Malaysian Journal of Microbiology, Vol 14(7) 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Cloning and expression of alkalophilic xylanase gene from an Indonesia local *Bacillus halodurans* CM1 in *Escherichia coli* and its application on deinking process of waste paper

Is Helianti[1]*, Maria Ulfah[1], Shafa Noer[2], Niknik Nurhayati[1] and Endang Saefudin[2]

[1]Center for Bioindustrial Technology-Agency of Assessment and Application of Technology (BPPT), Building 611, LAPTIAB-BPPT, Puspiptek-Serpong, Tangerang Selatan 15314, Indonesia.
[2] Department of Chemistry, Faculty of Mathematics and Natural Science, University of Indonesia, Depok, Jawa Barat, Indonesia.
Email: ishelianti@gmail.com, is.helianti@bppt.go.id

Received 17 January 2018; Received in revised form 2 August 2018; Accepted 4 August 2018

ABSTRACT

Aims: In recent studies, xylanase is the valuable enzyme in paper industry, and the demand of this enzyme is increasing. The finding out of the new xylanases with good properties and applicable for paper industries from Indonesian biodiversity is still required. In this study, we isolated, cloned, and expressed the gene encoding alkalophilic xylanase gene from Indonesia indigenous *Bacillus halodurans* CM1 in *Escherichia coli*, and applied the recombinant xylanase in deinking process.
Methodology and results: The open reading frame of the gene consist of 1191 bp, with nucleotide identity 99% with that of *B. halodurans* C-125 has been obtained by using PCR. The gene was submitted into GenBank with accession number KU759320. The gene was then subcloned and expressed in *E.coli* using pET 21d(+) vector, and we found that only extracellular that have significant activity. The gene product had optimum activity at 65 °C and pH 9. After purification using Ni-NTA, the single band was obtained, and the molecular mass was about 45 kDa based on SDS/PAGE analyses. The recombinant xylanase had been applied in deinking process of old news paper at laboratory scale of a paper industry. This recombinant xylanase application showed better brightness and whiteness, as well as higher brightness and whiteness gain of of product compared to the commercial one when using the same raw material.
Conclusion, significance and impact of study: The study is the first example of the cloning of industrially important enzyme (xylanase) from *B. halodurans* CM1 and showed potential application of the recombinant enzyme in deinking process of waste paper.

Keywords: Bacillus halodurans, thermoalkalophilic xylanase, deinking process, cloning

INTRODUCTION

The decreasing of forest based raw materials is a major problem in the growth of paper industry (Kumar and Satyanarayana, 2014). One alternative to overcome the scarcity and increasingly expensive raw materials of paper from the original pulp (virgin pulp) is the re-use of waste paper as raw material (Lee *et al.*, 2007). Deinking is an important process in waste paper recycling, because a major problem with recycled fiber is the removal of ink (Lee *et al.*, 2013). To obtain fiber from waste paper, deinking process is the first step to remove ink from the fibers. Conventional deinking process by using chemicals will affect the environment because it will produce possibly harmful waste. The use of enzymes in deinking process demonstrates the ability of a reduction in chemical use and wastewater treatment. As compared to the conventional chemical deinking process, enzymatic deinking has attracted a great value due to its minimum impact on the environment and cost savings (Rismijana *et al.*, 2003).

For applications in pulp bleaching, deinking and textile industries, the property of xylanase must be thermostable and alkalistable, because these processes are carried out at alkaline pH and high temperatures (Collins *et al.*, 2005). Bleaching paper pulps with xylanases was the first successful commercial application for these enzymes (Prade, 1996). The recombinant thermotolerant and alkalophylic xylanase (Xyn3F) was used for pre-bleaching of industrial eucalyptus pulp with no prior pH adjustment (Weerachavangkul *et al.*, 2012). *Bacillus halodurans* S7 was also reported as a producer of thermostable alkaline active endo-β-1-4-xylanase that potential in pulp and paper industry (Mamo *et al.*, 2006).

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14() 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

The production of thermo-stabile endoxylanase by a novel polyextremophilic *B. halodurans* TSEV1 in combination with commercial cellulase was also studied obtaining highly effective in deinking of waste paper at alkaline pH and elevated temperatures (Kumar and Satyanarayana, 2014).

Alkalophilic microorganisms have made a great impact in industrial application, since their capability to produce alkalophilic or alkaline-stable enzymes. Alkalophiles microorganism can be isolated from normal environments such as garden soil, although viable counts of alkalophiles are higher in samples from alkaline environments (Horikoshi, 2004). Many of the alkalophilic microorganisms have been found to produce xylanases which isolated in the near neutral region but relatively have high activities in alkaline conditions. Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.8) which catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan (Collins *et al.*, 2005). Xylan is the main polysaccharides found in the plant tissue cells and one-third of all renewable organic carbon on earth (Prade, 1996). The conversion of this plant residue involved enzyme into the value-added products such as biofuels, bio-plastics, small sugar: glucose, xylose and chemicals, pre-bleaching of paper pulps and deinking of waste paper are in high demand for industrial applications (Satyanarayana *et al.*, 2012).

Recently, we isolated a thermoalkalophilic bacterium from sediment of mineral rich, local hot spring, Cimanggu, West Java. The temperature of the spot was 60 °C and the pH was 8. Based on the analyses of 16S rRNA sequence similarity and biochemical characteristics, this strain was clustered into the same group of *B. halodurans* species with 99% identity to *B. halodurans* C-125. We designated the strain as *B. halodurans* CM1 (Ulfah *et al*., 2011). In this work we cloned the alkalophilic xylanase gene isolated from this strain and expressed in *E. coli* using pET21d(+) expression vector. The trial of the gene product into deinking application at the laboratorium scale in a paper industry was also conducted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

Bacillus halodurans CM1 was previously isolated. The 16S rDNA sequence was deposited at NCBI genbank (accession no. JN903769) (Ulfah *et al.,* 2011). Plasmid maintenance and cloning were done in *E. coli* DH5α (genotypes: *F−endA1 hsdR17* (*rk−mk+*), *supE44 thi1 recA1 gyrA* (*Nalr*), *relA1D* (*lacZYAargF*), *U169* (*ø80lacZDM15*)). The plasmid used for cloning was pGEM-T Easy (Promega, USA). 1.5% (w/v) agar Luria-Bertani (LB) plate media containing 100 µg/mL (w/v) ampicillin, 50 µg/mL (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 1 mM isopropyl-β-thiogalactopyranoside (IPTG) were used for selection of *E. coli* DH5α harbouring recombinant plasmids. LB agar medium containing ampicillin (100 µg/mL) and 1% (w/v) oat spelt xylan was used for monitoring xylanase expression (Noer, 2011). The plasmid used for expression was pET21d(+) with N-terminal T7-Tag sequence plus an optional C-terminal His-Tag sequence. Luria-Bertani (LB) media containing 100 µg/mL (w/v) ampicillin and 1 mM (IPTG) were used for expression in *E. coli* BL21 star (DE3) (Genotype: F^{-}*ompT hsdS*$_B$ (r_B^-, m_B^-) *galdcmrne131* (DE3)).

Xylanase gene amplification, cloning, sequencing, and subcloning into expression vector

The alkalophilic xylanase gene was amplified from *B. halodurans* CM1 genomic DNA using degenerated primer ½ Forward: 5'-ATG(C/A)T(A/T)A(A/C)A(A/C)(C/T)(G/T)TT (A/T)AGAAA(A/G)CC-3' and ½ Reverse: 5'-TTAATC(G/A)ATAATTCTCCA(A/G)(A/T)AAGC-3'. This alkalophilic xylanase gene was then cloned into pGEM-T Easy vector and generated pGEM-Alkxyn CM1 (Noer, 2011). The fragment then was sequenced to know the valid DNA sequence. The full length of alkalophilic xylanase gene of *B. halodurans* CM1 including its native signal sequence was subcloned into pET21d(+) expression vector under T7 RNA polymerase promoter using primer Forward 5'-CATGCCATGGAAATGATTACA CTGTTTAGAAAACC-3' and Reverse 5'-CCGCTCGAGAT CGATAATTCTCGAGTAAGC-3', with *NcoI* and *XhoI* restriction site, respectively. The gene was ligated in the vector which cut with the same restriction enzyme (Figure 1B). The positive pET21d(+)-Alkxyn CM1 recombinant plasmid generated (Figure 1C) then was used to further transform *E. coli* BL21 star (DE3). This recombinant *E. coli* then used further in expression experiment. Cloning, subcloning, PCR, and DNA plasmid preparation followed standard protocols as in the work of Sambrook and Russel (2001). Sequencing was done using primers in frame with vector sequences. The DNA sequence was analyzed by performing BLAST searches (http://www.ncbi.nlm.nih.gov/). The DNA sequence then was submitted into Genbank to obtain accession number.

Xylanase enzyme from recombinant *E. coli*

The recombinant *E. coli* containing pET21d-Alkxyn CM1 was cultured in LB media containing ampicillin (100 µg/mL) under 37 °C for 16-18 h. Five percent of seed culture was transferred into LB fresh medium containing ampicilin (100 µg/mL) until OD_{600} ~0.5, then induced by 1 mM IPTG. The enzyme was produced under aerobic fermentation in 28 °C for 24 h. The enzyme was recovered by centrifugation. The supernatant was collected for enzyme assay. The pellet was also investigated for the activity by resuspended with buffer sodium phosphate pH 7 with 1 mM mercapto ethanol then sonicated for 5 min with 20 sec on and 20 sec off.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14() 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

Enzyme purification

For xylanase purification, all operations were performed at 4 °C unless otherwise mentioned. After growth in liquid medium, supernatant was obtained by centrifugation at 12,000 rpm for 15 min at 4 °C. The crude extract of enzyme was purified by HisPur™ Ni-NTA Purification Kit (Thermo Scientific) according to the instructions provided by the manufacturer. The homogeneity of the purified enzyme was monitored by SDS-PAGE. SDS-PAGE was performed in a 12% (w/v) polyacrylamide gel. Protein bands were visualized by PageBlue™ Protein Staining Solution (Thermo scientific). Protein was determined by the Bradford assay (Biorad) according to the instruction provided by manufacturer using bovine serum albumin as a standard.

Assay of enzyme activities

The crude xylanase extract was obtained by recovering supernatant after centrifuging (3000× g, 10 min, 4 °C) the culture. The purified xylanase was obtained after purification. Both crude enzyme extract and purified one were used for enzyme assay. Xylanase activity was measured (each sample in triplicates) by Miller method using dinitrosalicylic acid to detect reducing sugar and D-xylose was used as a standard (Miller, 1959; Bailey *et al.*, 1992) with validated procedure as described previously (Helianti *et al.*, 2010). Protein concentration was measured by means of dye-binding assay method of Bradford and bovine serum albumin (BSA) was used as the standard protein (Bradford, 1976).

Effect of pH and temperature on xylanase activity

The optimum condition of temperature on xylanase activity was measured at 55-70 °C. The effect of pH on the activity at pH range 7-11 using the following buffers: 50 mM phosphate buffer (pH 7), 50 mM Tris-HCl (pH 8-9), and 50 mM Tris-Glycine buffer (pH 10-11). Xylanase activity was measured duplicates using the Miller (1959) method using dinitrosalicylic acid to quantify reducing sugar. D-xylose was used as a standard. Fifty microliter crude extract at appropriate dilutions in phosphate buffer was mix with 450 µL of 0.5% beechwood xylan in 50 mM of buffer at the indicated pH. The mixture was then incubated at the indicated temperature for 5 min. Subsequently DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, 20% (w/v) potassium sodium tartrate) was added to stop the reaction. Then the mixture was boiled at 100 °C for 5 min, and kept at room temperature. Afterwards 250 L of water was added. The absorbance was measured at 540 nm. As blank we used the same mixture as in the above sample; but the enzymes was added following addition of DNS into the reaction mixture. One unit xylanase activity was defined as the amount of enzyme that releases 1 µmol of xylose per min under the assay condition.

Application of recombinant enzyme in deinking process (laboratory scale)

This recombinant xylanase was used in laboratory scale of deinking process of old newspaper (ONP) based on protocol done at laboratorium of a paper industry in West Java, Indonesia. ONP were obtained by disintegrating the paper supplies at the mill. The pulp was treated in the presence of sodium hydroxide and surfactant, deinked by flotation and washing. The deinking process without enzymes, the one with commercially available enzyme, and the target process with our recombinant xylanase were tried. The improvement of brightness and the whiteness of the paper produced were measured and compared. Analysis of brightness and the whiteness properties were prepared by making hand sheets. The brightness and whiteness were determined with a spectrocolorimeter a common spectrophotometer used in paper industry (Datacolor ELREPHO).

RESULTS AND DISCUSSION

Xylanase gene amplification, cloning, and sequencing

B. halodurans C-125 is a halophilic bacterial strain whose genome have been completely sequenced so far. From this information several outstanding enzymes have been discovered and studied (Takami *et al.*, 2000). Our *B. halodurans* CM1 was also an alkalothermophilic bacterium that isolated from a hot spring in Cimanggu, West Java, Indonesia. From the morphology and biochemical properties there were several difference compared to the C-125 strain, however, the 16S rDNA analysis showed identity 99% (Ulfah *et al*., 2011). This bacterial strain produced xylanase that have optimum activity at pH9 and temperature 70 °C. Hence, we tried to isolate this alkalylanase from this same species but lived in different habitat.

The degenerate primers were designed based on the alignment of other DNA encoding xylanase family 10 glycosyl hydrolase from several species (Takami *et al*., 2000; Martinez *et al.*, 2005; Mamo *et al*., 2006; Chang *et al.*, 2007). A positive PCR amplicon (1.2 kbp) was obtained and continued onto ligation with pGEM-T Easy TA cloning vector (Figure 1A). This DNA sequences with 1191 bp length were submitted into GenBank with accession number KU759320. Nucleotide BLAST result showed that this xylanase has 99% identity with that from *B. halodurans* C-125, and 94% identitiy with that from *B. halodurans* S7 (data not shown). In the amino acid level the alignment with other xylanase revealed the xylnase had homology with other *B.* xylanase genes, showed similarity 98% with the one from *B. halodurans*, or 66% with the one from *B. campisalis* 66% (Figure 2).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14() 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

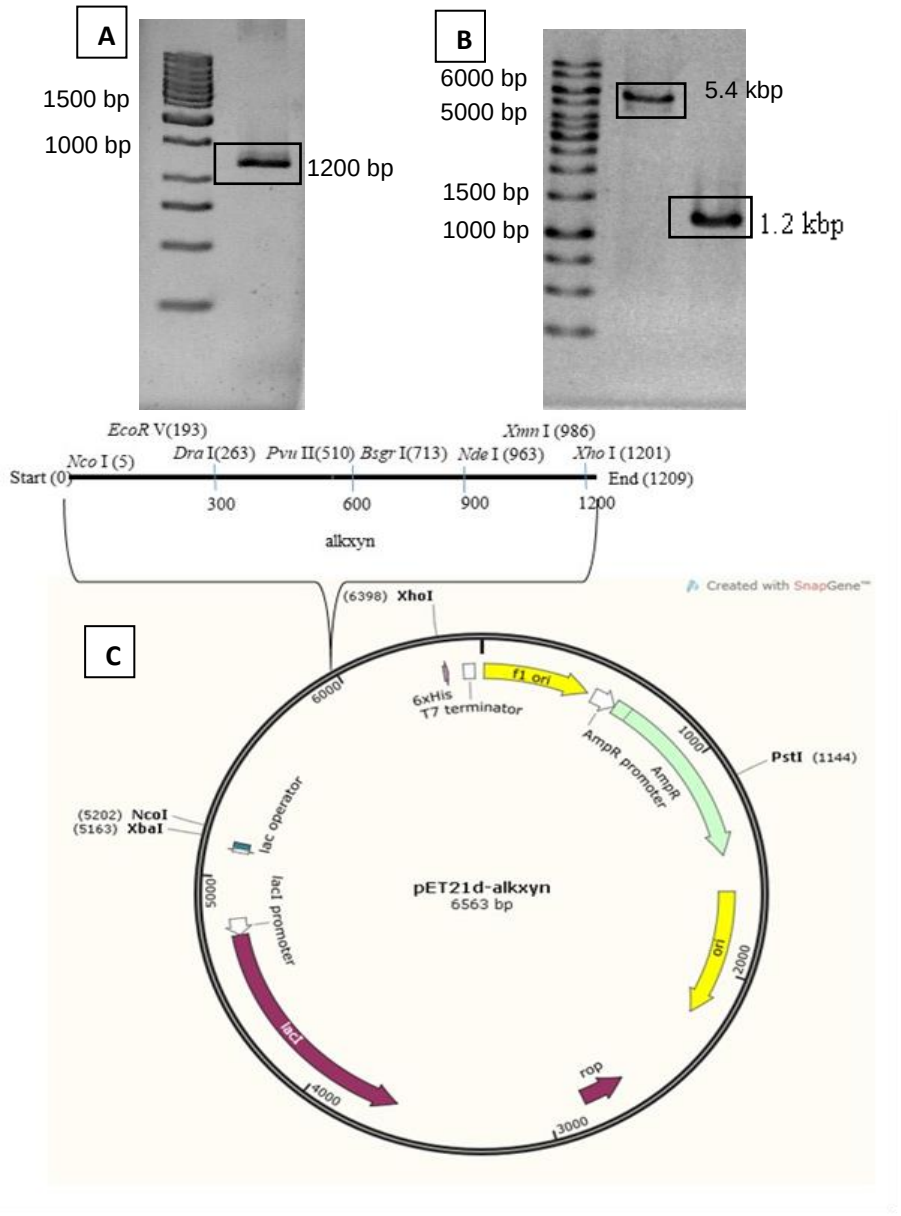

Figure 1: The cloning procedure of alkalophilic xylanase gene. (A) The PCR amplification of alkalophilic xylanase gene from *B. halodurans* CM 1 using degenerate primer mentioned in the materials and method and genome *B. halodurans* CM1 as the template. The specific PCR product is in the expected size (1.2 kbp), this product then was cloned into pGEM T Easy. (B) The PCR product using specific primer containing alkaline xylanase gene using specific primer containing *NcoI* and *XhoI* restriction site and pGEMT Easy containing alkaline xylanase gene fragment as the template (1.2 kbp) , and pET21d(+) vector after cutting using the same restricition enzyme (5.4 kbp). The gel picture was taken before ligation. (C) Map of recombinant plasmid pET21d. The 1211 bp fragment of alkaline xylanase was inserted by ligation at *NcoI* and *XhoI* site of the pET21d(+) vector.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14() 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

```
KU759320          MITLFRKPFVAGLAISLLVGGGIGNVAA---AQGGPPKSGVFGENEKRND--QPFAWQVA
AY376353          MITLFKKPFVAGLAISLLVGGGLGNVAA---AQGGPPKSGVFGENQKRND--QPFAWQVA
2UWF_A            ------------------------------------------------N--QPFAWQVA
AAB70918          MLKTLRKPFIAGLALSLLLTGGASSVFAQGNGQAGPPKGGIFKEGEKGNGNVQPFAWQVA
WP_046525547      MLKVLRKPLITGLALALLLPAAGTGAAASN---------------------APVSALEAA
                                                                     * :.*

KU759320          SLSERYQEQFDIGAAVEPYQLEGRQAQILKHHYNSLVAENAMKPESPQPREGEWNWEGAD
AY376353          SLSERYQEQFDIGAAVEPYQLEGRQAQILKHHYNSLVAENAMKPVSLQPREGEWNWEGAD
2UWF_A            SLSERYQEQFDIGAAVEPYQLEGRQAQILKHHYNSLVAENAMKPVSLQPREGEWNWEGAD
AAB70918          SLADRYEESFDIGAAVEPHQLNGRQGKVLKHHYNSIVAENAMKPISLQPEEGVFTWDGAD
WP_046525547      PLEERYKDSFNIGAAIEPHQLEGISGEVLKRHYNSIVAENVMKPINIQPEEGKFNFEEAD
                  .* :**::.*:****:**:**:* ..::**:****:****.*** . **.** :.:: **

KU759320          KIVEFARKHNMELRFHTLVWHSQVPEWFFIDEDGNRMVDETDPDKREANKQLLLERMENH
AY376353          KIVEFARKHNMELRFHTLVWHSQVPEWFFIDENGNRMVDETDPEKRKANKQLLLERMENH
2UWF_A            KIVEFARKHNMELRFHTLVWHSQVPEWFFIDENGNRMVDETDPEKRKANKQLLLERMENH
AAB70918          AIVEFARKNNMNLRFHTLVWHNQVPDWFFLDEEGNPMVEETNEAKRQANKELLLERLETH
WP_046525547      KIVKFARENDMDLRFHTLIWHSQVPDWFFLDKEGNKMVDETDPKKREKNKKLLLKRVETH
                   **:***::*:******:**.***:***:*::** **:**:  **: **:***:*:*.*

KU759320          IKTVVERYKDDVTSWDVVNEVIDDGG----GLRESEWYQITGTDYIKVAFETARKYGGEE
AY376353          IKTVVERYKDDVTSWDVVNEVIDDGG----GLRESEWYQITGTDYIKVAFETARKYGGEE
2UWF_A            IKTVVERYKDDVTSWDVVNEVIDDDG----GLRESEWYQITGTDYIKVAFETARKYGGEE
AAB70918          IKTVVERYKDDVTAWDVVNEVVDDGTPNERGLRESVWYQITGDEYIRVAFETARKYAGED
WP_046525547      VKTIVKRYKDDVDSWDVVNEVIDDYAPNGKGLRESPWYQITGTDYIKVAFETADRFVGKD
                  :**:*:****** :*******:**      ***** ****** :**:****** :: *::

KU759320          AKLYINDYNTEVPSKRDDLYNLVKDLLEQGVPIDGVGHQSHIQIGWPSIEDTRASFEKFT
AY376353          AKLYINDYNTEVPSKRDDLYNLVKDLLEQGVPIDGVGHQSHIQIGWPSIEDTRASFEKFT
2UWF_A            AKLYINDYNTEVPSKRDDLYNLVKDLLEQGVPIDGVGHQSHIQIGWPSIEDTRASFEKFT
AAB70918          AKLFINDYNTEVTPKRDHLYNLVQDLLADGVPIDGVGHQAHIQIDWPTIDEIRTSMEMFA
WP_046525547      AKLYINDYNTEVEVKRDHLYNLVKDLLDQGVPIDGVGHQAHIQLGWPSLQQMEDSFNKFA
                  ***:********  ***.*****:*** :**********:***:.**:::: . *:: *:

KU759320          SLGLDNQVTELDMSLYGWPPTGAYTSYDDIPAELLQAQADRYDQLFELYEELAADISSVT
AY376353          SLGLDNQVTELDMSLYGWPPTGAYTSYDDIPEELFQAQADRYDQLFELYEELSATISSVT
2UWF_A            SLGLDNQVTELDMSLYGWPPTGAYTSYDDIPEELFQAQADRYDQLFELYEELSATISSVT
AAB70918          GLGLDNQVTELDVSLYGWPPRPAFPTYDAIPQERFQAQADRYNQLFELYEELDADLSSVT
WP_046525547      SLGLDNQVTELDVSLYGWPPRPAYPTYDAIPESEFDRQAERYDQIFELYERLGDKISSVT
                  .***********:*******  *:.:** ** . :: **:**:*:*****.*   :****

KU759320          FWGIADNHTWLDGRAREYNNGVGIDAPFVFDHNYRVKPAYWRIID....100%
AY376353          FWGIADNHTWLDDRAREYNNGVGVDAPFVFDHNYRVKPAYWGIID.....98%
2UWF_A            FWGIADNHTWLDDRAREYNNGVGVDAPFVFDHNYRVKPAYWRIID.....98%
AAB70918          FWGIADNHTWLDDRAREYNDGVGKDAPFVFDPNYRVKPAFWRIID.....77%
WP_046525547      FWGIADNHTWLDDRAREFNNGVGKDAPFVFDINYNTKPAYWSIMD.....66%
```

Figure 2: Alignment deduced amino acid of xylanase gene from *B. halodurans* CM1 (this study, GenBank accession number KU759320) with other *Bacillus* xylanase genes: AY376353 (*B. firmus*), 2UWF_A (*B. halodurans*), AAB70918 (*Bacillus* sp. NG-27), WP_046525547 (*B. campisalis*).

The expression, purification, and partial characterization of the enzyme

The sequenced and confirmed DNA fragment then inserted into pET-21d between *NcoI* and *XhoI* site of the vector, expressed in *E. coli* BL21 as a host under the T7 lac promoter using IPTG as inducer (Figure 1 C). The result showed that expression of this xylanase in *E. coli* gave extracellular xylanase activity (10.1 U/mL). The activity in the intracellular form was not detected. It was different with that reported by other group's work. Yang *et al.* (1988) expressed the xylanase gene using the native promoter, the xylanase activity mostly found in periplasmic space. Mamo *et al.* (2006) also found the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14() 2018, pp. 655-662
DOI: http://dx.doi.org/10.21161/mjm.114217

xylanase they cloned was detected in the cytoplasm, periplasm, and the extracellular medium. The intracellular fraction could not be detected in our enzymes might be due to the inclusion body forming, although we didnot check the presence of it.

The purification using Ni-NTA purification kit gave almost single band on SDS-PAGE, with purity increased 27.5 fold with specific activity 543.24 U/mg (Table 1). The purified enzyme showed the molecular mass of 45 kDa (Figure 3). This is the same value as predicted by the deduced amio acid namely molecular weight 45,278 Da and pI 4.66. The enzyme has optimum activity at 65 °C and pH 9 (Figure 4). This value was a few different with the optimum value of the enzyme produced in wild type resource microorganism whose optimum activity at 70 °C and pH 9 (Ulfah *et al.*, 2011).

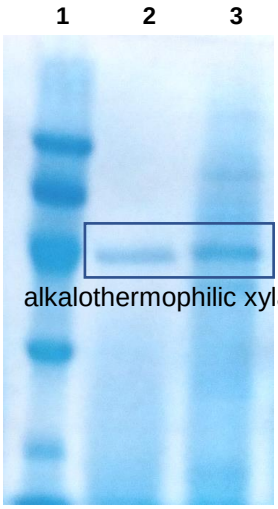

Figure 3: Purification result of recombinant *B. halodurans* CM1 xylanase cloned and expressed in *E. coli*. Lane 1, protein marker; Lane 2, enzyme after purification; Lane 3, crude extract.

Application in deinking process at laboratorium scale in the paper industri

This recombinant xylanase with volumetric activity 12.5 U/mL was tried to be applied in deinking process of paper industri at the laboratory scale. The result showed that our recombinant enzyme could be applied in deinking process of waste paper, and gave brightness and whiteness better compared to the process without enzyme, and gave comparable improvement in brightness and whiteness compared to commercial enzyme used already in the same process (Table 2). There are several studies reported xylanase were applied in several different deinking process. Thermo-alkali-stable laccase and xylanase by co-culturing of *Bacillus* sp. and *B. halodurans* gave increased in ONP pulp deinking, the treatment increased brightness (11.8 %) (Gupta *et al*., 2015). Xylanases and cellulases from *Trichoderma longibrachiatum* MDU-6 were used for the deinking of paper waste and deinked newspaper pulp sample shows brightness of 52%, which was 9.6% higher than its control sample (Chutani and Sharma, 2016).

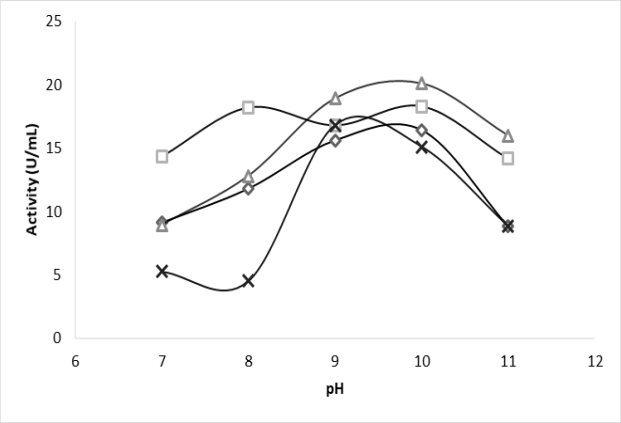

Figure 4: Temperature and pH profile of recombinant xylanase from *B. halodurans* CM1 cloned and expressed in *E. coli.* The effect of temperature on xylanase activity was measured at 55-70 °C. The effect of pH on the activity at pH range 7-11 using the following buffers: 50 mM phosphate buffer (pH 7), 50 mM Tris-HCl (pH 8-9), and 50 mM Tris-Glycine buffer (pH 10-11). Xylanase activity was measured duplicates using the Miller (1959) method using dinitrosalicylic acid to quantify reducing sugar. ◊ assayed at 55 °C, □ assayed at 60 °C, Δ assayed at 65 °C, **x** assayed at 70 °C.

Table 1: Purification summary of recombinant xylanase from *B. halodurans* CM1 cloned and expressed in *E. coli.*

Step	Volumetric activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Purity (fold)
Crude extract	10.08	0.511	19.73-	-
Ni NTA purification	20.10	0.037	543.24	27.5

Table 2. Results of deinking process using recombinant xylanase from *B. halodurans* CM1 cloned and expressed in *E.coli.*

Sample	Brightness	Whiteness	Delta Brightness	Delta Whiteness
Blank (without enzyme)	43.6	29.45	-	-
With commercial enzyme	45.1	30.27	1.5	0.82
With our recombinant xylanase	45.78	30.82	2.18	1.37

ISSN (print): 1823-8262, ISSN (online): 2231-7538

CONCLUSION

To conclude, in this study an alkalothermophylic xylanase gene from *B. halodurans* CM1 isolated from Indonesia habitat has been cloned and expressed in *E. coli.* This recombinant xylanase was proven to be applicable in deinking of waste paper ONP, promised its value for further engineering and production as well as application in the future.

ACKNOWLEDGEMENTS

Part of this work was funded by Insinas Research Incentives Program of Ministry of Research, Technology, and Higher education 2017 granted to IH.

REFERENCES

Bailey, M. J., Biely P. and Poutanen. K (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* **23, 257-270.**

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation on microgram quantities of protein in utilizing the principle of protein dye binding. *Analytical Biochemistry* **72, 248-254.**

Chang, P., Tsai, W. S., Tsai, C. L. and Tseng, M. J. (2004). Cloning and characterization of two thermostable xylanases from an alkaliphilic *Bacillus firmus*. *Biochemical and Biophysical Research Communications* **319, 1017-25.**

Chutani, P. and Sharma, K. K. (2016). Concomitant production of xylanases and cellulases from *Trichoderma longibrachiatum* MDU-6 selected for the deinking of paper waste. *Bioprocess Biosystem Engineering* **5, 747-58.**

Collins, T., Gerday, C. and Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Review* **29, 3-23.**

Gupta, N., Reddy, V. S., Maiti, S. and Ghosh, A. (2000). Cloning, expression, and sequence analysis of the gene encoding the alkali-stable, thermostable endoxylanase from alkalophilic, mesophilic *Bacillus* sp. strain NG-27. *Applied and Environmental Microbiology* **99(6), 2631-2635.**

Gupta, V., Garg, S., Capalash, N., Gupta, N. and Sharma, P. (2015). Production of thermo-alkali-stable laccase and xylanase by co-culturing of *Bacillus* sp. and *B. halodurans* for biobleaching of kraft pulp and deinking of waste paper. *Bioprocess Biosystem Engineering* **5, 947-56.**

Helianti, I., Nurhayati, N., Ulfah, M., Wahyuntari, B. and Setyahadi, S. (2010). Constitutive high-level expression of an endoxylanase gene from the newly isolated *Bacillus subtilis* AQ1 in *Escherichia coli*. *Journal of Biomedical and Biotechnology* **Article ID 980567, 1-12.**

Horikoshi, K. (2004). Alkaliphiles. *In: Proceedings of the Japan Academy.* Series B. **80,166-178.**

Kumar, V. and Satyanarayana, T. (2014). Production of endoxylanase with enhanced thermostability by a novel polyextremophilic *Bacillus halodurans* TSEV1 and its applicability in waste paper deinking. *Process Biochemistry* **49, 386-394.**

Lee, C. K., Darah, I. and Ibrahim C. O. (2007). Enzymatic deinking of laser printed office waste papers: Some governing parameters on deinking efficiency. *Bioresource Technology* **98, 1684-1689.**

Lee, C. K., Ibrahim D. and Omar, I. C. (2013). Enzymatic deinking of various types of waste paper: Efficiency and characteristics. *Process Biochemistry* **48, 299-305.**

Mamo, G., Rajni, H. K. and Mattiason, B. (2006). A thermostable alkaline active endo-β-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterization. *Enzyme Microbial Technology* **39, 1492-1498.**

Mamo, G., Thunnissen, M., Hatti-Kaul, R. and Mattiasson, B. (2009). An alkaline active xylanase: Insights into mechanisms of high pH catalytic adaptation. *Biochimie* **91, 1187-1196.**

Martínez, M. A., Delgado, O. D., Baigorí, M. D. and Siñeriz, F. (2005). Sequence analysis, cloning and over-expression of an endoxylanase from the alkaliphilic *Bacillus halodurans*. *Biotechnology Letter* **8, 545-50.**

Miller, G. L. (1959). Use of dinitrosalycylic acid as reagent for the determination of reducing sugars. *Analytical Chemistry* **31, 208-218.**

Noer, S. (2011). Kloning Gen Xilanase Alkalotermofilik pada *Escherichia coli* DH5α dan Karakterisasi Produk Gennya. [Cloning open reading frame of alkalothermophilic xylanase into *Escherichia coli* DH5α]. MSc. Thesis. Universitas Indonesia, Indonesia.

Prade, R. A. (1996). Xylanases: From biology to biotechnology. *Biotechnology Genetic Engineering Review* **13, 100-131.**

Rismijana, J., Indriani, I. N. and Pitriyani, T. (2003). Penggunaan enzim selulase dan hemiselulase pada proses deinking kertas koran bekas [The use of cellulase and hemicellulose in deinking process of waste news paper]. *Jurnal Matematika dan Sains* **8, 67-71.**

Sambrook, J. and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Satyanarayana, T., Sharma, A., Mehta, D., Puri, A. K., Kumar, V. and Nisha, M. (2012). Biotechnological applications of biocatalysts from the firmicutes *Bacillus* and *Geobacillus* species. *In*: Microor-ganisms in Sustainable Agriculture and Biotechnology. Satyanarayana, T., Johri, B. N. and Prakash, A. (eds). Springer, The Netherlands. **pp. 343-379.**

Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S. and Horikoshi, K. (2000). Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and

genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Reseach* **28, 4317-31.**

Ulfah, M., Helianti, I, Wahyuntari, B. and Nurhayati, N. (2011). Characterization of a new thermoalkalophilic xylanase-producing bacterial strain isolated from Cimanggu hot spring, West Java, Indonesia. *Microbiology Indonesia* **5, 139-143.**

Weerachavangkul, C., Laothanachareon, T., Boonyapakron, K., Wongwilaiwalin, S., Nimchua, T., Eurwilaichitr, L., Pootanakit, K., Igarashi, Y. and Champreda, V. (2012). Alkaliphilic endoxylanase from lignocellulolytic microbial consortium metagenome for biobleaching of eucalyptus pulp. *Journal of Microbiology and Biotechnology* **22, 1636-43.**

Yang, R. C. A., MACKenzie, C. R., Bilous D., Seligy V. L. and Narang, S. A. (1998). Molecular cloning and expression of a xylanase gene from *Bacillus polymyxa* in *Escherichia coli. Applied Enivronmental Microbiology* **54, 1023-1029.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538