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Direct Cloning of a Xylanase Gene from Pawan-Riau Hot Spring

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A functional gene containing an Open Reading Frame (ORF) encoding a β-1, 4-endoxylanase glycosyl hydrolase family 11 was cloned directly using metagenomic PCR-cloning method from Pawan Hot Spring sample in Riau. The gene consisted of 642 nucleotides, encoded for 213 amino acids. The amino acid sequence analysis using BLAST showed that the gene has high homology (93%) with xylanase gene from *Bacillus subtilis*. The gene showed its function when it was subcloned into an expression vector and overexpressed in *E. coli*. The crude extract of the recombinant enzyme had activity for 170 U/ml at 50 °C. The result of this work showed that metagenomic approach was a powerful short cut method to obtain recombinant biocatalyst that was useful for industrial application.

Key words: β-1, 4-endoxylanase, metagenomic DNA, Pawan-Riau hot-spring

INTRODUCTION

β-1, 4-Endoxylanases (Xylanases) (EC 3.2.1.8.) are enzymes that catalyze the cleavage of xylan backbone at 1-4 carbon linkage regularly to produce xylose and xylo-oligosaccharides. Xylanase is an important enzyme for biotechnology, because it is one of the xylanolitic enzymes which can degrade xylan, into useful substance such as xylose. Xylan is the second major of world abundant polymer waste. Xylanase is also a promising enzyme to replace the use of environmentally-harmful chemical in paper industry (Kulkarni *et al*. 1999).

Some researchers have isolated, purified, and characterized microbial xylanases and also cloned the genes using classical approach with pure microbial strains (Tsujibo *et al*. 1997; Chang *et al*. 2004; Huang *et al*. 2005). Unfortunately, this conventional method, limits analysis of microorganisms that grow under laboratory condition (Cowan *et al*. 2005). The number of prokaryotic microorganism cells on earth was estimated at 10^{30}, and consist of between 10^6 and 10^8 separate species (Cowan 2000). From this diversity, only 1% can be cultured and have been already studied using classical cultivation approach (Amann *et al*. 1995). Therefore, we lost the unculturable microorganisms that place 99% of microorganisms' population in biosphere. They remain as untapped resources for novel genes useful for industry (Cowan *et al*. 2005). When the final target is to obtain a recombinant enzyme, standard conventional microbiological methods sometimes becomes cumbersome. This is due to the researchers must conduct "double screening" i.e., screening the target isolate followed by screening the target genes, consecutively.

In this paper, the retrieving of a functional recombinant xylanase gene using metagenomic approach was described. In this study, I directly attained a xylanase gene from the environmental DNA sample from Pawan hot spring (Riau, Indonesia), which is a potential resource of thermophilic enzymes gene. The gene was retrieved by means of a Polymerase Chain Reaction (PCR)-cloning, then subcloned into pET101/D-TOPO expression vector and overexpressed in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Medium. Bacterial strains which were used as hosts for plasmid amplification and expression were *E. coli* DH5 α (Sony Suhandono, Department of Biology, Institute of Technology Bandung) and *E. coli* BL21 star (Invitrogen), respectively. Plasmids used in this experiment were pGEM-T easy (Promega) and pET101/D-TOPO expression vector (Invitrogen). The 1.5% agar LB medium containing antibiotic and X-gal/IPTG were used for selection *E. coli* harbouring recombinant plasmid. Agar LB medium containing antibiotic and 0.5% Oat-spelt xylan was used for confirmation of the gene expression in *E. coli*.

Sampling and Environmental DNA Extraction. Sample used in this work was a mixture of mud and water collected from Pawan hot spring in Riau with temperature approximately 55 °C and pH approximately 8. The taken samples were put into sterile bottles. The samples were stored at 4 °C, or in -80 °C for longer storage. Metagenomic DNA was extracted from 5 g (wet) sample using method proposed by Zhou (Zhou *et al*. 1996) with some modification. The procedure was modified by freezing and thawing before extraction. The thawed sample was mixed with DNA extraction buffer (containing 100 mM Tris-HCl, 100 mM sodium EDTA, and 100 mM sodium phosphate, 1.5 M NaCl, and 1% CTAB), heated, and then extracted with 10% SDS. The protein contaminant was removed by chloroform/isoamyl alcohol, then crude DNA was precipitated by isopropanol. The crude DNA extracted was then purified further using Gene Clean Kit (Q-Biogene, USA), low melting agarose (Invitrogen), or column cartridge

(Promega). The extracted DNA was analyzed using 0.8% agarose gel electrophoresis. Other than extraction method proposed by Zhou *et al*. (1996), the extraction using Soil DNA preparation Kit (Q-Biogene) was also performed.

Genetic Engineering Experiment. The protocols in genetic engineering experiment were performed based on the standard protocols (Sambrook & Russel 2001). Prior to the xylanase analysis, amplification of 16S-rDNA was performed to check the capability of the extracted DNA as the template. The extracted and purified DNA was then used as a template for PCR using 5'-AAT GCG GCC GCA ATG TTT AAG TTT AAA AAG AAT TTC T -3' as a forward primer and 5'-GC TCT AGA TTA CCA CAC TGT TAC GTT AGA ACT T -3' as a reverse primer. The primers and Taq polymerase were purchased from Invitrogen and primers were specific for endoxylanase glycosyl hydrolase family 11 as a result of alignment of xylanase family 11 from *Bacillus*. Taq polymerase PCR condition were 94 ^{o}C for three minutes for hot start, then 45 second, 52 ^{o}C 1 minute, 72 ^{o}C 2 minutes for 30 cycles using Thermal cycler (Eppendorf). The plasmid DNA containing the confirmed gene of xylanase family 11 from *Bacillus licheniformis* strain I5 in pBAD/gIII vector was chosen as the positive control. DNA marker used was 1 kb DNA ladder with size range from 100 bp-12 kbp (Invitrogen). The documentation of the agarose electrophoresis was conducted using Geldoc equipment (Kodak).

The PCR product with the predicted size then cut and purified with Gene Clean Kit, incubated at 72 ^{o}C for 30 minutes to add extra adenines (A) at the 3'-end of the PCR products, then ligated to pGEM-T easy vector at 4 ^{o}C over night. The ligation mixture was used to transform *E. coli* DH5 α using ampicilin and white/blue screening.

The positive clones then were analyzed using *Eco*RI restriction enzymes purchased from Invitrogen. The positive clones harbouring the predicted size were sequenced and analyzed. One sequenced gene then was subcloned into pET101/D-TOPO champion vector using proof reading PCR amplification with *Pfu* polymerase (Promega) under the same PCR condition but without hot start step. The forward primer used in the proof reading PCR was 5'-CACC ATG TTT AAG TTT AAA AAG AAT-3', whereas the reverse primer was the same as the first Taq-PCR amplification. The ligation then transformed to *E. coli* DH5 α, and the positive plasmid harbouring xylanase gene was chosen. This plasmid then was transformed again into *E. coli* BL21 star using electroporation (Gene pulser, Biorad). The positive colony with clear zone was checked in LB containing Oat Spelt xylan (0.5%) to observe the xylanase activity.

Bioinformatics Analysis of *Xylanase* Gene. The plasmid DNA was sequenced in the Center for Assessment of Biotechnology, BPPT. The DNA sequence encoding xylanase was analyzed using CHROMAS and Genetyx software. The search for similarity in nucleotides and amino acid level was performed using BLAST program in http://www.ncbi.nlm.nih.gov. The gene then was submitted into GenBank, USA. The multiple sequences analysis was performed through server http://www.genome.ad.jp.

The xylanase genes which were used in multiple alignment analysis were: *B. licheniformis* (Accession Number AAZ17387), *Paenibacillus macerans* (Acc. Num. AAZ17386), *Bacillus amyloliquefaciens* (Acc. Num. AAZ17388), *B. circulans* (Acc. Num. AAM08360), *B. subtilis* (Acc. Num. CAA84276), and *Thermobacillus xylanilyticus* (Acc. Num. CAJ87325). Signal peptide was predicted using http://www.cbs.dtu.dk server.

Recombinant Enzyme Preparation and Enzyme Assay. The colony of *E. coli* BL21 star which contained positive recombinant xylanase was cultivated at 50 ml LB containing ampicilin, and at OD_{600} = 0.6, IPTG was added and the cultivation was continued for four hours. The cells were pelleted by centrifugation and suspended in 5 ml 20 mM phosphate buffer pH 7 containing 1 mM mercaptoethanol, and disrupted by sonication on-off for five minutes. The crude extract containing the recombinant enzyme was recovered by centrifugation (13,000 rpm). Xylanase activity was measured (samples in duplicate) based on Miller method using dinitrosalicilic acid to detect reducing sugar (Miller 1959; Bailey *et al.* 1992) at temperature range between 30 and 70 ^{o}C. One unit of xylanase activity was defined as the amounts of enzyme to produce 1 μmol of xylose per min under the assay conditions.

RESULTS

The success of metagenomic approach is depending on the purity of DNA extracted from the environment. The extracted DNA from Pawan-Riau hot spring without further purification could not give any PCR amplification product. Several purification protocols such as using column cartridge or melting agar were carried out, however only purification with Gene Clean Kit provided the consistent result.

The extraction of environmental DNA from the same sample by using Soil DNA Extraction Kit, without recommended lyses equipment, gave clean DNA (Figure 1a). However, it resulted without large size DNA and gave negative amplification in 16S rDNA amplification experiment due the fragmented size. The extraction of environmental DNA from the sample by using method proposed by Zhou gave genomic DNA both in high molecular weight and in the fragmented size (Figure 1b). However, it provided PCR amplification product band mainly at the same size as the predicted one (approximately 650 bp) (Figure 1d). Thus, for this sample, the Zhou *et al*. (1996) method gave the better result compare to the soil DNA extraction kit.

A gene called *xyn* PW8 was cloned directly from this sample then was successfully subcloned into a pET101/D-TOPO expression vector. It was confirmed as a functional gene, which provided a positive recombinant *E. coli* BL21 star with xylanase activity (Figure 2). The activity of recombinant xylanase in the crude extract after cell disruption was 170 U/ml at 50 ^{o}C (Figure 3).

This PW8 xylanase gene had an ORF containing 642 bp nucleotide that encoded 213 amino acids (Figure 4). The gene obtained in this work had been submitted into GenBank with Acc. Num. DQ888232. The GC content of the gene was 41%;

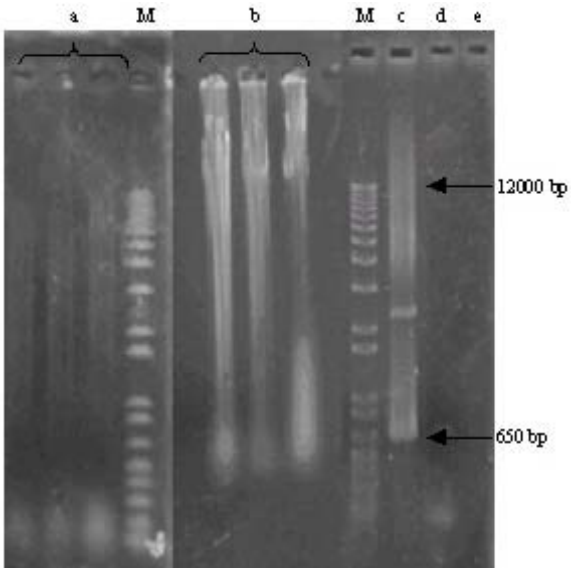

Figure 1. DNA extracted and the positive clones of *E. coli* BL21 star containing functional xylanase gene directly extracted. a. Extraction of environmental DNA from Pawan-Riau hot spring using soil DNA extraction kit, b. Extraction of environmental DNA from Pawan-Riau hot spring based on Zhou *et al*. (1996) method, c. Positive control (pBAD/gIII vector containing gene encoding of xylanase family 11 from *B. licheniformis* strain I5), d. PCR product using Pawan-Riau hot spring metagenomic sample as a template, e. Negative control. M: DNA marker 100 bp-12 kb.

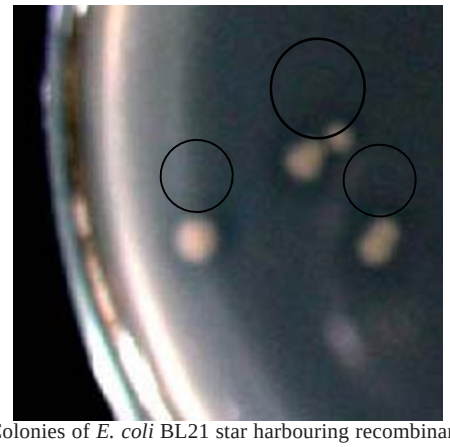

Figure 2. Colonies of *E. coli* BL21 star harbouring recombinant xylanase (PW8) with clear zone area (circled).

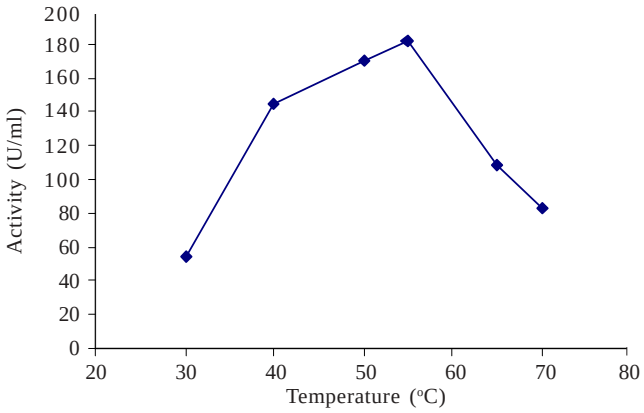

Figure 3. Effect of temperature on enzyme activity of crude extract of PW8 recombinant xylanase in duplo experiments. Enzyme sample was in 20 mM phosphate buffer pH 7 containing 1 mM mercaptoethanol.

whereas the calculated molecular mass and isoelectric point (pI) of gene product were 23,323 Da and 9.85 (including the signal peptide), respectively. The amino acid homology of this recombinant xylanase with other *xylanases* was analyzed as shown in Figure 5. This PW8 xylanase gene has differences in 13 amino acids from that of *B. subtilis* xylanase or has homology of 93% identity. Amino acid number 1 up to 30 were as predicted as a signal peptide based on signal peptide prediction server (http://www.cbs.dtu.dk).

DISCUSSION

The extraction of environmental DNA and the direct cloning of functional xylanase gene were undertaken. Two methods of the metagenomic approach were conducted; the first was the sequence-based approach (PCR-cloning) with the result of DNA sequences, and the second was functional based approach (metagenome library) with the result of functional clones (Cowan *et al*. 2004). In this work, we chose the first approach to discover xylanase genes from Riau hot spring samples, because it has several advantages. The experiment of PCR-cloning approach was not too complicated compared to the metagenome library. It was convenient and relatively not a time-consuming method. However, obtaining only partial genes and/or putative enzymes gene without any proven enzyme activity, becomes the disadvantages of PCR-cloning approach. In fact, there is less metagenomic DNA amount in more extreme habitat. Hence, I suggest that PCR-cloning is the best choice for utilization of environmental genetic of hot springs.

Metagenomics approaches can be used to explore the discovered of enzymes and secondary metabolites such as amylase, esterase, and antibiotic (Gillespie *et al*. 2002; Yun *et al*. 2004; Rhee *et al*. 2005). In these researches, abundant samples were used for metagenomic DNA extraction.

In this work, I carried out PCR-cloning of xylanase genes directly from Pawan-Riau hot spring environmental sample. I focused on xylanase gene since it has many biotechnological applications with relative high economically values (Kulkarni *et al*. 1999). Direct cloning of xylanase gene from human guts was carried out by Hayashi *et al*. (2005) and from rumen gut by Liu *et al*. (2005). Sunna and Bergquist (2003) described the PCR-cloning from environmental DNA of hot pool in New Zealand. Their studies reported direct cloning of xylanase genes from metagenomic sample using similar PCR-cloning approach. However, in their papers they reported different type of xylanase. Sunna and Bergquist (2003), and also Liu *et al.* (2005) isolated xylanase gene belong to glycosyl hydrolase family 10 using degenerate primers, whereas Hayashi *et al.* (2005) isolated gene of xylanase family 8 which they stated as a novel xylanase.

I designed and used specific primers for xylanase family 11. These specific primers covered the whole gene (ORF) of the xylanase gene. Actually, the other degenerate primers for other xylanase genes also designed and applied into PCR amplification. However, only specific primers of xylanase family 11 (*xyn 11*) gave positive results in this experiments. An example of the use of specific primer sets that are specific to

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        10        20        30        40        50        60
aatgcggccgcaatgtttaagtttaaaaagaatttcttagttggattaacggcagcttta
            M  F  K  F  K  K  N  F  L  V  G  L  T  A  A  L
        70        80        90       100       110       120
atgagcattagcttgttttcggcaaccgcctctgcagctagcacagactactggcaaaat
M  S  I  S  L  F  S  A  T  A  S  A  A  S  T  D  Y  W  Q  N
       130       140       150       160       170       180
tggactgatgggggcggaatggtaaacgctgtaaatgggtctggcgggaattacagtgtt
W  T  D  G  G  G  M  V  N  A  V  N  G  S  G  G  N  Y  S  V
       190       200       210       220       230       240
gattggtctaatacgggaaatttcgttgttggtaaaggttggactacaggttcgccatct
D  W  S  N  T  G  N  F  V  V  G  K  G  W  T  T  G  S  P  S
       250       260       270       280       290       300
aggacaataaactataatgccggagtttgggcgccgaatggcaatggatatttgacttta
R  T  I  N  Y  N  A  G  V  W  A  P  N  G  N  G  Y  L  T  L
       310       320       330       340       350       360
tatggttggacgagatcacctctcatagaatactatgtagtggattcatggggtacttat
Y  G  W  T  R  S  P  L  I  E  Y  Y  V  V  D  S  W  G  T  Y
       370       380       390       400       410       420
agacctactggaacgtataaaggtactgtaaaaagtgatgggggtacatatgacatatat
R  P  T  G  T  Y  K  G  T  V  K  S  D  G  G  T  Y  D  I  Y
       430       440       450       460       470       480
acaactacacgttataacgcaccttccattgagggcccaaaaactacttttacgcagtac
T  T  T  R  Y  N  A  P  S  I  E  G  P  K  T  T  F  T  Q  Y
       490       500       510       520       530       540
tggagtgttcgccagtcgaagagaccaactggaagcaacgctaaaatcactttcagcaat
W  S  V  R  Q  S  K  R  P  T  G  S  N  A  K  I  T  F  S  N
       550       560       570       580       590       600
catgttaaagcatggaagagtcatggaatgaacctggggagtatttggtcttaccaagtc
H  V  K  A  W  K  S  H  G  M  N  L  G  S  I  W  S  Y  Q  V
       610       620       630       640       650       660
ttagcgacagagggatatcaaagtagtggaagttctaacgtaacagtgtggtaa
L  A  T  E  G  Y  Q  S  S  G  S  S  N  V  T  V  W  *
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Figure 4. Nucleotides sequences of PW8 xylanase and the deduced amino acid (GenBank Acc. Num. DQ888232). The bold type showed the forward primer sequence for cloning in pGEM-T easy. The underlined nucleotides showed *Not* I restriction site for further experiment in different vector. The underlined amino acids showed the predicted signal peptide.

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PW8xyn      MFKFKKNFLVGLTAALMSISLFSATASAASTDYWQNWTDGGGMVNAVNGSGGNYSVDWSN
Bsub        MFKFKKNFLVGLSAALMSISLFPATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
Blich       MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
Paenib      MFKFKKNFLVGLTAAFMSISMFSATASAAGTDYWQNWTDGGGTVNAVNGSGGNYSVNWSN
Bamyl       MFKFKKNFLVGLSAALMSISLFSATASVASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
Bcirc       MFKFKKNFLVGLSAALMRIILFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN
Thermob     ------------------------------NTYWQYWTDGIGYVNATNGQGGNYSVSWSN
                                          . *** **** * ***.**.******.***

PW8xyn      TGNFVVGKGWTTGSPSRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
Bsub        TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
Blich       TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
Paenib      TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
Bamyl       TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG
Bcirc       TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWPRSPLIEYYVVDSWGTYRPTG
Thermob     SGNFVIGKGWQYGAHNRVVNYNAGAWQPNGNAYLTLYGWTRNPLIEYYVVDSWGSYRPTG
            :****:****  *:  *.:*****.* ****.*******.*.************:*****

PW8xyn      TYKGTVKSDGGTYDIYTTTRYNAPSIEGPKTTFTQYWSVRQSKRPTGSNAKITFSNHVKA
Bsub        TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFSNHVNA
Blich       TYKGTVKSDGGTYDIYTTTRYNAPSIDGDNTTFTQYWSVRQSKRPTGSNAAITFSNHVNA
Paenib      TYKGTVKSDGGTYDIYTTTRYNAPSIDGDNTTFTQYWSVRQSKRPTGSNAAITFSNHVNA
Bamyl       TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA
Bcirc       TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQTKRPTGSNATITFSNHVNA
Thermob     DYRGSVYSDGAWYDLYHSWRYNAPSIDG-TQTFQQYWSVRQQKRPTGSNVSITFENHVNA
             *:*:* ***. **:* : *******:*   ** ******* *******. ***.***:*

PW8xyn      WKSHGMNLGSIWSYQVLATEGYQSSGSSNVTVW
Bsub        WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
Blich       WKSHGMNLGSNWAYQVLATEGYKSSGSSNVTVW
Paenib      WKSHGMNLGSNWAYQVLATEGYKSSGSSNVTVW
Bamyl       WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW
Bcirc       WKSHGMNLGSNWAYQVMATGGYQSSGSSNVTVW
Thermob     WGAAGMPMGSSWSYQVLATEGYYSSGYSNVTVW
            * : ** :** *:***:** ** *** ******
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Figure 5. Alignment of xylanase studied in this work with other xylanase glycosyl hydrolase family 11. PW8 *xyn*; xylanase gene from uncultured bacteria Pawan-Riau hot spring (Acc. Num. DQ 888232, this study); Bsub: that from *B. subtilis* (Acc. Num. CAA84276); Blich: xylanase gene *B. licheniformis* (Acc. Num. AAZ17387); Paenib: *P. macerans* (Acc. Num. AAZ17386); Bamyl: *B. amyloliquefaciens* (Acc. Num. AAZ17388); Bcirc: *B. circulans* (Acc. Num. AAM08360); Thermob: *T. xylanilyticus* (Acc. Num. CAJ87325).

bacterial lipase and nitrile hydratase *á*-subunit sequences in amplifying metagenomic DNA extracts and obtaining putative gene fragments was described by Cowan group previously (Cowan *et al*. 2005). However, they only succeeded up to the putative gene but did not conduct the functional gene experimentally.

DNA sequence from this study gave a full ORF with no frame shift and had the initiation as well as the stop codon. Hence, from the DNA sequence it can be predicted that the DNA was a functional gene. This prediction was proven that the crude extract of recombinant enzyme showed its activity at temperature range between 30-70 ^{o}C and the highest activity was at 55 ^{o}C (Figure 3). This optimum temperature was similar to that of the habitat temperature. Purification and further characterization of the recombinant *xylanase* such as thermostability and pH profile are still on progress.

The results of this study showed that the endoxylanase family 11 might be an ubiquitous enzyme and widely distributed among the microorganisms inhabited the hot springs. Since the homology of this metagenomic xylanases was very high to xylanase from *B. subtilis* (approx. 93% amino acid identity), it was predicted that the ubiquity was at least among *Bacillus* species. The ubiquity of gene encoding lipaseA among *Bacillu*s species living in soil habitat was described by Ruiz *et al.* (2003). Thus, the same phenomenon might be found in xylanase family 11 of *Bacillus* species.

This hypothesis was also supported by the BLAST result that showed this xylanase genes of family 11 have very highly conserved region at N-terminal (or at signal peptide region) and C-terminal as well as at the internal amino acid sequence among *Bacillus* species (Figure 5).

Studies of direct cloning of xylanase gene from environmental sample is still limited, and this current study might be the first example of the direct cloning of xylanase gene from Indonesian thermal habitats. Using the direct cloning approach from the environmental sample, I succeeded to obtain the recombinant xylanase faster than that of the conventional approach. Hence, it was proven that the metagenomic approach was a very potential and faster way to obtain both novel genes and their products.

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