MICROBIOLOGY INDONESIA
ISSN 1978-3477

Volume 3, Number1, April 2008
p 23-26

Production and Purification of Xylanase From Indonesian Isolate *Bacillus* sp. AQ-1 Grown on Bunch Palm Oil

PUJI RAHAYU[1], SISWA SETYAHADI[2*], AND HARMITA[1]

[1]*Pharmacy Department, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia;*
[2]*Center for the Bioindustrial Technology, Badan Pengkajian dan Penerapan Teknologi, BPPT building 2, 15 floor, Jalan MH. Thamrin 8, Jakarta 10340, Indonesia*

Xylanase (endoxylanase, EC 3.2.1.8) is a commercial enzyme that has been applied in the industrial production of fuel, food, textiles and paper. Xylanase was isolated from the culture supernatant of *Bacillus* sp. AQ-1 grown on Nakamura medium containing 0.5% powder bunch palm oil. The optimum pH and temperature of xylanase activity were pH 7.0 and 60 °C, respectively. The enzyme was purified by anion exchange chromatography using DEAE-Sepharose-Fast-Flow column and gel filtration chromatography using Sephacryl S-300 column. The results showed that purification of xylanase produced two forms of xylanase, which were identified as xylanase A and xylanase B. Xylanase A can be separated from xylanase B by ultrafiltration using a 30 kDa polyethersulfone membrane. The molecular weight of xylanase A and B were 15.7 and 57.7 kDa, respectively.

Key words: xylanase, *Bacillus* sp., purification

Currently, xylanolytic enzymes (endoxylanase, EC 3.2.1.8) have been the focus of much attention in industry and agriculture. These enzymes have many important applications in the saccharification of agricultural wastes (Biely 1985), the improvement of bakery products (Gilbert and Hazlewood 1993), the food and feed industry (Kulkarni *et al*. 1999) as well as the textile industry (Querido *et al*. 2006). Xylanase can also be converted into liquid fuel and solvents (Woodward 1984; Biely 1985; George *et al*. 2001). Poutanen (1997) observed that both endogenous and added enzymes have an important effect on the quality of cereal foods. Xylanase is also required for the degradation of xylan (Sunna *et al*. 1997b). It is the second most abundant biopolymer (after cellulose) and the main component of hemicellulose, which is present in nature in large amounts and is a major by-product of the farming industry (Simpson *et al*. 1991). The hydrolysis of xylan to xylo-oligosaccaride and xylose is catalyzed by xylanases (Takahashi *et al*. 2000).

Xylanases are also applied for the bleaching of kraft pulp, increasing the brightness of the pulp in the pulp and paper industry (Purkarthofer *et al*. 1993; Viikari *et al*. 1994; Kulkarni *et al*. 1999). In the process of pulp bleaching, the pulp is usually treated at high temperature at alkaline pH. Therefore, thermostable xylanase would be preferred in biotechnological application, giving the advantage of running xylan digestion at high temperature. Another problem in enzyme treatment in the pulp and paper industry is the availability and cost of the enzyme, due to the high cost of the substrate for enzyme production (Hinnman 1994). Therefore, the use of low cost substrates such as solid agricultural wastes is one of the ways to reduce production costs. Bunch palm oil is one of the many solid agricultural waste materials that can be used as substrate in xylanase production. The purity of xylanase is also very important to ensure stability of enzyme in order to prevent enzyme protein denaturation. Purified xylanases have possible applications in the chemical industry, nutritional industry, beverage industry, food industry and also in the production of alternative artificial sweetener that is low in calories (xylitol) (Bhat 2000; Goulart *et al.* 2005). Aims of the present study were to produce and purify xylanase from *Bacilus* sp. AQ-1 using bunch palm oil as the substrate in the fermentation medium.

*Corresponding author:, Phone: +62-21-3169503, Fax: +62-21-3169510, E-mail: siswa59@yahoo.com

MATERIALS AND METHODS

Enzyme Production. *Bacillus* sp. AQ-1 (culture collection of Bioindustry Laboratoy, BPPT) was grown at 37 °C in Luria Broth medium containing 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract and 0.5% (w/v) sodium chloride. The culture of *Bacillus* sp. AQ-1 was incubated at 30 °C for 24 h in the Nakamura (1994) medium using powder bunch palm oil as the substrate. The substrate was prepared by sun drying for 2 days, and then was ground and sieved (35 mesh sieve). The composition of Nakamura (1994) medium was as follows: 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract, 0.1% (w/v) $K_2HPO_4 \cdot 3H_2O$, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.5% (w/v) substrate (Nakamura 1994). After 24 h, the cells were removed by centrifugation at 6 000 rpm using High-Speed Refrigerated Centrifuge Himac CR 21G and R10A3 rotor for 15 min at 4 °C and the supernantant from extracellular secretion was used for estimation of xylanase activity and protein content and also for xylanase purification.

Enzyme Activity and Protein Concentration. Xylanase activity was assayed by the dinitrosalicylic acid (DNS) method, according to Bailey *et al.* (1992). Oat spelt xylan was dissolved in 50 mM sodium phosphate buffer (pH 7.0). The reaction mixture containing 50 µL of crude enzyme and 450 µL of 1% (w/v) xylan was incubated for 5 min in the thermomixer at the optimum temperature for enzyme activity. The reaction was terminated by the addition of 750 µL of DNS reagent. The mixture was centrifuged at 14 000 rpm using High-Speed Refrigerated Centrifuge Himac CR 21G and R10A2 rotor for 2 min and the supernatant was boiled at 100 °C for 5 min. Reducing sugars released during incubation were measured as xylose equivalents by absorbance (A) at 540 nm. One unit of xylanase activity is defined as the amount of enzyme required to liberate 1 µmol of xylose per minute at the assay condition (Takahashi *et al*. 2000; Tanaka *et al*. 2005).

Protein concentration was determined by the folin-phenol method, with bovine serum albumin as a standard (Lowry *et al*. 1951). The protein content was measured at 750 nm.

Effect of Temperature and pH on Enzyme Activity. Xylanase activities were measured at temperatures ranging from 30-100 °C under the standard assay conditions. Enzymes activities were also assayed at pH values from 4.0 to 9.0 (universal buffers) at the optimum temperature.

Enzyme Purification. The cell-free supernantant of the culture was applied to the DEAE-Sepharose-Fast-Flow column (20×200 mm). Elution of the enzyme was carried out with 50 mM sodium phosphate buffer (pH 8.0) at a flow rate 1 mL min^{-1}. Each fraction was analyzed for xylanase activity and protein content. The active fractions were pooled and loaded onto Sephacryl S-300 column (16×600 mm) at a flow rate 0.5 mL min^{-1}. Equilibration and elution were performed with 50 mM sodium phosphate buffer (pH 8.0). Fractions were collected and analyzed for xylanase activity and protein content. The final steps of xylanase purification was carried out by ultrafiltration using a 30 kDa polyethersulfone membrane.

Electrophoresis. The molecular weight of the purified xylanase was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in 12% (v/v) gels using low molecular weight markers (14.4-97.0 kDa). A suspension of beechwood xylan at a final concentration 0.1% (w/v) was incorporated into the separating gel before addition of ammonium persulphate. Proteins were visualized by silver staining with silver nitrate solution.

Zymogram Analysis. The electrophoretic gels were washed in 2.5% (v/v) Triton X-100 for 60 min and then incubated at 60 °C for 15 min in 50 mM sodium phosphate buffer (pH 7.0). The reaction was stopped by incubation at 4 °C for 15 min. The gel was then incubated in 0.1% (w/v) congo red solution for 20 min at room temperature with gentle shaking and destaining was accomplished by washing the gel with 1 M NaCl until clear bands, indicating xylanase activity, became visible. The addition of 1 N HCl caused the background to turn dark blue, emphasizing the activity bands (Sunna *et al*. 1997a).

RESULTS

Enzyme Production. Microbial xylanases are usually inducible and secreted into culture medium containing xylan. The extracellular xylanase activity of *Bacillus* sp. AQ-1 grown on medium containing 0.5% (w/v) powder bunch palm oil in 24 h incubation was 65.90 U mL^{-1}, while its protein content was 0.5 mg mL^{-1}. Xylanase activity was determined at optimum temperature and pH.

Effect of Temperature and pH. Fig 1 shows the effect of temperature and pH on xylanase activity. The optimum temperature of xylanase at pH 7.0 was observed at 60 °C (Fig 1a), while the optimum pH for xylanase activity was observed at pH 7.0 (Fig 1b).

Enzyme Purification. Purification was carried out at room temperature (26-28 °C). Sodium phosphate buffer (50 mM, pH 8.0) was used throughout the purification procedure. The purification protocol involves two steps of column chromatography: ion exchange and gel filtration. Filtrate from the culture was applied onto a DEAE-Sepharose-FF column (20×200 mm). Xylanase was eluted from that column as a single protein peak that coincided with the peak of enzyme activity (Fig 2). The active fractions of anion exchange chromatography (fractions 1-30) were further purified by gel-filtration-chromatography using Sephacryl S-300 column (16×600 mm). Fig 3 shows the xylanase activity and protein content from the gel filtration chromatogram. The active fractions from that column (fractions 185-360) were subjected to ultrafiltration. Using this method, xylanase A had been separated from xylanase B. Xylanase A was in the permeate solution, while xylanase B was in the retentate solution. Table 1 summarizes the procedure for the purification of extracellular xylanase from *Bacillus* sp. AQ-1, indicating the degree of purification and yield for each step.

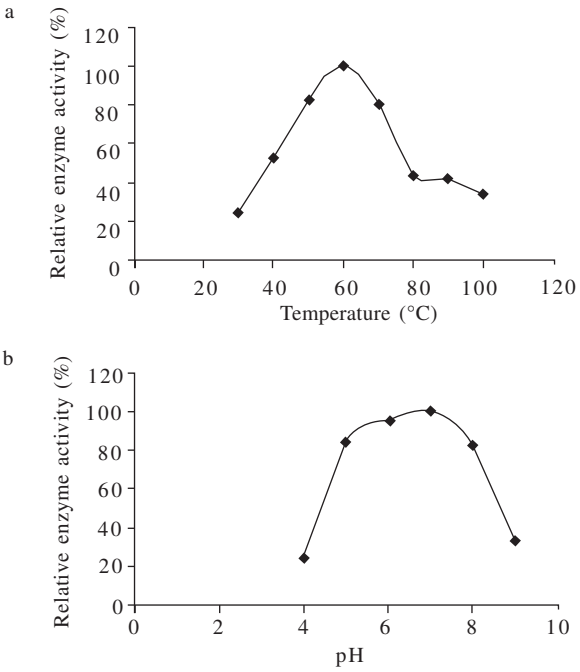

Fig 1 Influence of temperature (a) and pH (b) on the activity of xylanase from *Bacillus* sp. AQ-1. Optimum activity temperature was measured at pH 7.0, while optimum activity pH was determined at the optimum temperature. Buffers used for optimum pH determination were 50 mM sodium acetate (pH 4.0), 50 mM sodium citrate (pH 5.0), 50 mM sodium phosphate (pH 6.0-7.0) and 50 mM Tris-HCl (pH 8.0-9.0) at final concentration.

Electrophoresis. Electrophoretic studies of crude extract and purified xylanase showed the presence of multiple bands (Fig 4). A low molecular weight ranging from 14.4 to 97.0 kDa was also observed. The molecular weight standards were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa) and phophorylase b (97 kDa).

Zymogram Analysis. The purity of xylanase was demonstrated by SDS-PAGE using 12% (v/v) gel containing 0.1% (w/v) beechwood xylan (Fig 5). The permeate and retentate solution of ultrafiltration produced single activity band on each of the two gels. The molecular weight of these purified enzymes were estimated to be about 15.7 kDa (xylanase A) and 57.7 kDa (xylanase B) by standard curve of molecular weight.

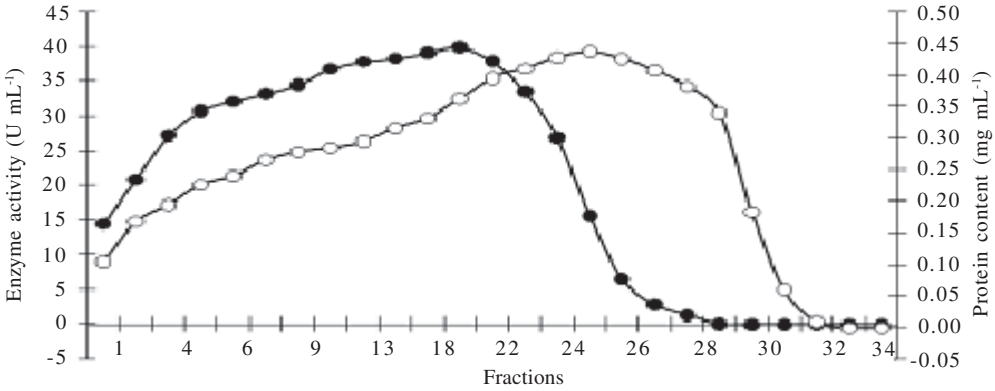

Fig 2 Elution profile of enzyme activity (●) and protein content (○) of xylanase cell-free extract by anion exchange chromatography in DEAE-Sepharose-FF column (20×200 mm). The column was eluted with 50 mM sodium phosphate buffer (pH 8.0).

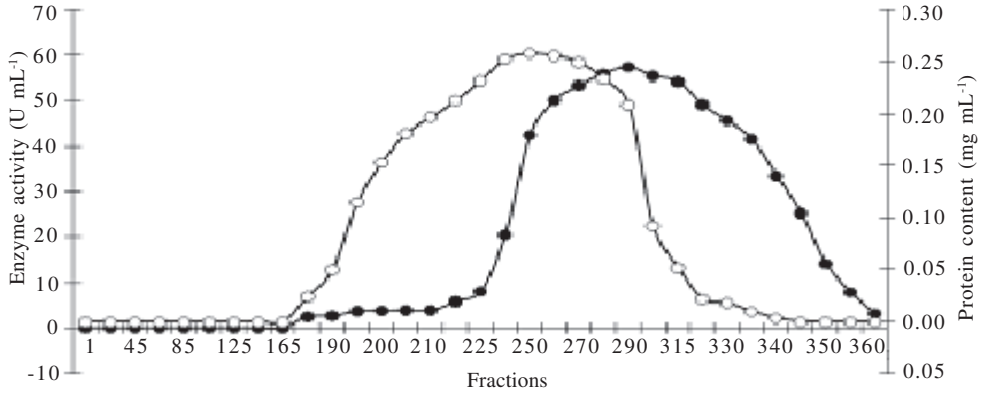

Fig 3 Elution profile of enzyme activity (●) and protein content (○) of pooled fractions from the first column by gel filtration chromatography in Sephacryl S-300 column (16×600 mm). The column was eluted with 50 mM sodium phosphate buffer (pH 8.0).

Table 1 Purification profile of xylanase from *Bacillus* sp. AQ-1

Purification steps	Volume (mL)	Total activity (U)	Protein (mg)	Specific activity (U mg^{-1})	Purification factor (fold)	Recovery[a] (%)
Culture fluid	50	3238.19	26.90	120.37	1.00	100.00
DEAE Sepharose FF	50	2736.06	13.60	201.23	1.67	84.49
Sephacryl S-300	40	2270.20	10.77	210.73	1.75	70.11
Ultrafiltration						
Permeate (Xyl A)	20	230.08	2.79	82.41	0.68	7.11
Retentate (Xyl B)	1	6.14	0.04	165.96	1.38	0.19

[a] recoveries are expressed as percentage of initial activity. Purification factors are calculated on the basis of specific activity

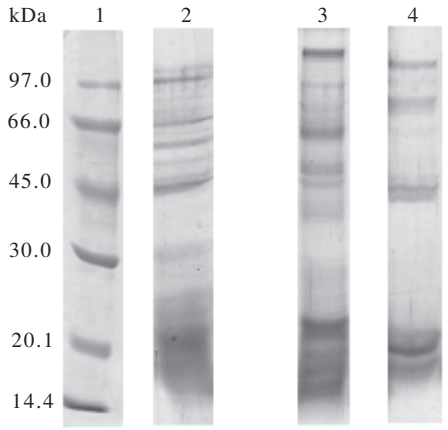

Fig 4 SDS-PAGE, of crude xylanase. Lane 1, protein standards; lane 2, xylanase crude extract; lane 3, permeate ultrafiltration; lane 4, retentate ultrafiltration.

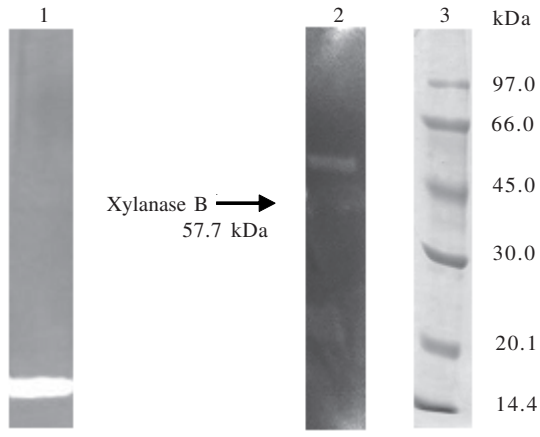

Fig 5 SDS-PAGE of purified xylanase. Lane 1, permeate solution; lane 2, retentate solution of ultrafiltration; lane 3, protein standards.

DISCUSSION

Hydrolysis of xylan requires multiple enzymes with different modes of action acting in cooperation. Different microorganisms produce different kinds of xylanolytic enzymes. In this work, we have produced xylanase from *Bacillus* sp. AQ-1 at an activity level of 65.90 U mL^{-1} using bunch palm oil as the xylan substrate. As shown in Fig 1a, the xylanolytic enzyme produced by *Bacillus* sp. AQ-1 was optimally active at 60 °C. Similar results were observed for other microorganism, an optimal temperature of xylanase produced by *Aspergilus oryzae* was at 60 °C (Kitamoto *et al*. 1999). The enzymes reported to date are optimally active in neutral pH. The highest activity was observed at pH 7.0 when incubated at the optimum temperature (60 °C). Although some microorganisms produce xylanases that have optimum activity at pH above 8, most alkaliphilic and alkali-tolerant microorganisms produce xylanases that are optimally active around neutral pH (Blanco *et al*. 1995).

Using ion exchange chromatography, gel filtration chromatography and ultrafiltration, purified enzymes can be separated from other proteins. Some proteins at a low concentration and lacking assayable xylanase activity, may be coeluted with the enzyme. Two forms of xylanases, produced from ion exchange chromatography and gel filtration chromatography, were identified as xylanase A and xylanase B. Ultrafiltration using a 30 kDa polyethersulfone membrane separated both of them, one enzyme was in the permeate solution (xylanase A) and the other enzyme was in the retentate solution (xylanase B).

A summary of purification steps is presented in Table 1. The overall levels of recovery were 7.11 and 0.19% (xylanase A and B), with 0.68- and 1.38-fold purification of xylanase A and B, and with specific activity of 82.41 and 165.96 U mg^{-1} protein, respectively. The purification factor of xylanase A was lower than expected (less than 1). The high specific activity of xylanase B suggests that it is the major extracellular xylanase produced by *Bacillus* sp. AQ-1. The apparent purity of the enzyme was demonstrated by SDS-PAGE and zymogram analysis with gel containing 0.1% (w/v) beechwood xylan, which showed the molecular weight of xylanase A and B were 15.7 and 57.7 kDa, respectively.

In conclusion, we report the production of xylanase from *Bacillus* sp. AQ-1 grown on medium containing powder bunch palm oil gave high assayable enzyme activity. Even though various materials can be used as a substrate in the xylanase production, bunch palm oil is a potential substrate to produce the enzyme. The results from xylanase purification also confirm that xylanase B, with an apparent molecular weight of 57.7 kDa is the major xylanase secreted by *Bacillus* sp. AQ-1. In the future, purified xylanase can be applied in the chemical, beverage and functional food industry or in xylitol production.

ACKNOWLEDGEMENT

We thank Badan Pengkajian dan Penerapan Teknologi for encouragement, financial support and facilities to carry out this work. We also appreciate the help given by all the staff of BPPT.

REFERENCES

Bailey MJ, Biely P, Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257-70.

Bhat MK. 2000. Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355-83.

Biely P. 1985. Microbial xylanolitic system. Trends Biotechnol 3:289-90.

Blanco A, Colom JF, Pastor FIJ, Vidal J. 1995. Purification and properties of xylanase A from alkali-tolerant *Bacillus* sp. strain BP-23. Appl Environ Microbiol 61:4468-70.

George SP, Ahmad A, Rao MB. 2001. A novel thermostable xylanase from *Thermomonospora* sp.: influence of additives on thermostability. Biores Technol 78:221-24.

Gilbert HJ, Hazlewood GP. 1993. Bacterial cellulose and xylanases. J Gen Microbiol 193:187-94.

Goulart AJ, Carmona EC, Monti R. 2005. Partial purification and properties of cellulase-free alkaline xylanase produced by *Rhizopus stolonifer* in solid-state fermentation. Braz Arch Biol Tech 48:327-33.

Hinnman RL. 1994. The changing face of the fermentation industry. Chem Technol 24:45-8.

Kitamoto N, Yoshino S, Ohmiya K, Tsukagoshi N. 1999. Purification and characterization of overexpressed *Aspergillus oryzae* xylanase, XynF1. Biosci Biotechnol Biochem 63:1791-94.

Kulkarni N, Shendye A, Rao M. 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411-56.

Lowry OH, Rosebrough PJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biochem 193:265-75.

Nakamura S, Nakai R, Wakabayashi K, Ishiguro Y, Aono R, Horokoshi K. 1994. Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus sp*. Strain TAR-1. Biosci Biotechnol Biochem 58:78-81.

Poutanen K. 1997. Enzymes: An important tool in the improvement of the quality of cereal foods. Trends Food Sci Tech 8:300-06.

Purkarthofer H, Sinner M, Steiner W. 1993. Cellulose-free xylanase from *Thermomyces lanuginosus*: optimization of production in submerged and solid-stable culture. Enzyme Microb Tech 15:677-82.

Querido ALA, Coelho JLC, Araujo EF, Chaves-Alves VM. 2006. Partial purification and characterization of xylanase produced by *Penicillium expansum*. Braz Arch Biol Tech 49:475-80.

Simpson HD, Haufler UR, Daniel RM. 1991. An extremely thermostable xylanase from the thermophilic eubacterium *Thermatoga.* Biochem J 277:413-17.

Sunna A, Prowe SG, Stoffregen F, Antranikian G. 1997a. Characterization of the xylanases from the new isolated thermophilic xylan-degrading *Bacillus thermoleovorans* strain K-3d and *Bacillus flavothermus* strain LB3A. FEMS Microbiol Lett 148:209-16.

Sunna A, Puls J, Antranikian G. 1997b. Characterization of the xylanolytic enzyme system of the extreme thermophilic anaerobic bacteria *Thermatoga martima*, *T. neapolitana*, *T. thermarum.* Comp Biochem Physiol 118A:453-61.

Takahashi H, Nakai R, Nakamura S. 2000. Purification and partial characterization of a basic xylanase produced by thermoalkaliphilic *Bacillus sp.* Strain TAR-1. Biosci Biotechnol Biochem 64:887-90.

Tanaka H, Nakamura T, Hayashi S, Ohta K. 2005. Purification and properties of an extracellular endo-1,4-b-xylanase from *Penicillium citrinum* and characterization of the encoding gene. J Biosci Bioeng 100:623-30.

Viikari L, Kantelinen A, Sundquist J, Linko M. 1994. Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Ref 13:335-50.

Woodward J. 1984. Xylanase: function, properties and applications. Top Enzyme Ferment Biotechnol 8:9-30.