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Characterization of Xylanase activity produced by *Paenibacillus* sp. XJ18 from TNBD Jambi, Indonesia

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Lignocellulose waste in nature is increasing due to the increasing activity of agroforestry. Up to 40% of lignocellulose biomass are consisted of xylan. Xylan complete breakdown requires the action of xylanase. Xylanase has been used to breakdown xylan into commercial product such as low calories sugar, prebiotic, and biofuel. Due to its wide application, several variation of xylanase characterization are needed. Our previous studies have collected *Paenibacillus* sp. XJ18 from TNBD forest, Jambi, Indonesia, to gain a unique enzyme characteristic. In this study the characteristic of crude xylanase from *Paenibacillus* sp. XJ18 was investigated. The highest activity of xylanase production was at 36 h. The xylanase showed activity in a broad range of pH (4.5-9.0). The highest activity showed at pH 5.0, 90 °C. Crude enzyme extract was unstable and had halftime at its pH and optimum temperature about 67 min. The xylanase activity was increased about 4.59 times after being concentrated by 70% acetone (2.4578 U/mL). Based on TLC result, xylanase from *Paenibacillus* sp. XJ18 was predicted to produce xylobiose exclusively from extracted corncob xylan.

Key words: corncob xylan, *Paenibacillus* sp., TNBD Jambi, xylanase characterization, xylan extraction

INTRODUCTION

The growth of agroforestry activity increases the agricultural and forestry waste which mainly consisted of lignocellulose. Lignocellulose is a sugar in non-degradable polymer form. About 25-40% of lignocellulose biomass consisted of xylan. Xylan is a complex highly branched heteropolysaccharide. Its general structure has a linear backbone consisting of 1.4-linked D-xylopyranose residues, a reducing sugar with five carbon atoms. These may be substituted with branches containing acetyl, arabinosyl, and glucuronosyl residues, depending on the botanic source and extraction method (Habibi & Vignon 2005). Corncob has the highest xylan content than other lignocellulose waste. It consists up to 40% of xylan (Yang *et al*. 2004).

Due to the heterogeneity and complex structure of xylan, its complete breakdown requires the action of xylanase. Xylanase is a complex enzyme, includes endoxylanase (E.C.3.2.1.8), β-xylosidase (E.C.3.2.1.37), α-glucuronidase (E.C.3.2.1.139), α-arabinofuranosidase (E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72) (Juturu & Wu 2011). Endoxylanases catalyzes the random hydrolysis of xylan to xylooligosaccharides, while β-xylosidase releases xylose residues from the non-reducing ends of xylooligosaccharides.

Xylanase attracts attention of many researcher due its action to breakdown xylan into commercial product such as low calories sugar-xylose and L-arabinose (Mikkelsen-Krog *et al.* 2011), prebiotic (Aachary & Prapulla 2011), and biofuel (Koppram *et al.* 2013). Xylanase are also used as bleaching agent in pulp and paper industries and increase the effectiveness of detergent cleaning. Due to its wide application, many variation of xylanase characters are needed. Our previous studies have collected xylanolytic bacteria (*Paenibacillus* sp. XJ18*)* from Taman Nasional Bukit Duabelas (TNBD) forest, Jambi. TNBD forest is a conservation area which has some endemic species. Exploration of xylanolitic from this area may gain unique characteristic of xylanase. The characteristics of xylanase from *Paenibacillus* sp. XJ18 are reported in this study.

MATERIALS AND METHODS

Xylan Extraction from Corncob. Xylan was extracted from corncob Dramaga Silangan 3

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varieties. The xylan extraction used alkali method (Richana *et al.* 2007). The corncob was dried and milled to the size of 40 mesh. Corncob powder was used as grits by soaking it in 1% NaOCl at 27 °C for 5 h. The grifts were rinsed by distillated water and centrifuged at 805.3 x g for 25 min to separate the lignin content from celluloses and hemicelluloses. The pellet was dried by incubation in oven at 60 °C for 48 h. The chemical composition of milled corncob powder and delignified corncob were analyzed using proximate analysis including moisture, ash, lipid, protein (AOAC 1990), lignin, cellulose, and hemicellulose (van-Soest 1963).

Xylan extraction was carried out by soaking the delignified corncob into 10% NaOH at 27 °C for 24 h and was filtered. Filtrate which contained xylan was neutralized by 6 N HCl and centrifuged at 591.65 x g for 25 min. Xylan was precipitated by addition of 95% ethanol into the filtrate with ratio 1:3 (v/v). The precipitated xylan was separated by centrifugation at 262.95 x g for 30 min. The extracted corncob xylan pellet was dried by incubation in oven at 60 °C until the moisture reach approximately 5%.

Microorganism. The culture of *Paenibacillus* sp. XJ18 was isolated from Taman Nasional Bukit Duabelas (TNBD) Jambi (coordinate: S 01°94'285" E 102°581'26") with xylanolytic index value about 4.25 (24 h incubation time), performed by Congo red staining method (Kurrataa'yun 2013). The soil pH was 4.60 and its temperature was 27 °C. The culture was maintained on xylan medium with pH 5 which composed of 0.5% xylan (Sigma Chemical), 0.5% bactopeptone, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.1% K_2HPO_4.

Determination of Optimum Time for Enzyme Production. *Paenibacillus* sp. XJ18 were grown in 0.5% xylan medium (w/v), pH 5.0 at 27 °C for 24 h. Three colonies of bacteria were inoculated into 10 mL 0.5% xylan broth-medium (pH 5.0) in 50 mL Erlenmeyer flask and then incubated in shaking incubator (121 rpm) at 27 °C for 8 h (A_{600nm} = 0.7; inoculum: 1.275 x 10^9 CFU/mL). About 1% (v/v) of pre-inoculated culture was inoculated into 100 mL xylan broth-medium (pH 5.0) in 500 mL Erlenmeyer flask. The culture was incubated in shaking incubator (121 rpm) at 27 °C and collected periodically (6 h interval) for 54 h. Bacteria growth was monitored by spectrophotometry using optical density (OD) at 600 nm. Extracellular xylanase activity was measured in the culture filtrate of the submerged fermentation. Xylanase assay was carried out by incubating appropriately diluted enzyme with 0.5% beech wood xylan in citrate phosphate buffer 0.2 M pH 5.0 at 27 °C for 30 min. The released reducing sugar was determined by 3.5-dinitrosalicylic acid (DNS) method using D-xylose as standard (Miller 1959). One Unit (U/mL) of xylanase activity was expressed as the amount of enzyme (mL) that produced one µmol of xylose equivalent per minute under assay conditions. The cell broth was centrifuged at 805.3 x g for 25 min to collect the crude enzyme.

Measurement of Xylanase Activity. The optimum pH for xylanase activity was obtained by assaying the crude enzyme extract at different pH (3.0-9.0, interval 0.5 unit). Citrate phosphate buffer (0.2 M) was used for pH 3.0-6.0, phosphate buffer (0.2 M) was used for pH 6.0-8.0, and tris-HCl (0.2 M) was used for pH 9.0. Xylanase assay was performed by DNS method (Miller 1959) at 27 °C. The optimum temperature was obtained by assaying the crude enzyme extract at different temperatures (30-90 °C), in its optimum pH, using DNS method (Miller 1959).

Thermal Stability of Enzyme Assay. Enzyme stability was obtained by incubating enzyme in different storage temperature. Various temperature storages (4, 27, 50, and 90 °C) were investigated due to the purpose of enzyme storage and application in industry. Crude enzyme extract was collected per hour for 5 h. The collected incubated enzymes were assayed using DNS method (Miller 1959) at pH 5.0 and temperature 90 °C.

Precipitation of Crude Enzyme Extract. Concentrating of crude enzyme extract was optimized using the comparing of Ammonium sulphate [$(NH_4)_2SO_4$] precipitation method and acetone precipitation method. Ammonium sulphate precipitation method were performed based on Kamble and Jadhav (2012) by modification. The precipitation was done by diluting 100 mL crude enzyme extract partially into cold ammonium sulphate with concentration 0-30%, 30-40%, 40-50%, 50-60%, 60-70%, and 70-80% (w/v) gradually. The pellet and filtrate were separated by centrifugation at 591.65 x g for 15 min at 4 °C. The precipitated enzyme in pellet was diluted with 10 mL of phosphate buffer (pH 6.0).

Acetone precipitation method was done by adding cold acetone into crude enzyme extract in various concentration (60-90%; 10% interval). This method was performed based on Sana *et al*. (2008) with modification. The pellet and supernatant were separated by centrifugation at 591.65 x g for 15 min at 4 °C. The pellet was diluted with 10 mL of phosphate buffer (pH 6.0). Precipitated enzyme from both method were assayed by DNS method (Miller 1959) at its optimum pH and temperature.

Analysis of Hydrolytic Products. 1 mL of concentrated enzyme (2.548 U/mL) was mixed

with 1 mL of 0.5% corncob xylan substrate (pH 6.0) and incubated at 40 °C for 5 h to analyze the hydrolytic products. The enzyme reaction was stopped by incubation at 100 °C for 15 min. The soluble hydrolytic product was separated from the pellet by centrifugation at 2,777.45 x g for 20 min. The hydrolytic product was analyzed using thin layer chromatography (TLC) method. TLC was performed using silica gel plate with acetic acid: n-buthanol: distillated water (1:2:1) as mobile system. Monosaccharides (glucose, xylose arabinose, and mannose) and disaccharides (cellobiose, sucrose, mannobiose, and maltose) were used as standards. Concentration of each standard was 10,000 ppm. Concentrated enzyme and corncob xylan substrate were used as control. One µL of standard was spotted while 4 µL of sample and controls were also spotted.

Degree of polymerization (DP) hydrolysis product was obtained from the reducing sugar and total sugar (Wang *et al*. 2011). Reducing sugar was measured by DNS method (Miller *et al*. 1959) and xylose was used as standard. Total sugar content in the water soluble fraction was measured by the phenol-sulfuric acid method (Dubois *et al*. 1956).

RESULTS

Extracted Xylan Description. Xylan extraction by alkali method had 10.82% xylan recovery (from 2,500 g of corncob powder to 270.40 g) (Table 1). Post delignification showed decreasing percentage of lignin to about 4.3% (Table 2). Delignified of corncob showed high yield of hemicellulose content (37.92%) (Table 2).

Optimum Time of Enzyme Production. Extracellular xylanase from *Paenibacillus* sp. XJ18 showed two peaks of enzyme activity with different incubation time. The first peak was found at 18 h of incubation time (early stationary phase) and the second peak at 36 h incubation time (late stationary phase). The enzyme activity at 18 and 36 h were 0.0003 and 0.0016 U/mL, respectively (Figure 1).

Effect of pH and Temperature on Xylanase Activity. Various pH affected the activity of extracellular xylanase from *Paenibacillus* sp. XJ18. A high xylanase activity was showed at pH 5.0, 6.0, and 7.5, respectively (Figure 2). Enzyme activity assay in various temperatures were performed on

Table 2. Corncob proximate assay at pre- and post-delignification

Content	Delignification (%)	
	Pre	Post
Raw Fiber	25.15	26.09
a. Lignin	21.00	16.70
b. Cellulose	33.10	34.07
c. Hemicellulose (xylan)	17.90	37.92
Water content	7.99	5.11
Ash content	5.11	1.46
Lipid	0.54	1.53
Protein	3.23	2.00

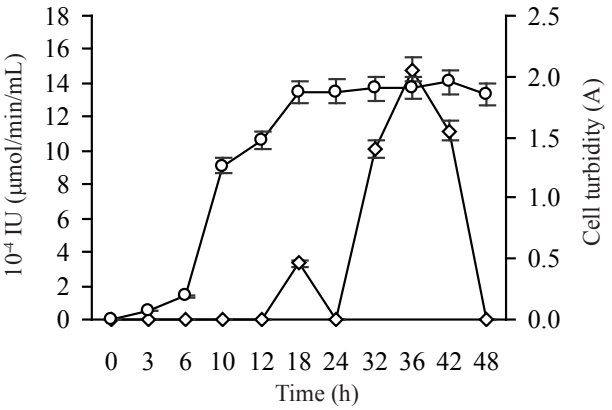

Figure 1. Production of xylanase based on *Paenibacillus* sp. XJ18 growth phase. The enzyme was produced by submerged fermentation using 0.5% xylan broth-medium (pH 5.0) in shaking incubator (121 rpm) at 27 °C. ◇ Enzyme Activity (IU), ○ Cell turbidity (A).

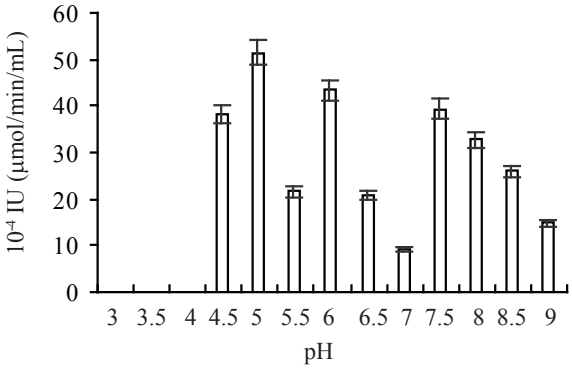

Figure 2. Effect of pH on xylanase activity from *Paenibacillus* sp. XJ18. Citrate phosphate buffer (0.2 M) was used for pH 3.0-6.0; phosphate buffer (0.2 M) was used for pH 6.0-8.0; and tris-HCl (0.2 M) was used for pH 9.0.

Table 1. Mass balance in xylan extraction by alkali method. The corncob was milled to the size of 40 mesh. Delignification was carried out by soaking the corncob in 1% NaOCl at 27 °C for 5 h. Xylan extraction was carried out by soaking the corncob in 10% NaOH at 27 °C for 24 h. The neutralized pH of corncob was precipitated by 95% ethanol (1:3) (v/v)

Process	Material	Total		Recovery percentage (%)
		Input (g)	Output (g)	
Mill	Dried corncob	4,800	2,900	60.42
Delignification	Corncob powder (40 mesh)	2,500	2,370	94.80
Extraction	Delignified corncob	2,370	270.40	11.40

afore mentioned three pH. The extracellular xylanase found as mesophile and thermophile xylanase. The highest activity enzyme of those three pH were found at temperature 90 °C. Another peak appeared at mesophile condition (pH 6.0, 40 °C), while at pH 5.0 had three other peaks at various temperatures (Figure 3). It showed that xylanase from *Paenibacillus* sp. XJ18 had broad range of characteristic enzyme.

Thermal Stability of Enzyme Assay. Extracellular xylanase from *Paenibacillus* sp. XJ18 was unstable and showed decreasing stability. Storage temperature at 90 °C had the shortest lifetime of enzyme. The enzyme activity at its optimum activity temperature had no activity at 4 h storage. The longest halftime of enzyme occurred at the 4 °C storage temperature, at about 185 min (Figure 4).

Precipitation of Crude Enzyme. The crude xylanase from *Paenibacillus* sp. XJ18 showed low activity of enzyme (0.55 U/mL). Instead of increasing, concentrated xylanase by 0-30% ammonium sulfate showed decreasing activity about 5.6 x 10^{-2} U/mL, while the specific activity did not increase significantly (0.13 U/mg). Precipitation of xylanase from *Paenibacillus* sp. XJ18 by 70% acetone showed an increased activity of enzyme nearly 5 times than the crude enzyme (4.25 U/mL). Concentrated xylanase from *Paenibacillus* sp. XJ18 by 70% acetone showed the most optimum condition with 39.60 purification fold and gain 231.82% of yield (Table 3).

Analysis of Hydrolytic Products. After 5 h incubation of xylanase from *Paenibacillus* sp. XJ18 with corncob xylan 0.5%, a product spot between monosaccharide and disaccharide was obtained on silica gel plate (RF: 0.5143). The product had the RF value lower than xylose standard (RF: 0.6514) (Figure 5). No xylooligosaccharide product was obtained on plate, but the spot of bigger product was observed at the spotted line. The polymerization degree of hydrolyzed product of extracted corncob xylan was reduced from 74 to 9.

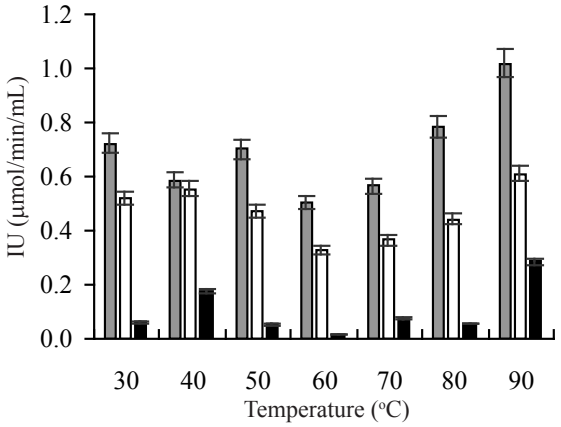

Figure 3. Effect of various temperature on xylanase activity from *Paenibacillus* sp. XJ18. Citrate phosphate buffer (0.2 M) was used for pH 5.0 and 6.0; and phosphate buffer (0.2 M) was used for pH 7.5. ■ pH 5.0, □ pH 6.0, ■ pH 7.5.

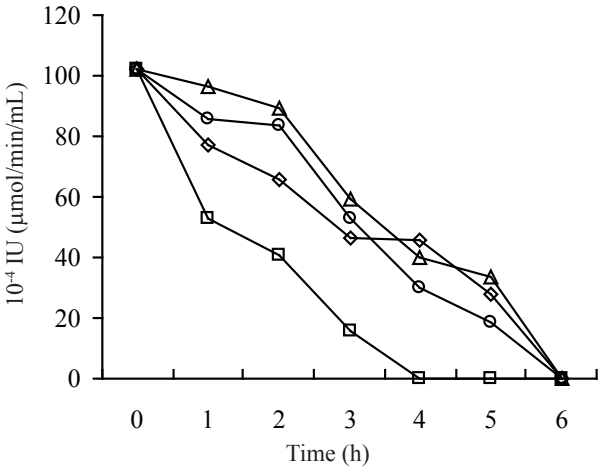

Figure 4. Thermal stability of xylanase from *Paenibacillus* sp. XJ18. About 0.5% xylan in 0.2 M buffer citrate phosphate (w/v) was used as substrate. 4 °C, 27 °C, 50 °C, 90 °C.

Table 3. Optimization concentrated method of xylanase from *Paenibacillus* sp. XJ18 using ammonium sulphate (Kamble & Jadhav 2012) and acetone precipitation method (Sana *et al*. 2010). Xylanase activity was obtained from the precipitated pellet and was measured by DNS method (pH 6.0, at 40 °C, for 30 min of incubation time)

Precipitation method	Total volume (mL)	Total enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Enzyme	100	55	513	0.11	1.00	100
0-30% Ammonium sulfate	100	5.6	42.34	0.13	1.23	10.18
30-40% Ammonium sulfate	95	0.00	40.88	0.00	0.00	0.00
40-50% Ammonium sulfate	93	0.00	55.80	0.00	0.00	0.00
50-60% Ammonium sulfate	89	0.00	54.82	0.00	0.00	0.00
60-70% Ammonium sulfate	86	0.00	82.04	0.00	0.00	0.00
70-80% Ammonium sulfate	83	0.00	88.25	0.00	0.00	0.00
60% Aseton	50	0.00	18.75	0.00	0.00	0.00
70% Aseton	50	127.5	30.03	4.25	39.60	231.82
80% Aseton	50	0.66	67.22	0.01	0.09	1.20
90% Aseton	50	0.50	103.23	0.00	0.05	0.90

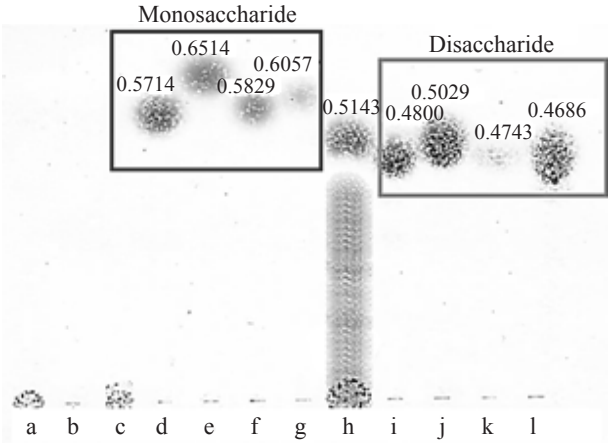

Figure 5. Hydrolysis product analyzed by TLC on silica gel plate. a. concentrated enzyme; b. phosphate buffer pH 6.0; c. 0.5% corncob xylan substrate (in phosphate buffer pH 6.0); d. glucose; e. xylose; f. arabinose; g. mannose; h. hydrolysis product of 0.5% xylan corncob with xylanase from *Paenibacillus* sp. XJ18 (1:1) for 5 h of incubation time; i. Cellobiose; j. sucrose; k. Mannobiose; l. Maltose.

DISCUSSION

Xylan extraction from corncob has been investigated by various methods. Yang *et al.* (2005) developed a dilute sulfuric acid pretreatment to enhance xylan extraction. It showed that higher steaming temperature results in higher extraction rate but also had higher reducing sugars content. Xylan extraction has also been investigated by power ultrasound treatment (Yang *et al.* 2009) and microwave treatment (Wanitwattanaramulg *et al.* 2012).

The investigation of various alkaline concentration treatment had been done by earlier studies. The increasing alkaline concentration showed higher xylan extraction rate. Richana *et al.* (2007) extracted 12.95% of xylan by 1% NaOH, while Yao (2011) used 25% NaOH and gained 24.39% yield of xylan. Corncob consists 73.04% holocellulose (34.45% α-cellulose, 18.73% β-cellulose, 19.84% γ-cellulose), 28.23% pentosans (xylan), and 16.03% total lignin (14.01% acid-insoluble lignin, and 2.02% acid soluble lignin) (Kumar *et al.* 2010). Xylan in corncob is covalently bonded with lignin and other carbohydrates, thus the raw material should be pretreated to break the bond and expose it. Delignification process in alkali treatment using NaOCl was done to solubilize majority of lignin (acid-insoluble lignin). Decoupling of xylan from lignin is important to access xylan, but complete lignin removal will result in loss of xylan from the sample. Ebringerová *et al.* (2005) found acid-chlorite bleaching as the most efficient delignification method without excessive deacetylation of xylan. The use of 0.5% NaOCl obtained the highest yield of extracted xylan, while 7.5% NaOCl could dissolved xylan and gained low yield of extracted xylan (Richana *et al.* 2007).

NaOH was used to dissolve xylan and removed α-celluloses which is insoluble in NaOH. Richana *et al.* (2007) discovered that xylan has higher solubility at 1% NaOH than hot water. Xylan extraction based on alkali method resulting in de-esterification of the acetyl groups which present on the xylan, leading to a water insoluble product. Rowley *et al.* (2013) has improved the method to gain a water-soluble product by using DMSO to dissolved xylan.

Xylanase is an inducible enzyme. Xylanase activity was observed to be induced by rich xylan, xylose, or xylooligosaccharide medium (Khucharoenphaisan *et al*. 2010). In general, although xylanases gene were transcribed in mid-log phase when cultured on xylan (Emami *et al.* 2002), the rate of xylanase production was usually found to be highest towards the late exponential or stationary phase (Emami *et al.* 2002; Pason *et al.* 2006; Knob *et al.* 2013). It is probably happened because other carbon source in medium was existed and played as repressor in xylanase production. Xylan or xylose combination with other readily metabolized carbon sources such as glucose or glycerol resulted in lower levels of xylanase production. Emami *et al.* (2002) found that no xylanase activity was detected in xylan medium supplemented with glucose until all of the hexasaccharide had been metabolized. Thus, it suggests that xylanase production can be repressed by other carbon sources. Rojas-Rejon *et al*. (2011) found cellobiose had more effect than glucose to repress xylanase activity in both mutant or parent strain of *Cellulomonas flavigena*. Beside other carbon source, D-xylose also could be repressor of xylanase production. At higher concentrations of D-xylose, a reduced level of xylanase gene expression of *Trichoderma ressei* was observed (Mach-Aigner *et al*. 2010).

Two peaks of enzyme activity with different incubation time occurred. It was predicted that the crude enzyme contained two different enzyme with two optimum condition. The appearance of multiple peaks also could be caused by isoenzyme and the existence of polysaccharide in medium. Jermyn (1962) found the combination of aryl-β-glucosidase and polysaccharide caused multiple peaks occurred at enzyme activity measurement.

Enzyme activity was affected by both pH and temperature. pH value affected the state of acidic ionization (carboxyl functional group in their side

chains) or basic amino acids (amine functional groups in their side chains). The altered ionization state of amino acids could change the ionic bonds that help to determine the 3-D shape of the protein and lead to the alteration of protein recognition that inactivated the enzyme. Crude xylanase from *Paenibacillus* sp. XJ18 showed multiple optimum pH at 5.0, 6.0, and 7.5. The optimum pH of enzyme activity was correlated with its producing-bacteria growth condition, while the others were found as neutral. It was predicted that crude xylanase from *Paenibacillus* sp. XJ18 contains at least three different protein characters.

Temperature can influence reaction rate of an enzyme. Higher temperature will lead to higher activity. But as protein, it will be limited by its thermal denaturation. Xylanase from *Paenibacillus* sp. XJ18 has various optimum temperature up to 90 ^{o}C with acidic condition, which was higher than thermophilic endo-1.4-beta-xylanase from acidophilic *Bispora* sp. MEY-1 that has been reported by Luo *et al.* (2009). Several optimum temperature and pH suggested that there are multiple kind of xylanases. It has been reported that some microorganisms produce multiple xylanases (Raj *et al*. 2013). Roy *et al.* (2013) also reported multiple xylanases and endoglucanases produced by *Simplicillium obclavatum* MTCC 9604 during growth on wheat bran. Xylanases may have diverse structures, physicochemical properties and rate of activities. It also showed the highest diversity and complexity in its enzyme family. This might be the result of a number of genes that affect xylan biosynthesis, redundancy in gene expression, and also could arise from post-translational modifications such as differential glycosylation or proteolysis, or both (Juturu & Wu 2011).

Thermostable enzymes are needed due to its potential applications in many types of industry. Xylanase from *Paenibacillus* sp. XJ18 showed shorter half life time at its optimum incubation temperature than xyl10C which had 3h of halftime (Luo *et al.* 2009). Zhang *et al.* (2014) discovered two regions in the N-terminus of thermophilic xylanase that were potentially important for the thermostability. They have constructed a mesophilic xylanase by introducing seven thermostabilizing residues from the extreme N-terminus of thermophilic xylanase. The best condition for precipitation of xylanase from *Paenibacillus* sp. XJ18 was found similar as the condition for xylanase from *Streptomyces* spp. 234P-16 (Meryandini *et al.* 2009). Acetone precipitated xylanase was more acidic and the protein was hydrophilic (Thongboonkerd *et al.* 2002). It probably showed that xylanase from *Paenibacillus* sp. XJ18 is more acidic and contains more hydrophilic residues.

The chemical structure of xylan in corncob is mainly composed of D-glucuronic acid, L-arabinose and D-xylose (2:7:19) (Ebringerová *et al.* 1994). Hydrolysis product analyses by TLC showed that the hydrolysis product was located between monosaccharide and disaccharide position. There was no xylose and arabinose product obtained. The difference of RF value of each disaccharides could showed the possibility of product as 4-O- β -D-xylopyranosyl- β -D-xylose (xylobiose). The big value of polymerization degree showed that there were many big products (polysaccharides) which unable to be hydrolyzed. These results indicated that the xylanase from *Paenibacillus* sp. XJ18 recognizes only two xylose units from one of the ends of the xylan backbone and mediates the hydrolysis of the adjacent β-1.4-linkage, producing xylobiose and bigger intermediates. Kubata *et al.* (1994) also discovered a xylanase from *Aeromonas caviae* ME-1 which produces xylobiose exclusively from oat spelt and birch xylans.

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