collected in the flow-through fraction, eluted at 0.5 mL/min with Tris buffer containing 20 mM Tris buffer (pH 7.0), 50 mM NaCl. Purity of rXyn11A was observed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to LaemmLi (1970) using 12% polyacrylamide running gels with 4% polyacrylamide stacking gels. A sample of the purified rXyn11A was subjected to Western blot analysis with a mouse anti-His antibody (Qiagen; Germantown, MD, USA) and to an enzyme activity assay.

Western blot analysis

The rXyn11A was analyzed using Western blotting with a mouse anti-His antibody (Qiagen; Germantown, MD, USA) diluted 1:3000 in blocking buffer After washing three times with phosphate buffered saline supplemented with Tween 20, signal was detected using horseradish peroxidase (HRP)-conjugated monoclonal anti-mouse antibody (Cell Signaling Technology Inc.; Beverly, MA, USA) with 3,3′, 5,5′-tetramethylbenzidine liquid substrate solution (Sigma–Aldrich Inc.; Beverly, MA, USA) according to the supplier's protocol.

Enzyme activity assays

The xylanase activity was measured by determining the amount of reducing sugar released from birch wood xylan (Sigma–Aldrich Inc.; Beverly, MA, USA). The reaction mixture consisted of 0.5 mL of 1% birch wood xylan in 100 mM Tris-HCl buffer (pH 7.0) and 0.1 mL enzyme (Kyu et al., 1994). After incubation for 10 min at 50 °C, reducing sugar was determined using the Somogyi-Nelson method with xylose as a standard (Nelson, 1994). One unit of the xylanase activity was defined as the amount of enzyme that yielded 1 μmole of reducing sugars in 1 min under the above conditions. The protein concentration was determined using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Characterization of the recombinant Xyn11A

The optimum temperature and pH of the purified recombinant xylanase were determined by assaying the xylanase activity at different temperatures (10–80 °C) and various pHs (pH 3.0–12.0). For the temperature profile, xylanase activity was determined by performing the enzyme assay at temperatures varying from 10 to

80 °C at 100 mM Tris–base buffer (pH 7.0). For the effect of pHs, the buffers used were 100 mM of the following buffer systems: acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0), Tris-HCl buffer (pH 7.0–8.0), Na_2CO_3–$NaHCO_3$ buffer (pH 9.0–10.0) and Na_2HPO_4–NaOH buffer (pH 11.0–12.0). The pH stability pre-incubation of the enzyme was at pH 5.0–8.0 and 60 °C for 8 h. The reaction mixture consisted of 1.0 U of enzyme solution and 0.5 mL of 0.5% birch wood xylan (Sigma–Aldrich Inc.; Beverly, MA, USA) suspension in 100 mM buffer of indicated pH. The reaction was incubated at the indicated temperature for 10 min and the increase in the amount of reducing sugar was determined using the Somogi-Nelson method (Nelson, 1994).

Adsorption experiment

The insoluble xylans and agricultural waste were used for the xylanase adsorption experiment. The reaction mixture contained 1.5 mg of insoluble xylans or 3.5 mg of agricultural waste and various amounts of purified rXyn11A (100–500 μg/mL of total protein) in a final volume of 1.0 mL of 100 mM phosphate buffer (pH 7.0). After the mixture was incubated at 4 °C for 30 min with slow vertical rotation, and following centrifugation, the concentration of the unbound rXyn11A protein ([P], μM) that remained in the supernatant was measured using the Lowry method. The amount of rXyn11A adsorbed to substrate (PC, in micromoles per gram of substrate) was measured by subtracting the rXyn11A in the supernatant from the total protein loaded. Adsorption parameters were estimated from Equation (1) according to Langmuir's adsorption isotherm (Hoshino et al., 1992):

$$\frac{1}{[PC]} = \frac{1}{K_a[PC]_{\max}} \times \frac{1}{P} + \frac{1}{[PC]_{\max}} \quad (1)$$

where $[PC]_{\max}$ is the maximum concentration of adsorption enzyme (micromoles per gram of substrate); K_a is the adsorption equilibrium constant (liters per micro mole).

The measurement of relative binding was performed by adding 250 μg of the purified rXyn11A to 1.5 mg of insoluble xylans or 3.5 mg of agricultural waste. The reaction mixtures had the same conditions used for the adsorption experiment. The ratio (as a percentage) of insoluble substrates-bound rXyn11A to the initial rXyn11A added into the reaction mixture was defined as the relative binding.

Kinetic analysis

Kinetic parameters (K_m and V_{max}) were determined by incubating the purified xylanase (1.0 U) with various concentrations of substrates (0.25–7.0 mg/mL of substrate, insoluble birch wood xylan (BWX), insoluble oat spelt xylan (OSX), corn husk and rice straw) in 25 mM phosphate buffer at pH 7.0 and 50 °C for 5 min. The values of K_m and V_{max} were estimated using the method of Lineweaver and Burk (1934).

Preparation of insoluble xylans and agricultural waste

The preparation of insoluble substrates was performed using the method of Irwin et al. (1994). An amount of 10 g of the commercial xylans such as insoluble BWX (Sigma–Aldrich Inc.) and insoluble OSX (Merck, Darmstadt, Germany), were suspended in 200 mL of distilled water and adjusted to pH 10 using 1 M NaOH. Then, the mixture was stirred for 1 h at room temperature and centrifuged at 10,000 × *g* for 10 min. The pellets were suspended and adjusted to pH 7.0 using 1 M acetic acid. The suspension was centrifuged under the above conditions and the pellets were washed twice with 10 volume of distilled water. Then, the pellets were filtered using Whatman No. 1 paper and freeze dried. Dried agricultural waste such as corn husk, and rice straw were prepared by cutting to a small size, then grinding in a blender and sieving to 80–100 mesh size. After they had been washed several times with warm distilled water to remove sugar residuals, the ground agricultural waste samples were dried at 50 °C and kept for later use.

Hydrolysis of insoluble xylans and agricultural waste

Hydrolysis of insoluble xylans and agricultural waste was performed by adding 1.0 U of the purified rXyn11A to the solutions of 0.5% weight per volume (w/v) insoluble xylans and 1% w/v agricultural residues in 25 mM sodium phosphate buffer (pH 7.0) and left at 50 °C. The hydrolysis products were taken at different time intervals, and the amounts of reducing sugars produced were determined using the Somogyi-Nelson method (Nelson, 1994).

Results and discussion

Expression of rXyn11A in E. coli

The *xyn11A* gene was cloned into an expression vector, pET15b, encoding a hexa-histidine tag and a TEV cleavage site with and without thioredoxin tag fusion at the N-terminal under the control of a strong bacteriophage T7 promoter. Both constructions were transformed into the host cell, *E. coli* BL21 (DE3) and the expression of the rXyn11A proteins was analyzed using SDS-PAGE. As shown in Fig. 1, only the construct with thioredoxin fusion showed signs of overexpressing of the thioredoxin-fused-Xyn11A (Thio-rXyn11A). In addition, Thio-rXyn11A was seen to express in a soluble form as indicated by a thick 30 kDa protein band in the supernatant fraction (Fig. 1A). For the construct without thioredoxin tag, no expression was observed in all the fractions tested (Fig. 1B). According to these results, it could be said that the expression of soluble Xyn11A from *B. firmus* K-1 is made possible by fusing the protein with thioredoxin.

Purification of rXyn11A

The Thio-rXyn11A was expressed in 1 L of *E. coli* stain BL21 (DE3) culture broth. The expression of Thio-rXyn11A was induced with 0.5 mM IPTG and purified by using Ni^{2+}-NTA bead that binds specifically to the hexa-histidine tag for separating the Thio-rXyn11A. The purified protein was eluted with 250 mM imidazole and analyzed using SDS-PAGE. The SDS-PAGE analysis indicated that Thio-rXy11A of good purity was easily obtained after passing through the Ni^{2+}-NTA column as indicated by a thick protein band corresponding to 30 kDa that appeared following SDS-PAGE (Fig. 2A). Western blot analysis confirmed that the 30 kDa protein band was Thio-rXyn11A as it could react with anti-His antibody (Fig. 2B). The thioredoxin tag was removed by incubating the protein with TEV proteinase. The rXyn11A without thioredoxin appeared on SDS-GAGE at the expected size of 19 kDa (Fig. 2C). The rXyn11A was subjected to gel filtration chromatography for further purification and it was seen that the protein produced a symmetric chromatogram, suggesting the conformation of the protein was highly homogeneous. The fraction corresponding to the peak of the chromatogram was analyzed using SDS-PAGE and the 19 kDa protein band of rXyn11A was found (Fig. 2D).

The purified rXy11A was analyzed for its xylanase activity and compared to the crude enzyme, the supernatant obtained after centrifugation of cell lysate, using birch wood xylan as a substrate (Table 1). It was found that the purity of rXyn11A increased around 6-folds compared to the crude. The improved purity resulted in an

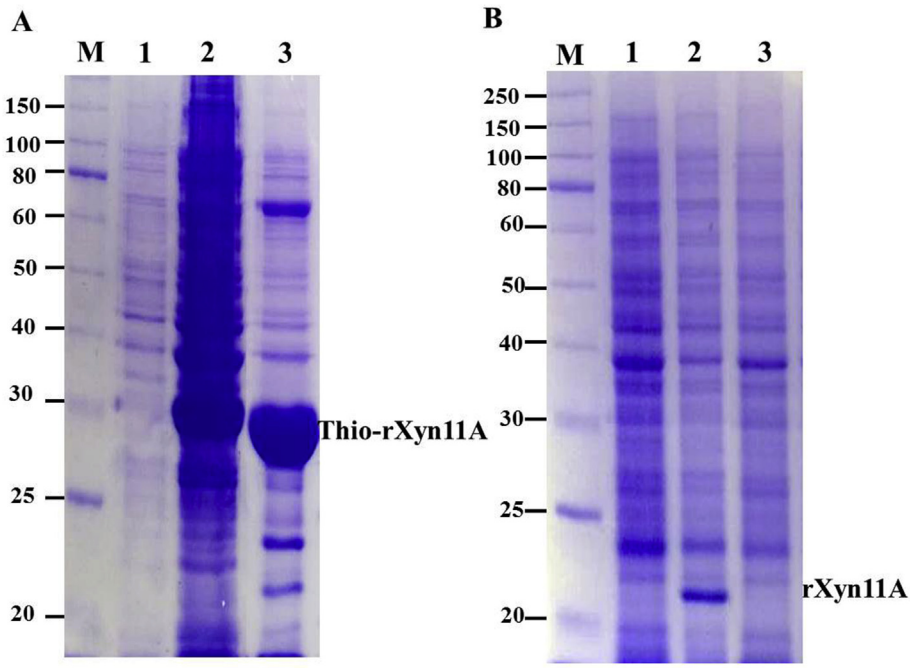

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of rXyn11A expressed from vector peT15b with (A) and without (B) thioredoxin fusion tag in *E. coli* BL21 (DE3) with lanes indicating: M, protein markers; 1, whole cell lysates without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction; 2, insoluble fraction (pellet) after IPTG induction; 3, soluble fraction (supernatant) after IPTG induction.

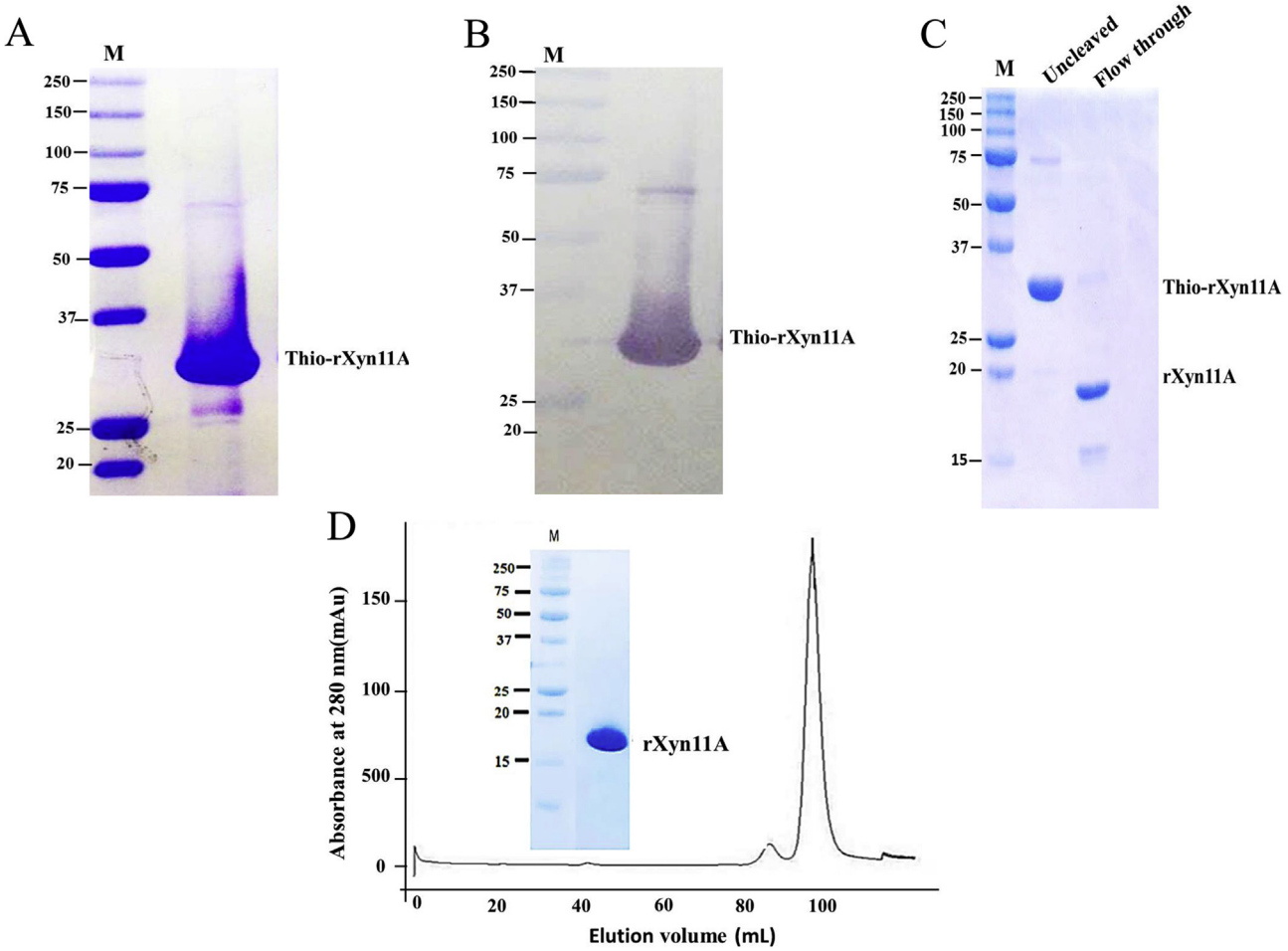

Fig. 2. Purification of rXyn11A from *B. firmus* K-1: (A) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the Thio-rXyn11A eluted from Ni^{2+}-NTA column; (B) Western blot analysis of Thio-rXyn11A obtained from Ni^{2+}-NTA column, where the blot was stained with anti-His antibody; (C) removal of thioredoxin tag using tobacco etch virus (TEV) cleavage, where the cleaved protein appeared on SDS-PAGE at 19-kDa after the thioredoxin tag has been removed; (D) gel filtration chromatography of rXyn11A, where the peak of the chromatogram, corresponding to the fraction eluted at 97 mL, was determined using SDS-PAGE.

Table 1
Summary of purification of rXyn11A from *B. firmus* K-1in *E. coli* BL21 (DE3).

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude enzyme	49.68	24,939	502	100.00	1.0
Purified Xyn11A	5.34	16,195	3034	64.93	6.0

impressive specific activity of rXyn11A in which 3034 U/mg was obtained. In comparison to the native Xyn11A isolated directly from *B. firmus* K-1 using corn husk column reported previously (Jommuengbout et al., 2009), the rXyn11A obtained from the current study showed a large improvement in specific activity which was 84-times higher than that of the native Xyn11A. A possible explanation for such a large difference in the specific activity between natural and recombinant Xyn11A is that whereas the expressed rXyn11A is retained in bacteria, the natural enzyme is secreted by the cells (Ratanakhanokchai et al., 1999; Jommuengbout et al., 2009) which could lead to degradation or denaturation by environmental conditions and consequently, reduce considerably its enzymatic activity as seen in other studies (Ahmed et al., 2009; Mergulhão et al., 2005; Le and Wang, 2014).

This result confirmed that the method used in the current study can produce rXyn11A that exhibits high specific activity. Fusion of Xyn11A with thioredoxin effectively enhanced the expression of Xyn11A in *E. coli* and the thioredoxin tag was easily removed without damaging the activity of the enzyme by incubating with TEV enzyme. The specific activity of 3034 U/mg seen in the rXyn11A in the current study can be considered very high when compared to the activity of the xylanases of the same family reported in other studies where birch wood xylan was used as a substrate. Alkaliphilic *Bacillus* SN5 exhibited a specific activity of 1646.6 ± 31 U/mg toward birch wood xylan (Bai et al., 2015). The specific enzyme activity of Xan11 from *Aspergillus niger* US368 was 415.1 U/mg (Elgharbi et al., 2015) while the activity of Xyl-II from *Aspergillus caespitosus* was 1314 U/mg (Sandrim et al., 2005). XynII from *Trichoderma inhamatum* had the highest specific activity toward birch wood, xylan at 1216.4 U/mg (Silva et al., 2015).

Optimum temperature, pH and stability

The effects of temperature and pH on the ability of rXyn11A to release reducing sugars from birch wood xylan are shown in Fig. 3A and B, respectively. The rXyn11A was active over a broad pH range (pH 3.0–12.0) with the highest activity obtained at pH 5. The rXyn11A was functional at temperatures as high as 70 °C in which more than 80% of relative activity was still retained and the highest activity was obtained at 60 °C. According to these results, the optimum temperature and pH for rXyn11A are similar to those seen in the native Xyn11A from *B. firmus* K-1 (Ratanakhanokchai et al.,

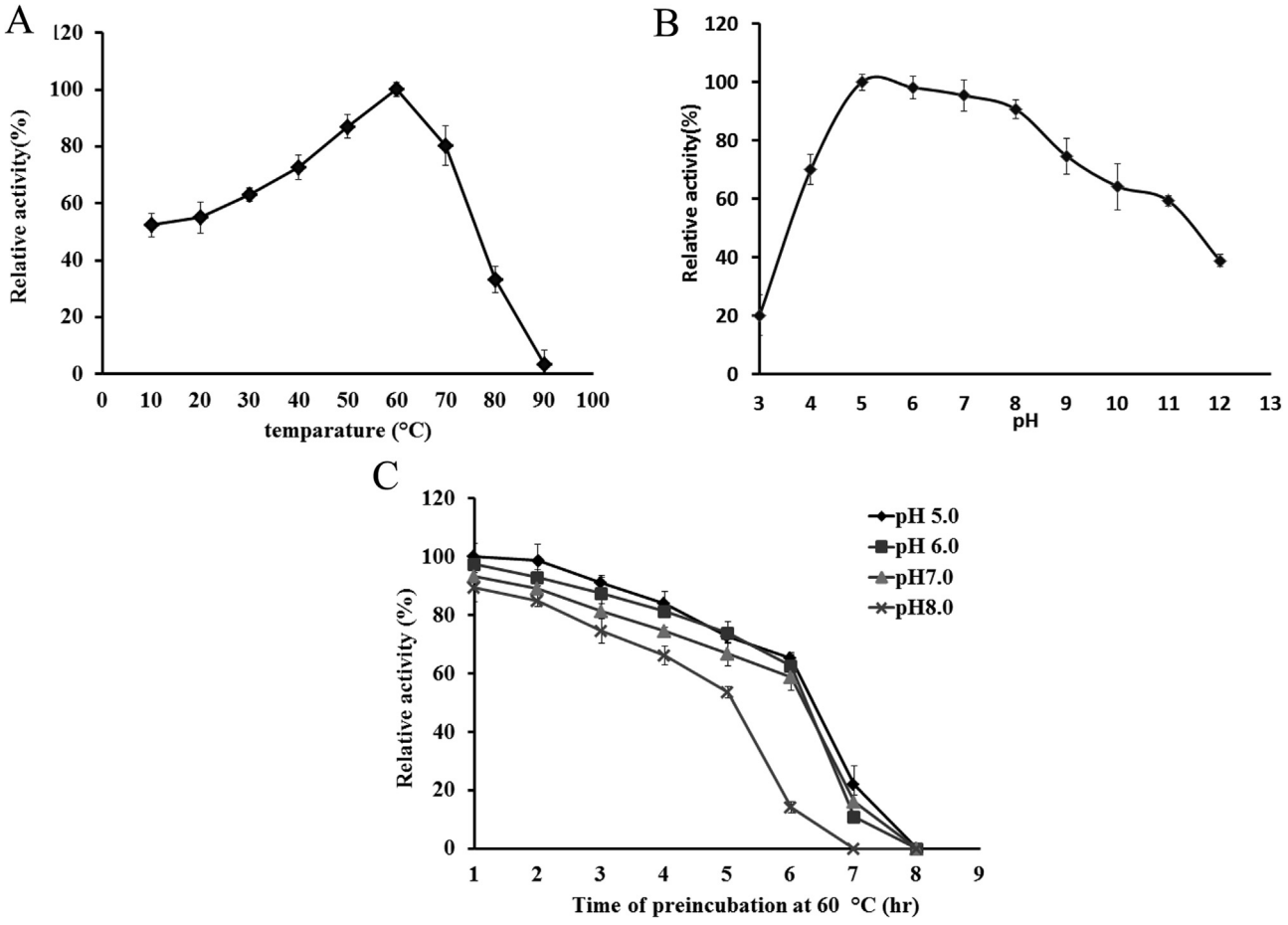

Fig. 3. Xylanase activity of rXyn11A as affected by temperature (A); pH (B); stability of the rXyn11A at different pH levels (C); where data are shown as mean ± SD (n = 3).

Table 2
Adsorption and kinetic parameters of Xyn11A against insoluble xylans and agricultural waste.

Substrate	K_a (10^4/M)	Relative adsorption (%)	K_m (mg/mL)	V_{max} (μmol/min·mg)
Insoluble xylans				
Birchwood xylan	11.98	77	0.19	7.14
Oat spelt xylan	8.81	72	0.28	6.44
Agricultural waste				
Corn husk	3.38	67	1.37	19.52
Rice straw	2.20	48	1.68	17.07

1999). This suggests that the xylanase properties observed in the native enzyme were preserved in the rXyn11A produced in the current study.

The stability of the rXyn11A at different pH levels was investigated and the results are shown in Fig. 3C. The enzyme was pre-incubated with the buffer of a specific pH at 60 °C for different time periods before transferring to xylanase activity assay (Fig. 3C). It was found that the relative activity of rXyn11A retained more than 70% after pre-incubation at all pH levels for 3 h. Moreover, the enzyme exhibited 60% relative activity at pH 8 even after 4 h of incubation. However, the enzyme was completely inactive after 8 h of pre-incubation at all pH levels. In comparison to other studies in which xylanases were tested for the ability to hydrolyze birch wood xylan, it could be said that the stability of the rXyn11A from the current study was comparable to the xylanase from *Paenibacillus campinasensis* (Zheng et al., 2012) and better than several other bacterial xylanases. The recombinant Xyn11A from alkaliphilic *Bacillus* sp. SN5 showed only 63.8% enzyme stability after pre-incubating at 60 °C in pH 8 for just 30 min (Bai et al., 2015) and the xylanase from *Bacillus pumilus* SV-205 retained 65% enzyme stability at 60 °C after preincubation in pH 6 for 2 h (Nagar et al., 2012). Apart from better stability, the rXyn11A from the current study also had the highest specific activity among these enzymes for hydrolyzation of the same substrate (birch wood xylan), suggesting potential application in the pulp industry as well as in bioenergy conversion.

Adsorption kinetic parameter analysis and reducing-sugar productivity of rXyn11A from insoluble xylans and agricultural waste

The binding affinity of rXyn11A for various insoluble substrates was evaluated by determination of the adsorption equilibrium constant (K_a) and the percentage of relative binding which are shown in Table 2. The K_a values were analyzed based on a double-reciprocal plot of substrate-bound rXyn11A, [PC] versus free rXyn11A, [P] (Zilliox and Debeire, 1998). The K_a value of rXyn11A for the insoluble BWX (11.98×10^4 M^{-1}) was approximately 1.4-folds higher than that for insoluble OSX (8.81×10^4 M^{-1}), corresponding to the relative adsorption and K_m and V_{max} value, indicating that rXyn11A prefers insoluble BWX over insoluble OSX, whose xylan structural unit contains a higher degree of substitution than the former (Liab et al., 2000). In a comparison between corn husk and rice straw, rXyn11A was shown to have a higher binding affinity toward corn husk than rice straw as reflected by a lower value in K_m and higher values in K_a, relative adsorption and V_{max}. The xylan content in corn husk (Tachaapaikoon et al., 2006) might be one of the reasons—along with some factors such as the lignin content, porosity, composition and structure that varied from plant to plant (Kuhad and Singh, 1993; Kaewintajuk et al., 2006)—that contributed to the better binding affinity by rXyn11A.

The results of the kinetic analysis clearly showed that rXyn11A had the ability to bind to all the insoluble xylans used in the experiment. Considering the fact that Xyn11A from *B. firmus* K-1 is a single domain, it was speculated that the rXyn11A from the current study may possess a secondary binding site (SBS) responsible for

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Bacillus subtilis          STDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT INYNAGVWAP 60
Bacillus circulans         -STDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT INYNAGVWAP 60
Bacillus firmus K-1        ANTYWQYWT DGGGTVNATN GPGGNYSVTW RDTGNFVVGK GWEIGSPNRT IHYNAGVWEP 60
                           :. *** ** **** ***.* *.******.*  :******** **  *** ** *:****** *

Bacillus subtilis          NGNGYLTLYG WTRSPLIEYY VVDSWGTYRP TGTYKGTVKS DGGTYDIYTT TRYNAPSIDG 120
Bacillus circulans         NGNGYLTLYG WTRSPLIEYY VVDSWGTYRP TGTYKGTVKS DGGTYDIYTT TRYNAPSIDG 120
Bacillus firmus K-1        SGNGYLTLYG WTRNQLIEYY VVDNWGTYRP TGTHRGTVVS DGGTYDIYTT MRYNAPSIDG 120
                           .********* ***. ***** ***.****** ***::*** * **********  *********

Bacillus subtilis          DRTTFTQYWS VRQSKRPTGS NATITFSNHV NAWKSHGMNL GSNWAYQVMA TAGYQSSGSS 180
Bacillus circulans         DRTTFTQYWS VRQSKRPTGS NATITFTNHV NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS 180
Bacillus firmus K-1        -TQTFQQFWS VRQSKRPTGN NVSITFSNHV NAWRNAGMNL GSSWSYQVLA TEGYQSSGRS 179
                              ** *:** *********. *.:***:*** ***:. **** **.*:***:* * ****** *

Bacillus subtilis          NVTVW 185
Bacillus circulans         NVTVW 185
Bacillus firmus K-1        NVTVW 184
                           *****
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Fig. 4. Multiple amino acid sequence alignment of Xyn11A from *Bacillus firmus* K-1 (AAQ14588.1), *Bacillus subtilis* (CAB13776.1) and *Bacillus circulan* (CAA30553.1), where * indicates positions which have a single fully conserved residue. indicates a strong conservation with a single group of amino acids, and the absence of an alignment character implies that an unrelated amino acid was substituted.

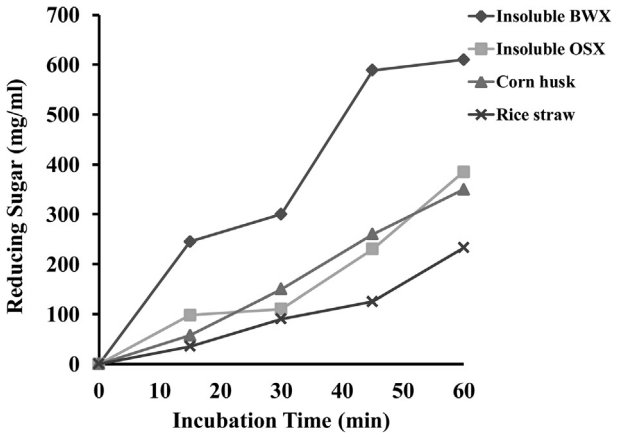

Fig. 5. Insoluble xylans and agricultural waste digestion by rXyn11A.

the binding to insoluble xylan. Amino acid sequence alignment was performed on the rXyn11A from the current study against other Xyn11A from *B. subtilis* and *B. circulan* using the CLUSTAL W alignment program on the GenomeNet server (Thompson et al., 1994) and the result is shown in Fig. 4. Previous study showed that residue N54, G56, N141, N181, T183 and W185 of Xyn11A from *Bacillus subtilis* were responsible for forming SBSs which bind to insoluble xylan and that mutation of these residues to alanine resulted in a very large decrease in the insoluble xylan adsorption of the enzyme (Cuyvers et al., 2011). According to the sequence alignment in Fig. 4, N54, G56, N141, N181, T183 and W185 were also conserved in Xyn11A from both *B. firmus* K-1 and *B. circulan* which are also capable of binding to insoluble xylan (Ratanakhanokchai et al., 1999; Ludwiczek et al., 2007). This result strongly indicates that Xyn11A from *B. firmus* K-1 might possess SBSs responsible for insoluble xylan binding.

The rXyn11A was also tested for its ability to digest insoluble substrates and the results are shown in Fig. 5. Insoluble xylan from birch wood was the most preferable substrate for rXyn11A as the highest amount of reducing sugar was released upon digestion assay. Insoluble oat spelt xylan, corn husk and rice straw were ranked second, third and fourth, respectively, as a preferable substrate for rXyn11A. This result shows that the rXyn11A obtained from this study has a similar substrate preference as seen in the native Xyn11A isolated from *B. firmus* K-1, again confirming that the rXyn11A from the current study can represent the properties of the native enzyme (Jommuengbout et al., 2009). More interestingly, rXyn11A digested these insoluble substrates around 100-times more effectively than the native enzyme directly isolated from the culture broth of the *B. firmus* K-1 growing on corn husk containing media reported previously (Jommuengbout et al., 2009). This could have been due to the fact that the purification method used in the current study effectively yielded rXyn11A with higher purity.

The current study has reported a method for the expression and purification of rXyn11A from *B. firmus* K-1 using an *E. coli* expression system. The rXyn11A obtained from was shown to have the same optimal conditions as the native enzyme isolated directly from the K-1, which was observed to be active in the pH range 5–12 at temperatures as high as 70 °C. The rXyn11A exhibited 84-times higher specific activity and hydrolyzed insoluble substrates around 100-times more effectively than the enzyme directly isolated from K-1. Sequence analysis revealed that the Xyn11A from K-1 might possess SBS that helps the enzyme bind and hydrolyze insoluble xylan. In summary, the results from this study demonstrated that the method developed can produce rXyn11A from K-1 that contains the same properties as seen in the native enzyme, but the purity and specific activity of the enzyme were greatly improved which makes it attractive for industrial applications.

Conflict of interest

There is no conflict of interest.

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