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Characterization of fusing CBM6 within *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 and its effectiveness on insoluble degradation

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ABSTRACT

Xylanase is rapidly gaining attention as suitable for applications in a wide variety of industrial processes such as food additives, animal feed, pulp bleaching and ethanol or xylitol production. Endo-1,4-beta-xylanase GH11 in *Pichia pastoris* expression, *xyn11*-CBM1 and *xyn11*-CBM1-CBM6 were constructed and characterized to investigate the influence of CBM6 on *Penicillium oxalicum*. Multiple copy genes of recombinant *Pichia* strains were selected on YPD agar +2,000 μg/mL of Zeocin^{TM} and xylan-Congo red clearance plate assay. Highest activities of Xyn11-CBM1 and Xyn11-CBM1-CBM6 were 39.07 ± 0.79 and 47.48 ± 0.33 units/mg protein, respectively at optimal conditions (citrate buffer pH 5.0, at 45°C). Xyn11-CBM1 and Xyn11-CBM1-CBM6 showed an acidic-stable property at pH 4.0-6.0 and 4.0-6.5, respectively (more than 100% of activity remaining) when kept at room temperature (RT) for 240 minutes. Xyn11-CBM1-CBM6 exhibited more than 85% and 75% of activity remaining in phosphate buffer pH 6.0 at 45°C for 60 and 120 minutes, respectively and significantly higher than Xyn11-CBM1. The activity of both enzymes was strongly inhibited by 1 mM of Hg^{2+} and showed over-estimated sizes (about 22.96 and 57.36 kDa) when separated by SDS-PAGE and zymogram analysis. Degradation of complex materials indicated that the highest reducing sugar release was produced by Xyn11-

CBM1-CBM6 activity. TLC analysis revealed that xylobiose, xylotriose and xylotetraose were produced by both enzymes when they hydrolyzed corncob, corn leaves and stalks, and rice straw. Results indicated that Xyn11-CBM1-CBM6 was a suitable candidate for application in various industries.

Keywords: *CBM6, Penicillium oxalicum endo-1,4-beta-xylanase GH11, Pichia pastoris*

INTRODUCTION

Hemicellulose, as the second most abundant lignocellulosic biomass, is found in plant cell walls as a branched polymer of pentose (xylose and arabinose), hexose sugars (mannose, glucose and galactose) and sugar acids (4-*O*-methyl-d-glucuronic acid and glucuronic acid) (Saha, 2003; Zhang *et al.*, 2006). Hemicelluloses are classified according to their backbone sugar composition as xylan, mannan and glucan. In nature, hardwood hemicelluloses contain mostly xylans whereas softwood hemicelluloses contain mostly glucomannans (McMillan, 1993; Saha, 2003). The structure of hemicellulose is an amorphous cross-linked polymer with a low molecular weight that can be dissolved in aqueous alkali (Timell, 1967; Huffman, 2003). Xylan is the major component of hemicelluloses in hardwood and annual plants. The polymer consists of the main chain of xylose monomer with β-1,4-glycosidic bond linkages and branches of small amounts of other saccharides that are closely associated with cellulose fibril and have lignin covering the fiber surfaces (Whistler and Richards, 1970; Reis and Vian, 2004. Thus, the effective degradation of xylan into fermentable sugar is a key process for utilization of hemicellulose materials. For complete hydrolysis of xylan, several enzymes are required including endoxylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, ferulic acid esterases and acetyl xylan esterases (Dodd and Cann, 2009). Xylanase is an important enzyme for the cleavage of internal glycosidic bonds in xylan to generate short xylo-oligosaccharides (Biely, 1985). Xylanases are a large group of hydrolytic enzymes that catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in the xylan backbone of hemicellulose (Zhang *et al*., 2011).

Most xylanases are identified into glycosidic hydrolase (GH) family 10 and 11 and many xylanases contain a catalytic domain and one or more non-catalytic carbohydrate-binding modules (CBMs) (Tomme *et al.*, 1995). The role of CBMs is to enhance catalytic efficiency and encourage targeting enzymes of insoluble polysaccharides (Henrissat *et al.*, 2000). Furthermore,

CBMs have been classified into three types based on their structure, function and ligand specificities: type A CBMs (e.g. CBM1, CBM2a, CBM10) bind the surfaces of crystalline polysaccharide, type B (e.g. CBM4, CBM17, CBM28) display an open groove that binds internally on glycan chains (*endo*-type), while type C or lectin-like CMBs (e.g. CBM13, CBM9-2) bind to the termini of glycan chains (*exo*-type) (Boraston *et al*., 1999). Teo *et al*. (2019) found that truncation of CBM in xylanase GH10 of *Roseithermus sacchariphilus* stain RA affected enzyme- specific activity and thermostability. Several reports found that the fusion of family 6 CBM into xylanase could enhance catalytic activity and adsorption on cellulose and insoluble xylan (Sun *et al*., 1998; Mangala *et al*., 2003).

Endo- 1, 4- beta- xylanase B (Xyn11) from *Penicillium oxalicum* (accession no. ADV31286.1) contains GH11 catalytic domain and a family 1 CBM that displays specificity for highly crystalline cellulose and/or chitin (Boraston *et al*., 2004). *Penicillium oxalicum* Xyn11B is an acidic- stable xylanase that is suitable for application in agricultural waste and food and feed industries (Juan *et al*., 2013; Liao *et al*., 2014). CBM family 6 displays a diversity of substrate specificities that bind to cellulose, xylan, mixed β-(1,3)(1,4)-glucan and β-1,3-glucan, agarose, chitin, lichenan, laminarin and mannan (Pires *et al*., 2004; Michel *et al*., 2009). Thus, this study aimed to investigate the effect of family 6 CBM on *Penicillium oxalicum* endo- 1,4- beta- xylanase GH11 and determine the influence of CBM family 6 on *Penicillium oxalicum* endo- 1,4- beta- xylanase GH11 in characterizing the recombinant enzyme and investigate insoluble degradation capability when they were overexpressed in *Pichia pastoris*.

MATERIALS AND METHODS

Materials

Two derivative genes of *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 accession no. ADV31286.1 (Xyn11-CBM1) and *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 fusion with CBM family 6 from GenBank accession no.WP_012488881.1 (Xyn11-CBM1-CBM6) to C-terminal were constructed and cloned in the pPICZ alphaA vector (Invitrogen). Cloned-target gene insertion was checked by DNA sequencing (ThermoFisher). Yeast nitrogen base (YNB) without amino acids was obtained from BD/Difco Laboratories (Franklin Lakes, NJ, USA). Biotin, methanol, glycerol, mono- and dibasic potassium phosphate, yeast extract, peptone and glycerol were purchased from Merck (Darmstadt, Germany). Congo red, sodium dodecyl

sulfate and Tris base were purchased from Sigma-Aldrich (MO, USA) while xylan (Beechwood) was obtained from Megazyme (USA). Restrict enzyme *Pme*I, Zeocin™ was obtained from Thermo Fisher (Invitrogen). Dextrose and other chemicals were purchased from Himedia (Mumbai, India).

Transformation and expression

Two genes (*xyn*11-CBM1 and *xyn*11-CBM1-CBM-6) were overexpressed in *P. pastoris* SMD1168H following the Invitrogen protocol. Briefly: *Escherichia coli* DH5α (Invitrogen) was used for storage and prolongation of plasmids. The pPICZA plasmids were transformed to *E. coli* DH5α strain using the heat-shock method. The *E. coli* transformant was selected on low salt Luria-Bertani agar (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.5) +30 μg/mL of Zeocin™. The plasmid was extracted by ZymoPURE™ Miniprep Kit (Irvine, CA, USA). A total of 5-10 μg of plasmids were linearized by restriction enzyme *Pme*I at 37°C for 15 minutes and the reaction was stopped at 65°C for 10 minutes. Complete linearization of plasmid was checked by 1.2% agarose gel electrophoresis and linearized-plasmids were transformed into *P. pastoris* SMD1168H through electroporation according to the parameters 400Ω, 1.8 kV and 25 uF. *Pichia* transformants were grown on YPD agar +100, +1,000 and +2,000 μg/mL of Zeocin™, respectively for multiple copies of plasmid screening (quantity selection). A single colony of recombinant *Pichia* was grown in 5 mL of BMGY medium at 30°C for 16-18 h and then diluted to OD_{600} = 1.0 before adding 5% v/v of cell suspension to 200 mL of BMMY medium. The mixture was shaken at 180 rpm for 4 days at 22°C. Methanol was fed into the medium to obtain a final concentration of 0.5% after 24 h and 1% for the remaining cultivation time. Cell suspensions were centrifuged at 6,000 rpm for 10 minutes and pellet cells were discarded. Secreted protein activity was determined using the xylan-Congo red clearance plate assay (quality selection) (Wood *et al*., 1988) and kept at -20°C until required for enzyme assay.

Optimal pH and temperature

Xylanase activity was determined using 1% beechwood xylan as substrate according to Bailey *et al*. (1992). Optimal pH values of Xyn11-CBM1 and Xyn11-CBM1-CBM6 were estimated at 40°C for 30 minutes in different buffer solutions (50 mM) as citrate buffer (pH 3.0-6.0), phosphate buffer (pH 6.0-8.0), and Tris-HCl buffer (pH 8.0-10.0). The optimal

temperature for xylanase enzyme was obtained by assaying enzyme activity at optimal pH at different temperatures (30-65°C) for 30 minutes. To determine pH stability, the enzyme was incubated at diverse pH values (3.0-10.0) at room temperature (RT) for 1, 2, 3 and 4 h. Thermal stability was determined at 30-65°C for 1, 2, 3 and 4 h, and remaining enzyme activity was then measured with 1% beechwood xylan at optimal pH and temperature for 30 minutes.

Xylanase activity assay

Xyn11-CBM1 and Xyn11-CBM1-CBM-6 were assayed in 50 mM citrate buffer pH 5.0 at 45°C using 1% beechwood xylan as a substrate. One unit of activity enzyme was defined as the activity of enzyme releasing 1 μ mol of reducing sugar per minute under these conditions. Release of xylose (incubated for 30 minutes) was measured by the dinitrosalicylic acid (DNS) method with D-xylose as standard (Miller, 1959). Concentrations of secreted proteins were determined using the Bradford method with bovine serum albumin as a standard (Bradford, 1976). The activity enzyme was expressed as μ mol min^{-1} mg^{-1} protein and relative enzyme activity was calculated by comparing with the positive control (set as 100%).

Effect of metal ions on xylanase activity

Xyn11-CBM1 andXyn11-CBM1-CBM6 activities were assayed at optimal pH and temperature for 30 minutes with 1% of substrate, and metal ions (Fe^{2+}, Cu^{2+}, Ba^{2+}, Ca^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+}) were presented at 1 mM of final concentration (or ranging between 149 and 278 ppm metal ions).

SDS-PAGE analysis and protein identification

SDS-PAGE was performed on a 6% (w/v) polyacrylamide gel and 5% stacking gel with mini-vertical gel electrophoresis (Amersham Biosciences, Buckinghamshire, England). Protein patterns were visualized by Coomassie Brilliant Blue R-250 staining (Amresco, Radnor, PA, USA) and destained with a solution containing 10% (v/v) methanol and 10% (v/v) acetic acid. The zymographic analysis was conducted using 0.5% substrate incorporation with 8% polyacrylamide gel and visualized by Congo red staining. The excess color was then washed with 1 N NaCl.

Degradation of insoluble substrate

Ten units of Xyn11-CBM1 andXyn11-CBM1-CBM6 were estimated for insoluble degradation and 1% corncob, rice husk, corn leaves and stalks, and rice straw were used as substrates. Reactions were incubated in a water bath shaker at 100 rpm, 45°C for 60 minutes. Reducing sugar release was determined by the DNS method. Each experiment was replicated at least three times with data displayed as mean ± SD. Statistical analysis was performed by SPSS (version 18). Differences between the means were analyzed by independent t-test with the level of significance defined at p-value=0.05.

Thin-layer chromatography (TLC) analysis

Xylan hydrolysis was estimated by TLC on plates of silica gel 60 F_{254} (Merck, Germany). One percent of the insoluble substrate (corncob, rice husk, corn leaves and stalks, and rice straw) was digested with 10 units of Xyn11-CBM1-CBM6 at optimal conditions for 1 h. The reaction was stopped by boiling for 15 minutes and filtered using Whatman™ paper no.1. Then, 15 μl of each sample was spotted onto TLC plates and developed with the solvent system (n-propanol: ethyl acetate: water = 6:1:3 v/v). Visualization was investigated by spraying with an ethanol-sulfuric acid mixture (95:5 v/v) containing 0.5% α-napthol and heating at 100°C for 5 minutes (Joseph and Murrell, 1983). A xylose (x1), xylobiose (x2), xylotriose (x3), xylotetraose (x4) and xylooligosaccharide mixture was used as the standard.

RESULTS

Recombinant *xyn11*-CMB1 and *xyn11*-CBM1-CBM6 expressions

Schematic of origin *Penicillium oxalicum* endo-1,4-beta-xylanase GH11(*POxyn11*-CBM1) represented the GH11 catalytic domain as a CBM1 and a signal peptide sequence. The artificial *xyn11*-CBM1and *xyn11*-CBM1-CBM6, the signal peptide was deleted and CBM6 and dockerin were added into the C-terminal of *POxyn11*-CBM1 (Figure 1). Gene target sequences were synthesized and incorporated into pPICZ alphaA and checked by DNA sequencing technique**.** A full-length plasmid carrying of *xyn11*-CBM1 and *xyn11*-CBM1-CBM6 were 3,926 and 4,904 bp, respectively. Preliminary screening of transformant clones indicated that 3 and 4 colonies of recombinant *Pichia* strains could grow on YPD agar +2,000 μg/mL of *xyn11*-CBM1 and *xyn11*-CBM1-CBM6, respectively. A single colony of recombinant *Pichia* was induced for gene expression in BMMY medium using 5% v/v of OD_{600}=1.0 in 5 mL cultures. Colonies from each recombinant gene which produced highest width haloes (3.0 and 1.4 cm of Xyn11-CBM1 and

Xyn11-CBM1-CBM6) in 1.0% beechwood xylan agar, staining with 0.1% Congo red, were selected as producing-enzyme colonies.

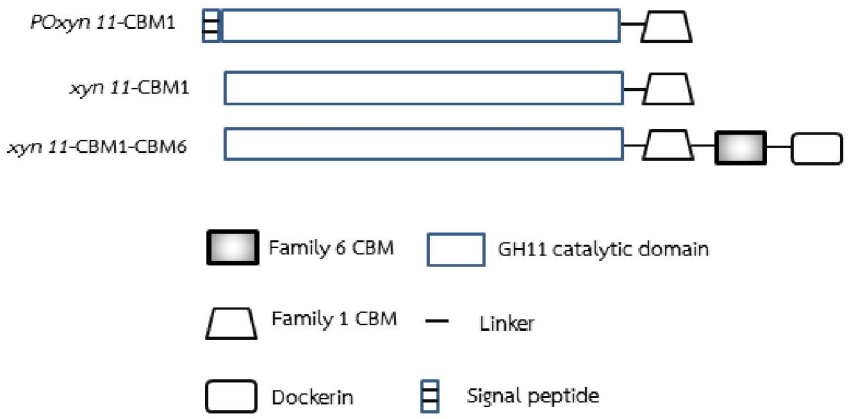

Figure 1. Schematic diagram of *Penicillium oxalicum* (accession no. ADV31286.1) gene and its derivatives.

Effects of pH, temperature and stability on Xyn11-CBM1 and Xyn11-CBM1-CBM6

Optimal pH and temperature of Xyn11-CBM1 and Xyn11-CBM1-CBM6 activity with 1% beechwood xylan were determined in pH range 3.0-10.0 and measured by the DNS procedure following the process previously described. Both enzymes showed similar reaction trend activity with pH 5.0 and 45 °C as the most suitable (Figure 2a, 2b), showing 39.07 ± 0.79 and 47.48 ± 0.33 units/ mg protein of Xyn11-CBM1 and Xyn11-CBM1-CBM6, respectively. The pH stability profiles after incubation in citrate buffer (pH 3.0-6.0), phosphate buffer (pH 6.0-8.0), and Tris-HCl buffer (pH 8.0-10.0) for 240 minutes at room temperature showed that Xyn11-CBM1 and Xyn11-CBM1-CBM6 activities were highly stable under extremely acidic conditions (showing more than 100% activity remaining at pH 4.0-6.0 and 4.0-6.5, respectively) (Figure 2c). Furthermore, Xyn11-CBM1-CBM6 activity was stable at 45°C, showing more than 85% and 50% activity remaining after incubation in phosphate pH 6.0 for 60 and 240 minutes, respectively and significantly higher than Xyn11-CBM1 (Figure 2d). Results indicated that family 6 CBM had a significant effect on *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 (*POxyn11*-CBM1) by increasing thermostability.

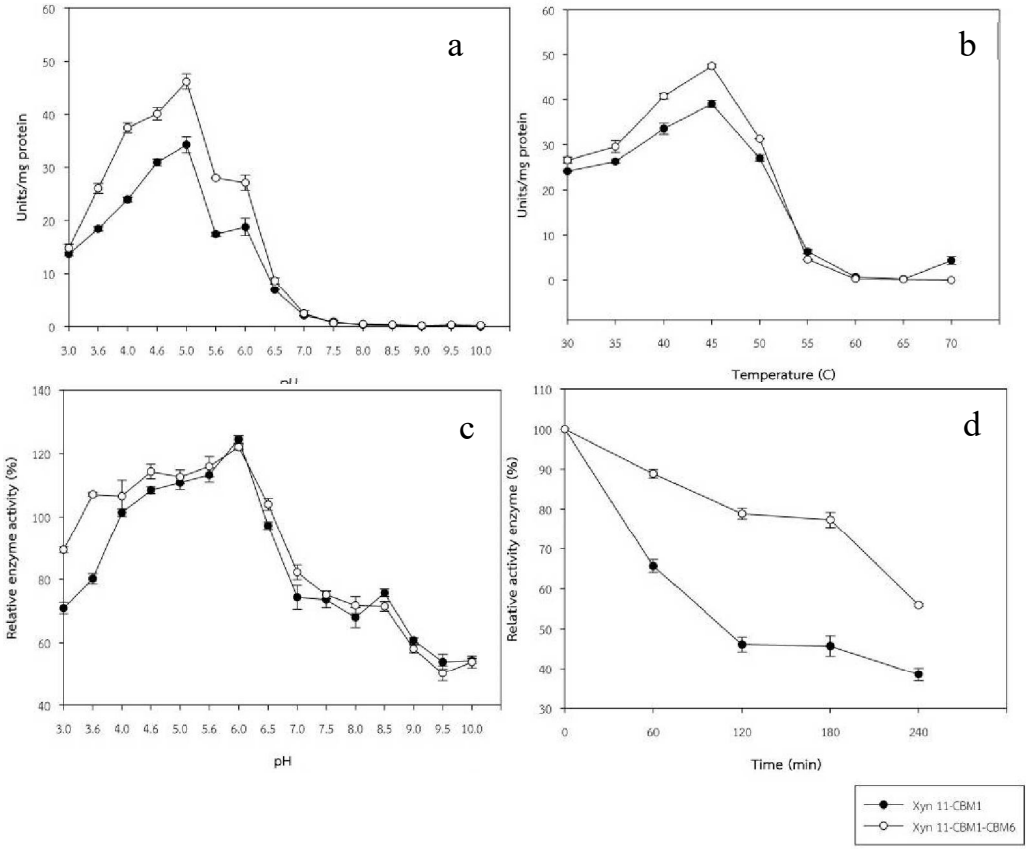

Figure 2. Optimal pH (a) and temperature (b) for activity of recombinant Xyn11-CBM1 and Xyn11-CBM1-CBM6. pH stability (c) after incubation at pH range from 3.0-10.0 for 240 minutes and thermal stability (d) at 45°C.

Effects of metal ions on enzyme activity

A previous study indicated that the concentration of metal ions at 1 mM was enough to determine positive or negative effects on xylanase activity (Liao *et al.*, 2014). Thus, here, concentrations of metal ions at 1 mM (or ranging between 149-278 ppm of metal ions) were evaluated on Xyn11-CBM1 and Xyn11-CBM1-CBM6 activity. Results showed that both Xyn11-CBM1 and Xyn11-CBM1-CBM6 were slightly inhibited in the presence of Ba^{2+}, Co^{2+}, Cu^{2+} and Fe^{3+} showing more than 80% of initial activity. In addition, Ca^{2+}, Fe^{2+} and Mg^{2+} significantly affected Xyn11-CBM1-CBM6 more than Xyn11-CBM1 (Table 1). The Mn^{2+} was an activator for Xyn11-CBM1-CBM6 at the highest value of 113.01±2.28% and significantly higher than Xyn11-CBM1, while Hg^{2+} strongly inhibited the activity of both enzymes. Thus, the common trend of metal ions on Xyn11-CBM1 and Xyn11-

CBM1-CBM6 activity was similar (decreasing) in the presence of Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+} and Hg^{2+}, while activities of both enzymes were enhanced by Fe^{3+} and Mn^{2+}. Maximum Xyn11-CBM1 and Xyn11-CBM1-CBM6 activities were 106.13±1.12 and 113.01±2.28% in the presence of Fe^{2+} and Mn^{2+}, respectively. A previous study revealed that xylanases from *P. oxalicum* GZ-2 and *P. oxalicum* B3-1(2) were strongly inhibited by Hg^{2+}, Fe^{3+} and Mn^{2+} (Juan *et al.*, 2013; Liao *et al.*, 2014). Generally, heavy metals were found in soils, water, and air and has let to contamination of food crop and the heavy metals contents between 0.10-80 ppm were found in soils of Thailand (Bernhard *et al.*, 2004; Obodai, *et al.*, 2014). Thus, before appliance enzyme in food, animal feed, agricultural and municipal waste degradation, it is necessary to ensure that the presence of metal ions could not influence on enzyme activity.

SDS-PAGE and zymogram analysis

Molecular masses of Xyn11-CBM1 and Xyn11-CBM-CBM6 were estimated at 22.96 and 57.36 kDa by amino acid sequences (data not shown). However, protein separations in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were not sharp and clear (Figure 3a). In addition, *P. pastoris* enabled some post-translational modifications which probably generated recombinant protein size higher than estimated. Activities of native proteins confirmed that the molecular weight of Xyn11-CBM1-CBM6 was higher than Xyn11-CBM-1 (Figure 3b).

Table 1. Effects of metal ions on activity of Xyn11-CBM1 and Xyn11 CBM1-CBM6

Metal ion (1 mM)	Relative activity (%) of Xyn11-CBM1	Relative activity (%) of Xyn11-CBM1-CBM6
Control	99.99±0.24	99.99±6.72
Ba^{2+}	82.31±4.61[a]	83.19±1.52[a]
Ca^{2+}	98.68±0.79[a]	90.38±2.71[b]
Co^{2+}	94.87±1.63[a]	93.34±4.98[a]
Cu^{2+}	93.25±6.39[a]	90.96±6.32[a]
Fe^{3+}	102.43±3.00[a]	105.88±0.12[a]
Fe^{2+}	106.13±1.12[a]	91.83±0.40[b]
Hg^{2+}	1.00±1.03[a]	1.69±0.00[a]
Mg^{2+}	92.00±1.61[a]	76.81±1.96[b]
Mn^{2+}	102.26±2.94[a]	113.01±2.28[b]

*All experiments were performed in triplicate (n=3) with results expressed as mean±SD. Difference between the means was analyzed by independent t-test with level of significance defined at p-value=0.05. Superscripts [a] and [b] refer to groups with different means in each row.

Degradation of insoluble materials and TLC analyses

Four insoluble substrates (corncob, rice husk, corn leaves and stalks, and rice straw) were digested with Xyn11-CBM1 and Xyn-CBM1-CBM6 to assess the hydrolysis of complex materials. Reducing sugar was evaluated by DNS. Results indicated that Xyn11-CBM-1CBM6 showed the greatest ability to digest all insoluble substrates with significant differences (p-value<0.05) (Table 2). To analyze the products of insoluble hydrolysis corncob, rice husk, corn leaves and stalks, and rice straw were digested at optimal conditions for 60 minutes. Results indicated that Xyn11-CBM1 and Xyn-CBM1-CBM6 hydrolyzed corncob, corn leaves and stalks, and rice straw into xylobiose, xylotriose and xylotetraose, respectively while xylooligosaccharide profiles of rice husk did not appear in the TLC plate. Possibly the main products of rice husk were not short-chain xylooligosaccharides (Figure 3c). Furthermore, no xylose was found in all productions, confirming *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 as an endoxylanase.

Table 2. Insoluble material degradation of Xyn11-CBM1 and Xyn11-CBM1-CBM6

Substrate	Reducing sugar (mg/mL)	
	Xyn11-CBM1	Xyn11-CBM1-CBM6
Corncob	0.05±0.01[a]	0.16±0.01[b]
Rice husk	0.07±0.003[a]	0.10±0.001[b]
Corn leaves and stalks	0.06±0.02[a]	0.14±0.02[b]
Rice straw	0.03±0.003[a]	0.10±0.01[b]

*Data are mean± SD. Difference between means was analyzed by independent t-test with level of significance defined at p-value=0.05. Superscripts [a] and [b] refer to groups with different means in each row.

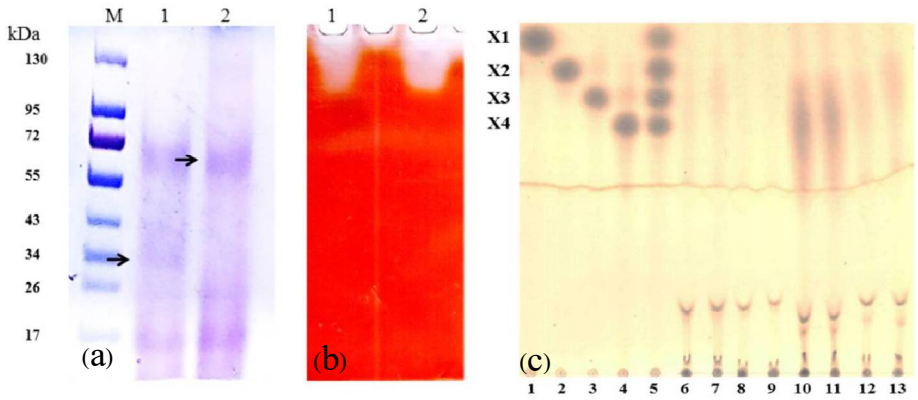

Figure 3. SDS-PAGE (a) and zymogram (b) analyses of Xyn11-CBM1 (lane 1) and Xyn11-CBM1-CBM6 (lane 2) from *Pichia pastoris* expression (arrows refer estimated size). TLC analysis of corncob (6, 7), rice husk (8, 9), corn leaves and stalks (10, 11), and rice straw (12, 13) when digested with Xyn11-CBM1 and Xyn11-CBM1-CBM6, respectively compared with xylo-oligosaccharide standard (c).

DISCUSSION

Xylan is the main composition of hemicellulose and is gaining increasing interest for sustainable resource utilization. Xylanase is widely used in food, feed, textile, and pulp and paper industries (Subramaniyan and Prema, 2002). However, the industrial application of xylanase is still limited because of its recalcitrant low activity, a complex structure of the lignocellulosic substrate and high production cost. Results suggested that family 6 CBM affected *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 by enhancing acidity tolerance and thermostability from pH 4.0-6.0 to pH 4.0-6.5 together with high temperature tolerance (showing more than 75% of initial activity after incubation at 45°C for 180 minutes). Furthermore, Xyn-CBM1-CBM6 showed more efficiency in digesting insoluble substrates. *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 has a CBM1 specific domain to bind the surfaces of crystalline structures (cellulose-binding modules) while family 6 CBM also recognizes substrates like beta-1,3-glucans, mixed beta-(1,3)(1,4)-glucans, beta-1,4-mannan, glucomannan, and galactomannan, amorphous cellulose and arabinoxylan (Michel *et al.*, 2009;

Guillén *et al.*, 2010). Thus, adding Xyn11-CBM1 with CBM6 at the C-terminal improved insoluble xylan-cellulose complex digestion by enhancing surface adsorption. In addition, main hemicellulose compositions of corncob, rice husk, corn leaves and stalks, and rice straw are arabinoxylan and arabino-glucuronoxylan which were recognized by CBM6 (Whistler and Lauterbach, 1958; Dutton and Kabir, 1972; Xiao *et al.*, 2001). Hence, this was a possible reason why Xyn11-CBM1-CBM6 showed increased potential for degradation of the complex structure of xylan than Xyn11-CBM1. Likewise, the adsorption and hydrolysis activity of xylanase A and B from *Clostridium stercorarium* on insoluble xylan were improved by the presence of two family 6 and one family 9 CBM (Ali *et al.*, 2001; Mangala *et al.*, 2003). However, the potential to encourage targeting enzymes of CBM6 and their associated glycoside hydrolase depends on substrate specificity and fusion events (the CBM position at catalytic modules). Tajwar *et al.* (2017) found that fusing CBM6 to the N-terminal of *Thermotoga maritime* xylanase did not increase activity on soluble and insoluble substrates, while fusing CBM6 to the C-terminal and CBM22 to the N-and C-terminals increased activity on insoluble substrates. Similarly, in a previous study on *Cellvibrio japonicus* endo-β-1, 4 mannanase (*Cj*Man5A), we showed that fusing CBM6 to the C-terminal did not influence mannanase activity (Dathong *et al.*, 2018). In addition, highest reducing sugar content was found when insoluble substrates were digested with cellulase and subsequently digested with recombinant Xyn-CBM1-CBM6 (data not shown). Results revealed that Xyn11-CBM1-CBM6 was suitable for converting the cellulose-hemicellulose complex into fermented sugar when using with cellulase. TLC analysis indicated that the main products of enzyme hydrolysis were xylobiose, xylotriose and xylotetraose. For hydrolysis completion, the reaction time must be extended to more than 60 minutes.

CONCLUSIONS

Family 6 CBM impacted on *Penicillium oxalicum* endo-1,4-beta-xylanase GH11 by improving acidic thermostability and insoluble degradation. Recombinant Xyn11-CBM1 and Xyn11-CBM1-CBM6 showed 39.07 ± 0.79 and 47.48 ± 0.33 units/mg protein, respectively at optimal pH and temperature. Furthermore, Xyn11-CBM1-CBM6 exhibited more than 85% and 75% of activity remaining which was significantly higher than Xyn11-CBM1 (showing less than 70% and 50% remaining) in phosphate buffer pH 6.0 at 45°C for 60 and 120 minutes, respectively. In addition, Xyn11-CBM1

and Xyn11-CBM1-CBM6 activity were slightly affected by Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+} and Mg^{2+} and strongly inhibited by Hg^{2+}. Enzyme activity was enhanced by Fe^{3+}, Fe^{2+}, Mn^{2+} and Fe^{3+}, Mn^{2+} for Xyn11-CBM1 and Xyn11-CBM1-CBM6, respectively. The capability of Xyn11-CBM1-CBM6 for complex materials hydrolysis was significantly higher than Xyn11-CBM1. TLC analysis confirmed that both recombinant enzymes were endoxylanases. These results suggested recombinant Xyn11-CBM1-CBM6 as a suitable candidate for various industries.

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