Purification and Characterization of a Xylanase from the Newly Isolated *Penicillium rolfsii* c3-2(1) IBRL

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An extracellular xylanase was purified from the mesophilic fungus *Penicillium rolfsii* c3-2(1) IBRL. After three consecutive purification steps, the extracellular cellulase-free xylanase was successfully purified to homogeneity with a recovery yield of 24%. A single protein band of 35 kDa was detected by SDS-PAGE, which had an optimum catalytic activity at pH 5.0 and 50 °C. This purified enzyme was stable at pH 5 to 7, thermostable up to 55 °C, and retained up to 83% of its activity after 4 hours of pre-incubation. A kinetic study yielded estimated K_m and V_{max} values of 5.73 mg/mL and 691.6 µmol/min/mg, respectively. Thin layer chromatography experiments showed that the purified xylanase was capable of hydrolyzing xylotriose, xylotetraose, xylopentaose, and xylohexaose but not xylobiose, suggesting it is an endo-xylanase. Enzymatic hydrolysis of oil palm trunk residues by commercial enzymes supplemented with the purified xylanase showed a considerable increase in total sugar conversion compared with the commercial enzymes alone, suggesting that xylanase is a key enzyme in the hydrolysis of oil palm trunk residues.

Keywords: Xylanase; Thermostability; Xylo-oligosaccharide; Oil palm trunk; Penicillium rolfsii

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INTRODUCTION

Much of the agricultural and agro-industrial waste produced on Earth is composed of hemicellulose. Hemicellulose is therefore an abundantly available renewable organic biomass (Fadel and Fouda 1993; Terrasan *et al.* 2010). One such hemicellulose is xylan. Xylan comprises various sugar units (glucose, xylose, mannose, galactose, arabinose, fructose, and galacturonic acid) contributed by attached side chains and side groups, such as α-D-glucuronosyl and α-L-arabinosyl units, depending on the natural source (Knob and Carmona 2010; Manju and Singh Chadha 2011). Because of the complex heterologous structure of xylan, the action of a wide spectrum of enzymes with diverse catalytic specificities and modes of action are required for its hydrolysis. Generally, two main enzymes are involved in the hydrolysis of xylan: β-1,4 endoxylanase (EC 3.2.1.8) that cleaves the β-1,4-linked xylose backbone, and β-xylosidase (EC 3.2.1.37) that cleaves xylose polymers from the nonreducing end of xylooligosaccharides and xylobiose (Beg *et al.* 2001; Manju and Singh Chadha 2011; Bajaj *et al.* 2012).

Xylanolytic enzymes have attracted a great deal of attention in recent years because of their potential application in biotechnology. Xylanase has been studied extensively in

various industrial processes, such as the food and feed industries, in textile processes, in pulping and bleaching processes, in the enzymatic saccharification of lignocellulosic materials and waste treatment processes, and in the production of several valuable products such as xylitol and bioethanol in the most economical way (Damiano *et al.* 2006; Ko *et al.* 2010; Nagar *et al.* 2012). The industrial application of xylanases has several advantages, enabling processes to be carried out using less chemicals, less harsh conditions, and less undesirable side reactions, all of which are beneficial for society and the environment (Sa-Pereira *et al.* 2003). A variety of microorganisms from nature, including fungi, bacteria, and yeasts, which are widely available, are capable of producing xylanases (Bajaj *et al.* 2012; Verma and Satyanarayana 2012). Filamentous fungi in particular have attracted a lot of attention for their potential industrial application as they produce a wide range of xylanases at high titer compared with enzymes derived from bacteria and yeast (Knob *et al.* 2010). The genera *Aspergillus*, *Trichoderma*, and *Penicillium* are considered rich sources of enzymes for xylan biodegradation (Li *et al.* 2007; Knob and Carmona 2010).

Here, we report the purification and characterization of the xylanase produced by *Penicillium rolfsii* strain c3-2(1) IBRL. The synergistic effect of adding purified xylanase, together with the commercial enzymes Celluclast 1.5L and Accellerase 1500, on the saccharification of oil palm trunk residues was also investigated.

EXPERIMENTAL

Microorganism and Culture Conditions

P. rolfsii c3-2(1) IBRL was isolated from a soil sample taken from an oil palm farm in the northern part of the Malaysian Peninsula. This strain was maintained in a potato dextrose agar slant (Difco, USA). The fungus was grown in a basal medium that contained: sodium nitrate, 1.0 g; potassium dihydrogen phosphate, 1.0 g; magnesium sulfate heptahydrate, 0.5 g, and yeast extract, 0.5 g, which were dissolved in 1 L of distilled water at pH 5.0. A portion of nuclear rDNA (ITS), comprised between the 18S and 28rDNA genes, was amplified using primer ITSF (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'). The nucleotide sequence of *Penicillium rolfsii* c3-2(1) IBRL was deposited in the GenBank nucleotide sequence database, with provided accession number of KF980897. The medium also contained 1% (w/v) xylan from birchwood (Sigma, USA) to induce the production of enzymes. The culture was incubated with agitation at 200 rpm for 7 days at 30 °C.

Enzyme Purification

All of the purification steps were performed at 4 °C to conserve the enzyme activity, unless stated otherwise. The procedure used to purify xylanase from *P. rolfsii* c3-2(1) IBRL involved the following four steps.

Step 1: Ammonium sulfate precipitation

After 1 week of fermentation, the culture broth was collected by filtration to remove the fungal mycelium. The supernatant was precipitated with 80% saturated ammonium sulfate at 4 °C. Precipitation was allowed to continue overnight, which was followed by centrifugation at 8000 rpm in a refrigerated centrifuge for 30 min. The precipitate was then dissolved in 50 mM sodium acetate buffer (pH 5.0), and any excess salt was removed using an Econo-Pac 10 DG desalting column (Bio-Rad). The filtrate was concentrated by

centrifugal ultrafiltration (Vivaspin 20, 5 kDa cut-off, Sartorius) and used as the starting material for purification.

Step 2: Anion-exchange chromatography

The concentrated enzyme solution was applied to an anion exchange column (RESOURCE™ Q, 6 mL; GE Healthcare Bio-Sciences AB, Sweden), which was pre-equilibrated with 20 mM 3-morpholinopropanesulfonic acid (MOPS) (Dojindo, Wako Pure Chemical, Japan) buffer (pH 7.0) containing 1.0 M NaCl. The sample was eluted at a flow rate of 4 mL/min. The eluted fractions were collected and screened to determine their xylanase activities and protein content. The active fractions were pooled and concentrated by ultrafiltration.

Step 3: Gel filtration

A portion of the pooled ion-exchange chromatography fractions was passed through a gel filtration column (HiPrep™ 16/60 Sephacryl S-100 High Resolution; GE Healthcare Bio-Sciences AB). The gel filtration column was pre-equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. The sample was eluted at a flow rate of 0.8 mL/min. The fractions were collected and analyzed to determine their xylanase activity and protein content. The active fractions were pooled and used in the next purification step.

Step 4: Hydrophobic-interaction chromatography

The pooled fractions with xylanase activities were dialyzed against 50 mM MOPS buffer containing 2.0 M ammonium sulfate (pH 7.0). Subsequently, the pooled fraction was passed through a hydrophobic column (RESOURCE™ PHE; GE Healthcare Bio-Sciences AB) at a flow rate of 1 mL/min. The column was pre-equilibrated and eluted with 50 mM MOPS buffer (pH 7.0) with a linear ammonium sulfate gradient (0 to 2 M). The fractions were collected and analyzed to determine their xylanase activity and protein content.

Enzyme Assay and Protein Determination

Xylanase activity was assayed by determining the amount of reducing sugar released from xylan derived from birchwood (Sigma). The reaction mixture consisted of 0.5% xylan, in pH 5.0 acetate buffer, and enzyme to give a final volume of 0.2 mL. After incubation for 15 min at 50 °C, the amount of reducing sugar contained in the sample was determined according to the Somogyi-Nelson method (Somogyi 1945). One unit of xylanase was defined as the amount of enzyme that hydrolyze 1 µmol of glycoside bonds of the substrate per minute. The concentration of protein in the crude enzyme preparation was determined using the Lowry method, with bovine serum albumin as the standard (Lowry *et al.* 1951). The broth containing the enzymes was used for enzymatic activity determination, and experiments were performed in triplicate.

Activity Detection by Zymography

Zymography was performed according to Zhang and colleagues with some modifications (Zhang *et al.* 2009). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the protein purity and the molecular mass of the purified enzyme under denaturing conditions. SDS-PAGE, using a 10% gel, was performed after incorporating xylan solution (final concentration = 0.1% w/v) into the separating gel. Ammonium persulfate and tetramethylethylenediamine were added to

facilitate polymerization. Then, the gel was soaked with washing buffer and shaken gently for 15 min. This washing procedure was repeated four times to remove the SDS. The gel was rinsed with distilled water and transferred to sodium acetate buffer for 30 min, which was repeated twice. The gel was rinsed with distilled water and incubated overnight in 50 mM sodium acetate buffer at 50 °C. The gel was stained with Congo red solution at room temperature for 30 min. The gel was rinsed with distilled water and destained in 1 M NaCl solution at room temperature for 1 h. Finally, the gel was transferred to 5% (v/v) acetic acid to enhance the visualization of a clear zone band in the gel against a dark background.

Effects of pH and Temperature on Enzyme Activity and Stability

The xylanase activity was measured under various pH conditions (pH 3.0, McIlavine buffer; pH 4.0 to 5.0, sodium acetate buffer; pH6, phosphate buffer; pH 7.0 to 8.0, sodium phosphate buffer; pH 9.0, Tris-HCl buffer) according to the method described earlier. To determine the pH-dependent stability, the purified enzymes were diluted in each of the different buffers and incubated at 4 °C for 24 h. Next, the xylanase activity was assayed after incubating at 50 °C for 15 min to compare the residual activity with the initial activity. To determine the optimal temperature, the xylanase activity was investigated at a temperature range of 30 to 75 °C. To evaluate the thermal stability, the purified enzymes were incubated at different temperatures with the optimal pH for various periods of up to 4 h. After incubation, the remaining activity was measured using the standard assay conditions.

Determination of Kinetic Parameters

The initial reaction rate (xylanase activity) was determined using xylan from birchwood as the substrate at 2 to 30 mg/mL with 50 mM acetate buffer, under optimal assay conditions (70 °C, pH 5.0, 15 min). The kinetic constants, *i.e.*, K_m and V_{max}, were estimated from the Michaelis–Menten plot and linear regression based on double-reciprocal plots according to the Lineweaver–Burk plot method using Sigma Plot 10 software. The maximum rate was expressed as μmol/min/mg of protein.

Substrate Specificity of Xylanase

The activity of the purified xylanase was tested using various polymers such as xylan from birchwood (Sigma), xylan from beechwood (Sigma), xylan from oat spelt (Sigma), carboxymethyl cellulose (Sigma), Avicel® (Sigma), curdlan (Sigma), gellan gum (Kanto Chemical, Japan), xanthan gum (Mp Biomedicals, LLC), and p-nitrophenyl β-D-xylopyranoside (Sigma). Enzymatic hydrolysis was conducted by incubating each selected substrate with the purified enzyme at the optimal pH and temperature. In each case, degradation was assayed based on the production of reducing sugars, which was measured as described earlier.

Analysis of the Hydrolysis Products

The hydrolysis products of xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose, were analyzed using thin layer chromatography (TLC). The purified xylanase (0.1 U) was added to 5 mg/ml xylobiose, xylotriose, xylotetraose, xylopentaose, or xylohexaose, prepared in 50 mM sodium acetate buffer (pH 5.0), and incubated at 50°C overnight (17 to 20 h). Another tube containing xylose was prepared as a control. The xylanase hydrolysis products along with xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose, were fractionated on a TLC plate (Silica gel 60; Merck;

Darmstadt, Germany). The solvent used for developing the chromatogram was chloroform:acetic acid:water (6:7:1) (Lee *et al.* 2009). The products were visualized by spraying a chromogenic reagent, a mixture of ethanol and sulfuric acid (95:5), onto TLC plates followed by drying at 100 °C for 5 to 10 min.

Effect of Xylanase Supplementation on the Enzymatic Hydrolysis of Oil Palm Trunk Residues

Oil palm trunk residues at 5% (w/v) were used as substrates and saccharified by commercial enzymes Celluclast 1.5L and Accellerase 1500 (6 FPU/g of substrate), which were suspended in 50 mM sodium acetate buffer solution (pH 5.0). Purified xylanases (0.1 mg/g of substrate) derived from *P. rolfsii* c3-2(1) IBRL were added to each commercial enzyme mixture with the substrate for evaluation of the saccharification effect. This experiment was performed in 50 mL Schott bottles at 50 °C and rotated at 180 rpm for 72 h. Samples were taken from the reaction mixture periodically for sugar analysis using the phenol-sulfuric acid method. Briefly, 1 mL of sample was added to 1 mL of 4% phenol and 5 mL of 96% sulfuric acid in a test tube. The solution was transferred to a cuvette, and the absorbance was measured at (λ) 490 nm.

RESULTS AND DISCUSSION

Purification of Xylanase

Data on the purification of the xylanase at various steps are summarized in Table 1. The purification scheme resulted in a five-fold purification of xylanase to homogeneity relative to the crude broth and a substantial increase in the specific activity compared with the initial value, *i.e.*, of 100.12 U/mg to 488.17 U/mg of protein. The crude extract was concentrated by ultrafiltration and purified by a simple three step process involving ion-exchange, gel filtration, and hydrophobic interaction chromatographies. The anion exchange chromatography elution profile of the ammonium sulfate-precipitated enzyme using RESOURCE™ Q is shown in Fig. 1. A single major xylanase activity peak was found corresponding to the protein fractions 3 to 5. These fractions did not adsorb to the positively charged solid support. The active fractions were eluted initially, before manipulating the gradient with increasing NaCl concentrations. Subsequently, the active fractions were pooled and concentrated, before further purification by gel filtration on a Sephacryl S-100 column (Fig. 2). The gel filtration chromatography showed a single major xylanase activity peak in fractions 43 to 65. Subsequently, the active fractions were eluted using a phenyl–hydrophobic column (Fig. 3). The active enzymes in fractions 30 to 43 were desorbed from the column with a decreasing ammonium sulfate concentration. These active fractions were pooled, and excess salt was removed by ultracentrifugation. The retentate was stored at 4 °C for later use. In these purification procedures, only a single major peak of xylanase activity was focused. In Fig. 1, a minor xylanase activity peak (fractions 12 to 20) is evident, which is also seen in Fig. 2 (fractions 15 to 40). These results showed that *P. rolfsii* c3-2(1) IBRL was able to produce multiple forms of xylanase, possibly contributing to the low xylanase yield (23.8%) shown in Table 1. However, it has been reported that the presence of other degrading activities, in which enzymes work synergistically for complete hydrolysis of xylan, cause the yield and fold values to be underestimated during the purification procedures (Lucena *et al.* 2004). Xylanase multiplicity has been reported to be a common feature of fungi and bacteria as a result of

differential mRNA processing and posttranslational modification (Flint *et al.* 1994; Okazaki *et al.* 1984; Nair *et al.* 2008). However, single xylanases have been described only for yeast (Wong *et al.* 1988). It would appear that the production of multiple xylanases is largely dependent upon the different types of carbon source used or the use of a mixture of carbon sources as growth substrates (Chavez *et al.* 2006). Generally, increasing the number of purification steps contributed to the decrease in xylanase recovery, and low enzyme recoveries (approximately 20%) are observed with more than three purification steps (Sa-Pereira *et al.* 2003).

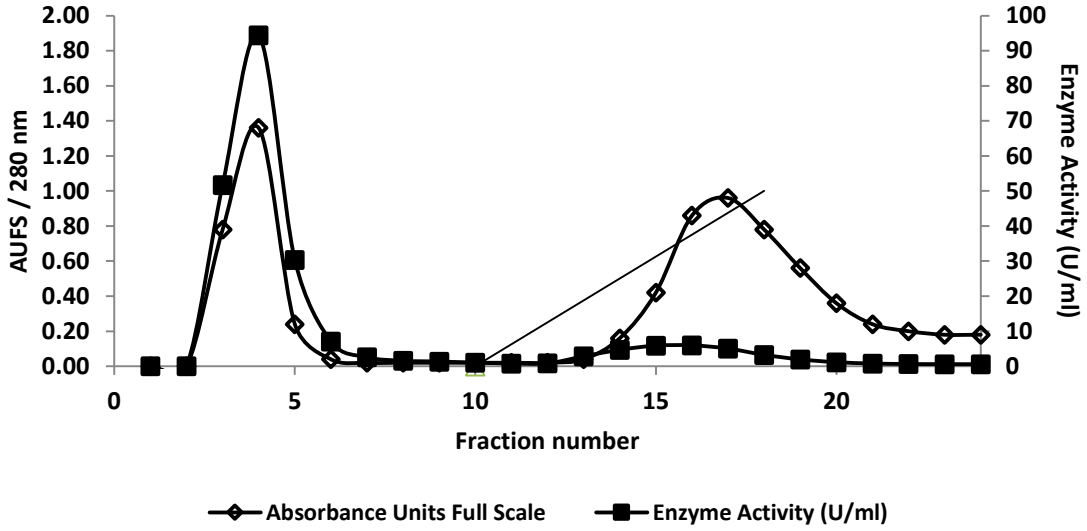

Fig. 1. Anion exchange chromatography showing the elution profile of protein and xylanase activity. NaCl (1.0 M) gradient (continuous line)

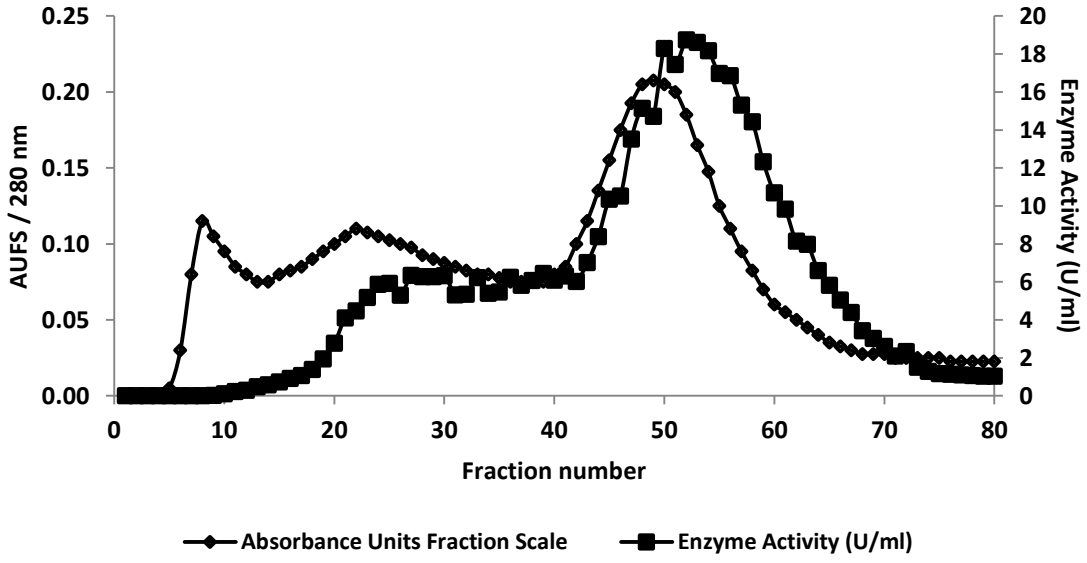

Fig. 2. Elution profile of xylanase purification by gel filtration HiPrep™ 16/60 Sephacryl S-100 High Resolution

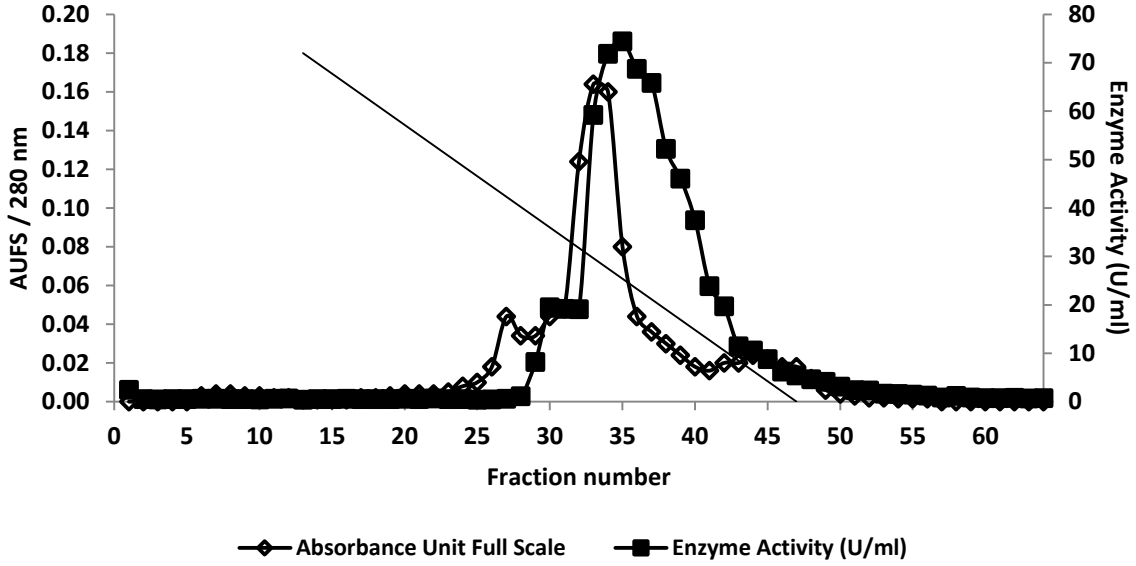

Fig. 3. Hydrophobic-interaction chromatography of xylanase on a phenyl Resource™ PHE column after gel-filtration column chromatography. $(NH4)_2SO_4$ (2.0 M) gradient (continuous line)

Table 1. Summary of the purification scheme for the xylanase from *P. rolfsii* c3-2(1) IBRL

Fraction	Volume (mL)	Total protein (mg)	Activity (units)	Total activity (units*ml)	Specific activity (units/mg)	Fold purification	% Yield
Cell-free supernatant	5.0	75.0	1501.76	7508.80	100.12	1.0	100.0
RESOURCE™ Q, 6 mL	1.0	21.03	3051.51	3051.51	145.10	1.45	40.6
HiPrep™ 16/60 Sephacryl S-100 high resolution	5.0	7.43	445.91	2229.55	300.07	3.00	29.7
RESOURCE™PHE	8.0	3.66	223.34	1786.72	488.17	4.86	23.8

SDS-PAGE and Zymogram Analysis of Purified Xylanase

The aforementioned purification steps provided apparently homogenous preparations of xylanase from *P. rolfsii* c3-2(1) IBRL. Coomassie blue staining of 4.57 µg

of the purified enzyme in 10% polyacrylamide gels separated by SDS-PAGE detected a homogeneous single protein band with an estimated molecular mass of 35 kD, as shown in Fig. 4. The zymogram of the xylanase preparation demonstrated the presence of a zone of hydrolysis in lane 4 that correlated to the single protein band in lane 2. The molecular mass of xylanases purified from the genera *Penicillia* has generally been reported to be ≤ 35 kDa, except for the xylanase derived from *Penicillium verruculosum*, which has a molecular mass of 65 kDa (Ryan *et al.* 2003; Lu *et al.* 2008). We analyzed the protein band purified from *P. rolfsii* c3-2(1) IBRL by nanoLC-MS/MS (data not shown). There was 29% homology between the amino sequence derived from the purified protein band from P. rolfsii c3-2(1) IBRL and XynA, a family 10 xylanase from *P. purpurogenum.* The molecular weight of the xylanase from P. rolfsii c3-2(1) IBRL was similar to that of XynA from *P. purpurogenum* (33 kDa).

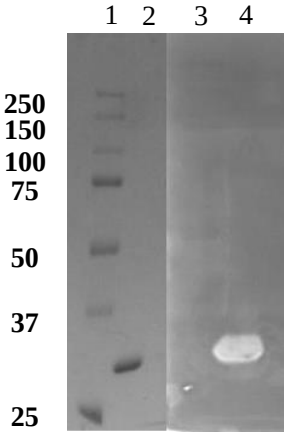

Fig. 4. Development of a xylanase zymogram. Lanes 1, 3: protein marker (Precision PlusProtein™ Standards – BIO-RAD); lanes 2, 4: 4.57 µg of purified enzyme. Lanes 1, 2: stained with Coomassie blue; lanes 3, 4: developed as zymogram with gel impregnated with 1% birchwood xylan, stained with Congo-red solution followed by destaining with 5% acetic acid (v/v)

Effect of pH and Temperature on Xylanase Activity

The effect of pH on xylanase activity was determined in a buffer ranging from pH 3.0 to pH 9.0 (Fig. 5). The favorable pH range for enzyme activity of the *P. rolfsii* c3-2(1) IBRL xylanase was 5.0 to 6.0, with an optimum of pH 5.0. A significant drop in xylanase activity was found below pH 5.0. However, xylanase activity gradually decreased when the pH was above pH 5.0, with low activity above pH 8.0. For its stability, the enzyme exhibited 90 to 100% of its maximum activity in the range pH 5.0 to 7.0. Enzymatic stability was lost below pH 5.0 and above pH 7.0. In recent studies, most of the xylanases of fungal origin showed higher activity at a pH between 3.5 and 5.5, and stability over a wide pH range (3.0 to 10.0) (Murthy and Naidu 2012). Bajpai reported that most of the fungal xylanases exhibited their optimum pH for xylan hydrolysis around pH 5, and stability between pH 2 to 9 (Bajpai 2009). Lee and colleagues supported this finding, reporting that most xylanases derived from fungi showed high activity under slight acidic conditions (Lee *et al.* 2009). One report on the xylanase produced by *P. chrysogenum* showed that the enzyme exhibited 90 to 100% of its maximum activity in the broader range of pH 5.0 to 7.0 (Haas *et al.* 1992). On the other hand, optimum activity at pH 2.5 and stability of xylanase I over 80% at high acidity (pH 1.6 to 3.0) has been reported in the fungi *P. sclerotiorum*, while xylanase II showed its optimum activity at pH 4.5, retaining

more than 60% of its maximum activity at pH 3.5 to 7.0 (Knob and Carmona 2010). Belancic and colleagues reported that the enzymes with small molecular weights (≤ 35,000 Da) mostly required acidic conditions for optimum activity, thereby sharing similar properties with the purified xylanase from *P. rolfsii* c3-2(1) IBRL, with a molecular weight of 35 kDa and an optimum pH of 5.0 (Belancic *et al.* 1995).

The effect of various temperatures, ranging from 30 °C to 70 °C, on the enzymatic hydrolysis of xylan from birchwood by the purified xylanase was studied (Fig. 6A). The optimum temperature for the xylan hydrolysis reaction was 50 °C. Hence, this enzyme can be classified as mesophilic, showing its optimal activity within a temperature range of 40 to 60 °C (Polizeli *et al.* 2005; Lee *et al.* 2009). At temperatures ranging from 30 °C to 60 °C, the xylanases retained up to 50% relative activity. However, at 65 °C the enzyme activity was 18%, and at 70 °C it was 6% of its maximum. Similar results were recorded for xylanases derived from other genera of *Penicillium*, in which optimum xylanolytic activities were achieved at 50 °C (Kimura *et al.* 2000; Andre *et al.* 2006; Murthey *et al.* 2012). The stability of purified xylanase was studied at temperatures of 50, 55, 60, and 65 °C (Fig. 6B). The purified xylanase retained its maximum activity (100%) even after 4 h preincubation of the enzyme at 50 °C. For the enzyme that was preincubated at 55 °C, maximum activity was maintained for 2 h and then decreased gradually to 83%. At a temperature of 60 °C, the purified xylanase activity sharply decreased within 1.5 h, and enzyme activity was lost within 20 min after preincubation at 65 °C. These findings were supported by the fact that most of the xylanases of fungal origin showed optimal xylanolytic activity at 50 °C and became inactivated above 60 °C (Gaspar *et al.* 1997). Bajpai reported that most fungi produce xylanases that are able to tolerate temperatures of 40 to 50 °C (Bajpai 2009). There are a few factors known to play a part in determining the thermostability of xylanases such as disulfide bridges, salt bridges, aromatic interactions, the content of arginine and proline, and the domain present in the N- or C- terminal (Bajpaj 2009). The broad pH and thermal stability of the purified xylanase from *P. rolfsii* c3-2(1) IBRL make it an attractive enzyme for use within the animal feed industry, because the pH and temperature of the digestive tract of livestock are approximately 4.8 and 40 °C, respectively (Collins *et al.* 2005; Chantasingh *et al.* 2006). Other biotechnological applications for xylanases, such as biomass hydrolysis and an animal feed additive, usually require optimum pH and temperature ranges of 4.8 to 5.5 and 40 to 50 °C, respectively (Teixeira *et al.* 2010).

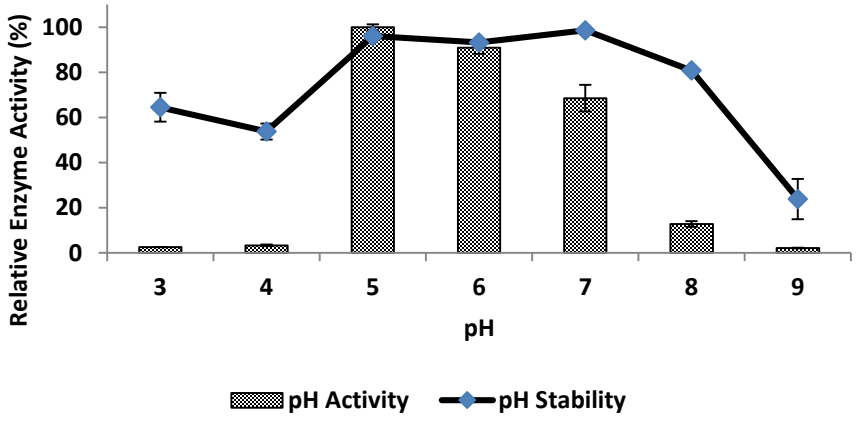

Fig. 5. Effect of pH on the activity and stability of *P. rolfsii* c3-2(1) IBRL xylanase

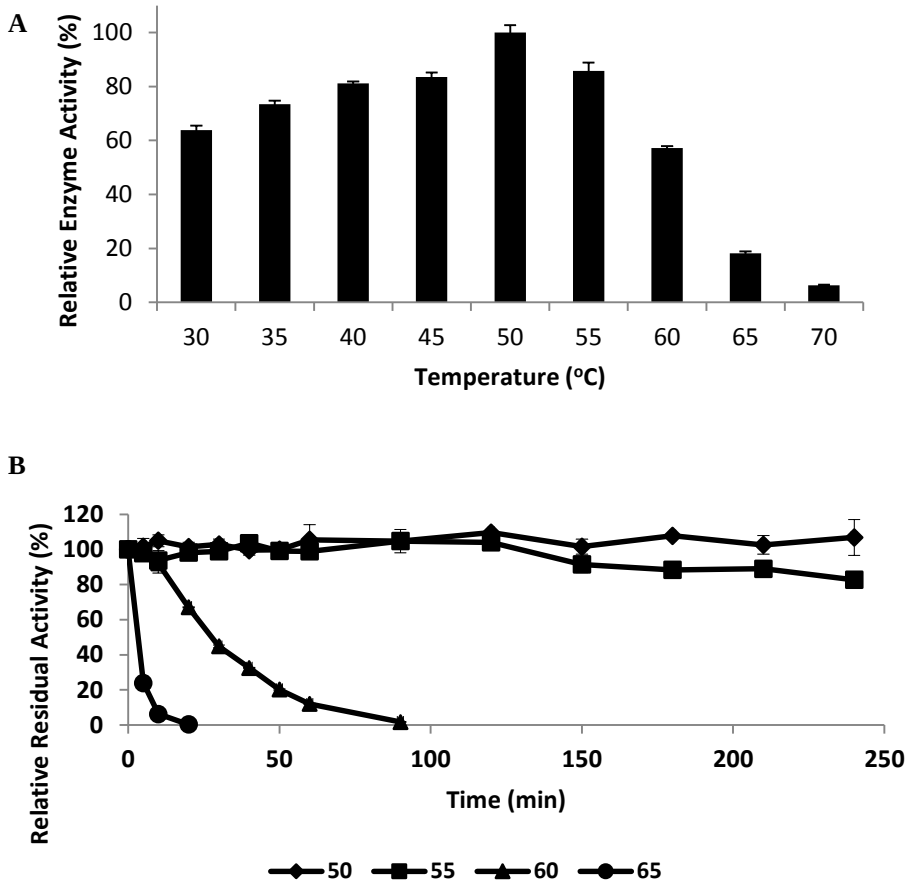

Fig. 6. Influence of temperature on the activity (A) and stability (B) of the *P. rolfsii* c3-2(1) IBRL xylanase

Kinetic Parameters

An enzyme must display the appropriate kinetic parameters if it is intended to be employed for industrial application (Bajaj *et al.* 2012). The kinetic parameters K_m and V_{max} of the purified xylanase were determined from the Michaelis–Menten and Lineweaver–Burk plots of xylanase activity at 50 °C using various concentrations of birchwood xylan as substrate. The K_m and V_{max} values for the purified xylanase from *P. rolfsii* c3-2(1) IBRL were 5.73 mg/mL and 691.6 µmol/min/mg, respectively, which were in agreement with the values observed for other fungal xylanases which range from 0.15 to 49.5 mg/mL for the K_m and 0.017 to 6300 µmol/min/mg for the V_{max} (Beg *et al.* 2001). The kinetic results for the *P. rolfsii* c3-2(1) IBRL xylanase were also supported by the findings of Murthy and Naidu who reported that the purified xylanase from *Penicillium* sp. exhibited a K_m value of 5.6 mg/mL and a V_{max} value of 925 µmol/min/mg using birchwood xylan as substrate (Murthy and Naidu 2012).

Analysis of Xylanase Hydrolysis Products

When a series of xylo-oligosaccharides (X3–X6) were incubated with purified xylanase overnight (18 h) at 50 °C, the major hydrolysis product was xylobiose with a trace amount of xylose (Fig. 7). Xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) were efficiently hydrolyzed to xylobiose as indicated by the TLC results. This purified xylanase is an endo-type xylanase because xylobiose was the major product

when X3–X6 were used as substrates for hydrolysis on TLC plates (Lee *et al.* 2009). Hydrolysis of oat spelt xylan by xylanase derived from *P. chrysogenum* revealed that xylose and xylobiose were the major products and it was concluded that the *P. chrysogenum* enzyme is a typical endoxylanase (Haas *et al.* 1992). Bajpai reported that endoxylanases show the highest activity against polymeric xylan, and the hydrolysis rate of xylan usually tends to decrease with decreasing chain length of the oligomeric substrate (Bajpai 2009). Xylotriose hydrolysis in most cases is negligible and at most limited. However, in contrast to the findings reported by Bajpai, the purified xylanase from *P. rolfsii* c3-2(1) IBRL was shown to efficiently hydrolyze xylotriose, as shown by the TLC data (lane X3) (Polizeli *et al.* 2005).

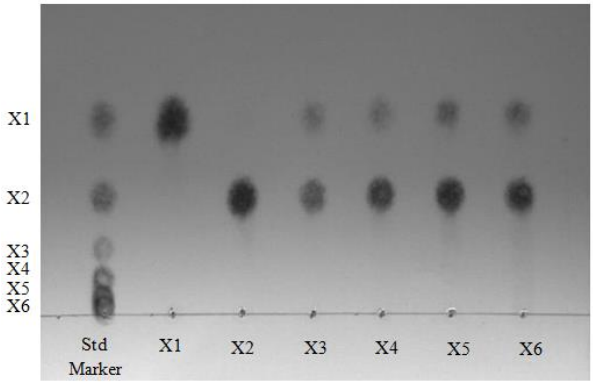

Fig. 7. TLC analysis of the hydrolysis products released from xylo-oligosaccharides

Substrate Specificity

The purified enzyme was investigated for its ability to hydrolyze various polysaccharide substrates (Table 2). Beechwood xylan was found to be most easily hydrolyzed by the purified xylanase, which showed a relative activity of 135%, followed by birchwood xylan (100%) and oat spelt xylan (84%). However, no detectable activity on carboxymethyl cellulose, Avicel®, curdlan, gellan gum, and xanthan gum could be found. Because this purified xylanase did not show any cellulase activity, we propose it is a cellulase-free xylanase. Additionally, these results showed that the purified xylanase of *P. rolfsii* c3-2(1) IBRL catalyzes the hydrolysis of 1,4-β-D-xylosidic linkages in xylan derivatives. The purified enzyme acted on *p*-nitrophenyl β-D-xylopyranoside indicating that it possesses β-xylosidase activity that liberates xylose from short oligosaccharides (Chavez *et al.* 2006). Haltrich *et al*. (1996) reported that different xylan preparations can differ both in the type and degree of substitution, contributing to the significant variation in activity values measured. The different specific activities observed for this purified xylanase may be accounted to the structural differences between xylan substrates. In general, the structural characteristics of xylan from beechwood and birchwood are similar, consisting of more than 90% β-1,4-linked xylose residues, whereas oat spelt xylan is a kind of arabinoxylan, the main chain of which is largely branched with arabinose residues (Gruppen *et al.* 1992; Kormelink *et al.* 1993; Morais *et al.* 2011). Oat spelt xylan contained a high percentage of xylose (84%), with water solubility varying greatly depending on the temperature and extent of centrifugation. Regarding solubility, birchwood xylan is more than 90% soluble in water whereas beechwood xylan is approximatively 95% insoluble in water (Morais *et al.* 2010). Additionally, beechwood xylan was found to have a 3% lower hexuronic acid content compared with birchwood xylan. Beechwood xylan was most

utilized by *Butyrivibrio fibrisolvens*, which is one of the most commonly isolated ruminal xylanolytic bacteria (Hespell and Cotta 1995). Similarly, *P. rolfsii* c3-2(1) xylanase favored beechwood xylan. This is probably owing to the short-chain xylose polymer structure of beechwood xylan, which on aggregation of the chains forms cellulose-like semicrystalline fibers that cause little obstruction to hydrolysis by *B. fibrisolvens* enzymes. In this case, the solubility properties of xylan seemed not to affect the hydrolysis of xylan.

Table 2. Relative Substrate Specificity of Purified Xylanase from *P. rolfsii* c3-2(1)

Substrate (1%)	Main linkage	Specific activity (U/mg)	Relative activity (%)*
Birchwood xylan	β-1,4	257.79 ± 6.35	100 ± 2.5
Beechwood xylan	β-1,4	351.49 ± 5.72	136 ± 2.2
Oat spelt xylan	β-1,4	228.16 ± 2.62	88 ± 1.0
CMC	β-1,4	0	0
Avicel	β-1,4	0	0
Curdlan	β-1,3	0	0
Gellan gum	β-1,3; β-1,4 and α-1,4	0	0
Xanthan gum	β-1,4	0	0
p-Nitrophenyl β-D-xylopyranoside		0.76 ± 0.09	0.29 ± 0.03

Effect of Xylanase Supplementation on the Enzymatic Hydrolysis of Oil Palm Trunk Residues

Oil palm trunks are felled and exhausted in large quantities in Malaysia and Indonesia because productivity of oil decreases after the trees reach more than 25 years of age. In this work the oil palm trunk residues were used as natural substrates. To investigate the influence of purified xylanase supplementation during the enzymatic hydrolysis of the oil palm trunk residues, hydrolysis experiments were performed for 72 h in the absence and presence of purified xylanases with commercial enzymes Celluclast 1.5L and Accellerase 1500 (Fig. 8). Without supplementation of purified xylanase, oil palm trunk residue hydrolysis with Celluclast 1.5L and Accellerase 1500 reached a plateau of 27% and 25% total sugar conversion at 24 h, respectively. The maximum hydrolysis of oil palm trunk residues achieved by Celluclast 1.5L and Accellerase 1500 enzymes was 33% and 29% after 72 h, respectively. When the oil palm trunk residues were hydrolyzed by Celluclast 1.5L and Accellerase 1500 with supplementation of purified xylanase, a considerable increase in total sugar conversion was observed after 24 h. The maximum degree of oil palm trunk residue hydrolysis by Celluclast 1.5 L and Accellerase 1500 in the presence of purified xylanase was about 40% and 43% after 72 h, respectively, with 6 FPU/mg of substrate. Interestingly, the supplementation of both commercial enzymes with

purified xylanase showed increased oil palm trunk hydrolysis. The Celluclast 1.5L preparation contained low levels of endoxylanase activity, and the Accellerase 1500 preparation predominantly contained endoglucanase and β-glucosidase activities, indicating low hydrolytic capability towards xylan for both commercial enzymes (Hu *et al.* 2011; Song *et al.* 2012). Purified xylanase therefore appears to be one of the key enzymes playing an important role in the hydrolysis of oil palm trunk residues, in which xylan is one the main components. Enhanced hydrolysis of cellulase cocktails when xylanase is added to the pretreated lignocellulosic substrates has previously been reported (Banerjee *et al.* 2010; Hu *et al.* 2011). It has been proposed that xylanase supplementation of cellulase enzyme mixtures results in the removal of the xylan coat on the surface of the pulp fiber, thus increasing cellulose accessibility to cellulase enzymes (Bura *et al.* 2009; Kumar and Wyman 2009; Hu *et al.* 2011). The unproductive binding of cellulase to lignin components can be reduced by xylanase, which was suggested to increase the proportion of substrate available for productive cellulase interactions (Ryu and Kim 1998; Kumar and Wyman 2009; Hu *et al.* 2011). Furthermore, xylanase has been suggested to solubilize the lignin fractions from lignocellulosic biomass by demolishing the lignin-carbohydrate complex, thus enhancing substrate digestibility (de Jong *et al.* 1997; Suurnäkki *et al.* 1997; Hu *et al.* 2011). As reported by Hu and colleagues, steam pretreated corn stover was mostly hydrolyzed completely by making use of the synergistic interaction that occurs between cellulase and xylanase (Hu *et al.* 2011). By manipulating the synergistic effect between xylanase and cellulase enzymes in an attempt to improve hydrolysis efficiency of pretreated lignocellulose at low protein loadings, the enzyme costs can potentially be reduced. Hence, the purified xylanase investigated here shows great potential and further studies into its synergistic effect with cellulase at low protein loadings are required to achieve maximum hydrolysis.

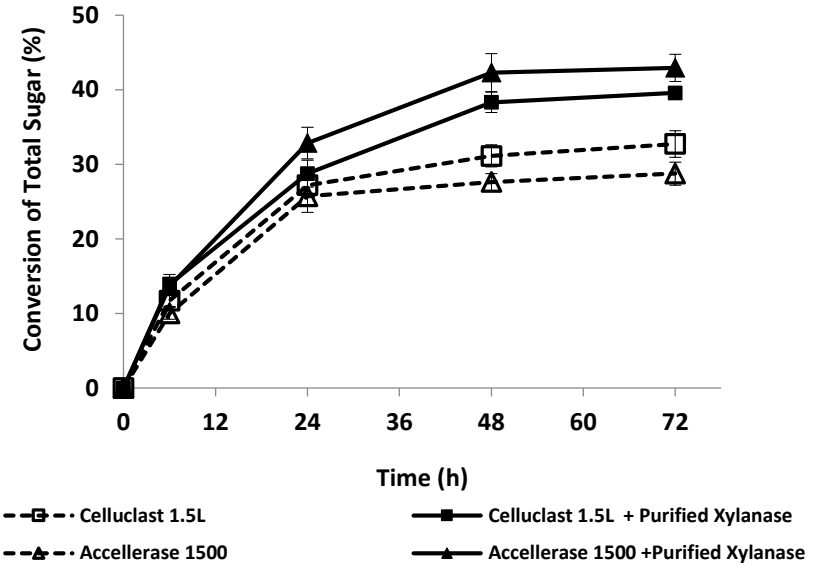

Fig. 8. Effect of purified xylanase supplementation (0.1 mg/g substrate) of the commercial enzymes Celluclast 1.5L and Accellerase 1500 on the conversion of oil palm trunk residues after 72 h hydrolysis

CONCLUSIONS

1. This is the first report on the identification, purification, and characterization of a xylanase from *P. rolfsii* c3-2(1) IBRL.
2. It was demonstrated that this enzyme is temperature tolerant and stable over a wide range of pH values. The broad pH stability and remarkable thermostability at 50 °C of this purified xylanase is advantageous for xylan saccharification.
3. Xylanase from *P. rolfsii* c3-2(1) IBRL is an endo-type xylanase.
4. Also, this xylanase showed synergistic effects with commercial lignocellulose degradation enzymes, and thus it could also be used as an enzyme supplement.

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