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Research Article

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Cloning and characterization of gene endo beta-1,4-glucanases *Bacillus* sp RP1-M3 from hot springs

Armaini, Abdl Dharma and Marniati Salim

Laboratory of Biochemistry, Department of Chemistry, Faculty of Mathematics and Natural Science, Andalas University, Kampus Limau Manis, Padang, Indonesia

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ABSTRACT

Isolates of 1-M3 isolated from a hot spring Rimbo Panti, has been identified using 16S rRNA gene. Based on the results of BLAST and kinship analysis, found that isolates RP 1-M3 including Bacillus sp. Amplification with primers specific for the gene endo-1,4 β-Glucanases obtained ± 1500 bp PCR product. PCR products were cloned into plasmid pGEM-T Vector obtained recombinant plasmid. Recombinant plasmid transformation into E. coli host, and selection results of transformation using media containing antibiotics, IPTG and X-GAL to get the positive transformation that carries a recombinant plasmid with target DNA. To observe the success of the transformation carried out colony PCR with primers T7SP6, obtained ± 2.000 bp PCR product consisting of 1,500 bp, indicated as DNA gene coding the enzyme endo-β1,4-Glucanases and ± 500 bp of sequence T7SP6. A result of gene sequence was translated into amino acids obtained Egl protein of 500 amino acid sequence. Based on the results of BLAST in the GenBank data base, the genes that have been isolated from Bacillus sp. RP 1-M3 includes genes endo-β1,4-Glucanases with 99.4% similarity with genes endo-β1,4-Glucanases from Bacillus megaterium (AGW99981.1) at the amino acid level.

Keywords: Cloning, Isolation, genes, endo β-1,4-Glucanases, *Bacillus sp*

INTRODUCTION

Free Cellulases has a characteristic consisting of a carbohydrate or a cellulose-binding domain (CBM) in the C-terminal region joined with poly-linker, short to the catalytic domain at the N-terminal. There are only two types of action for cellulose hydrolysis by the enzyme Cellulases, namely inversion and retention of a numeric carbon configuration. At least two amino acids with a carboxyl group is located in the active site to catalyze the acid-base catalyst [1]. Model action for Cellulases to the polymer are exo and endo, all cellulases targeting specific cleaved the bond of β-1,4-glycosidic [2].

Based on the classification, Cellebiohydrolases (exo-Glucanases) were classified as exo-acting based on assumptions able to cleave all the bonds β-1,4-glycosidic from the end of the chain. Endo-Glucanases while on the other hand, are classified as endo-acting Cellulasess because cleave the β-1,4-glycosidic internal only and have an open form of active sites [3] Glucanasess usually active at amorphous regions soluble more than crystalline cellulose [3]. Exo-Glucanases or cellobiohydrolases (exo-1,4-Glucanases, EC.3.2.1.91) serves to liberate glucose or cellobiose from the ends of the chain cellulosic fragments as a result of hydrolysis of glycoside bond. Exo-Cellulasess cleave 2-4 units of the chain ends of polysaccharides, the resulting by endo-Cellulases to produce tetra

saccharides and disaccharide, celebiose [4]. There are 2 types of exo-Cellulases (Celebiohydrolase / CBH) (a). The type of action at the end of the reduction of cellulose (b). The type of action at the end of the non-reduction of cellulose [5]

Endo Enzyme beta-1,4-Glucanases including glycosyl hydrolase family big family 5 (GH5), a group of enzymes that work on β-link oligo, polysaccharides, and gliko-conjugation. GH5 is one of the greatest of all family glycoside hydrolase as contained in CAZy (Carbohydrate Active Enzymes). Formerly known as Cellulases family clan A [6] the classification of families into groups larger, so called clan [7] Clan is a group of families has significant similarities in tertiary structure, and mechanisms of catalytic residues

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EXPERIMENTAL SECTION

2.1 Apparatus

The equipment used in this study; PCR machines, Biometra-German, High Pressure Steam Sterilizer BS-245, Water Bath Shaker-UV-1600, UV-Vis Spectrophotometer Shimazu, Taitex.Personal-11 Clean Bench - Hitachi (Laminar Flow), Incubator Shimazu, Eppendorf Centrifuge 5415- R, Gel Electrophoresis, Colony counter, Heater Block and laboratory glassw

2.2 Reagents

Reagents used; Media (solid and liquid) for growth of Bacillus sp RP1-M3 based on the optimization of the composition of the nutrient medium [8], Nutrient Broth, Tris-EDTA, Agar Rose, CelF (5'-ATGAAGCGCAGCATCAGCATCTTG-3 ') and CelR (5'GTTCGGCTG GGTGCCCAGCCAAATGGT-3 '), RTG-PCR, plasmid pGEM-T, amphicillin, chloroampheni col, isopropyl-β-Thiogalacto pyranoside (IPTG) and 5-bromo-4-chloro-3- indoly-β-D-galactophyranoside (X-Gal).

2.3 Procedures

2.3.1 Isolation of genomic DNA and identification of thermopiles bacteria

Isolation of DNA genome using DNA-Kit and identification of bacteria by 16S rRNA gene PCR amplification is done with genomic DNA using primers 27F and 1525R. PCR products were identified by electrophoresis agar rose, after that PCR products were sequenced and BLAST done online at the web site of NCBI (National Center of Biotechnology Information) http://www.ncbi.nlm.nih.gov/BLAST . Analysis of phylogenetic reconstruction, using methods Bootstraps (repetition of 1000) with the model parameters and estimates of Kimura 2 Evolutionary Divergence between sequences, all analyzes using MEGA5.10 program [9]

2. PCR amplification using Specific Primer for Endo- gene beta -1,4Glukanase

Operating conditions for the PCR amplification of DNA Endo- gene beta -1,4Glukanase as follows; denaturation (94 ° C) for 1 minute, annealing (69.4 ° C) for 1.5 minutes, the extension (72 ° C) for 1 minute, and extension (72 ° C) for an additional 5 minutes, 30 cycles of amplification performed by PCR machine (Biometra-Germany). The total volume of 25 µl PCR reaction consisting of DNA template 2 µl, 2 µl of primer (Primer Cel-F 5'-ATGCTCAAGATCGCCACGCTCG-3' and 5'-Cel-R GCAGGAAGAGAACAAG TTGGTGG-3') and 21 µl of ddH2O PCR in RTG- PCR Bead. To control the success of the amplification reaction, 5 µl, PCR products in the gel electrophoresis at concentration of agar rose 1% in 0.5 x TBE buffer [10] Visualization use the UV - Transluminator stained with ethidium bromide

2.3. Recombinant Plasmid

The process of making a recombinant plasmid DNA, amplification product insert into a plasmid. with ligation process This activity is carried out using a kit pGEM®-T Easy Vector Systems in accordance with the procedure Promega (Promega-USA). Composition ligation as follows: 2x Rapid Ligation Buffer 5 µl, pGEM®-T Easy Vector 1 µl, T4 DNA Ligase1 µl, 3 µl PCR products, total10µl, then incubated overnight at a temperature -4^{o}C.

2.4. Transformation of plasmids into E.coli BL21 (DE3)

Transformation is done by heat shock method, E. coli BL21 (DE3) as host which had been prepared, taken 50 µl, was mixed with 5 µl of recombinant DNA in 1.5 µl eppendorf tube and then incubated in ice for 2 minutes. Then put in a water bath temperature of 42 °C for 2 minutes to give a heat shock. Subsequently immediately cooled in ice for 2 minutes, the tube is moved, added to 250 µl SOC medium. Included in the mix-shaking incubator for 1 hour at a speed of 150 rpm, 37 ° C, then do the selection of result transformation

2.5. Selection results of the transformation on solid media LB

Selection of transformation is done using solid LB media are added antibiotics, namely 250 µl ampicillin and Chloroampenicol, IPTG and X-Gal, and homogenized. .Pipette 75 µl, the results of transformation included in the petridish that already contains media leveled to the entire surface of the LB medium until dry. Petri incubated overnight at 37 ° C, until obtained a single colony. Selection of the bacterial colonies result of tranformation used blue-white colony selection. Bacterial colonies with white color indicates the hosts that carry plasmids and DNA targets [11,12].

2.6. PCR Colonies

Colony PCR is done to look at the success of the transformation by means of bacterial colonies growing (white) was used as DNA template and using the primers T7 and SP6 with the composition; primer 10 pmol / µl, DNA amplification when using RTG, ddH2O 23 µl PCR Grade, Mix T7SP6 2 µl, and the DNA template, a total of 25 µl. PCR operating condition following initial denaturation 94ºC (5 minutes), the next denaturation 94ºC (1 min), annealing 55ºC (1 min), the extension 72ºC (1.5 minutes), final extension 72ºC (1 min).PCR products were sequenced to obtain the DNA sequence...

2.7. DNA sequencing analysis of Endo- beta -1,4-Glucanases

PCR products were sequenced to obtain sequence of DNA bases of a gene Endo- beta -1,4-Glucanases. Data of sequence DNA were then used for homology analysis using BLAST (Basic Local Alignment Search Tool) on the website website http://www.ncbi.nih.gov with sequence data that already exists in the databank to determine variations in gene sequence analysis and phylogenetic tree construction using software MEGA5 [9]

RESULTS AND DISCUSSION

3.1. Identification of Bacteria Thermopiles with 16S rRNA

Amplification of DNA from bacteria thermopiles isolates 1M-3 use gen16S rRNA with primer combinations 27F and 1525R produces PCR products with the size of about 1500 bp. Result of sequencing analysis using BLAST done online at the web site of NCBI (National Center of Biotechnology Information) http://www.ncbi.nlm.nih.gov/BLAST . Results obtained from the analysis of BLAST show that isolates 1M-3 has some similarities with Bacillus sp. H06 (AY461747,2) with similarity 98%, Bacillus cereus SBD2-1 (HQ236941.1) with 97% similarity, Bacillus cereus HNT6 (HQ156459.1) with 96% similarity, and Bacillus sp. TRP71H (FN993946.1) with 98% similarity. Based on these results it can be stated that the isolate 1M-3 including Bacillus sp [13}

3.2. Gene amplification and cloning of beta 1,4-Glucanases Endo

Amplification using specific primers Cel-F and Cel-R has size ± 1500 bp (Figure 1). PCR product (± 1500 bp) insert into pGEM-T easy vector with ligation process, the insertion can be easily done because the PCR amplification product is equipped with overhangs-A and the vector pGEM-T is equipped with overhangs-T. Vector carrying DNA target as results ligation is transformed into competent cells E. coli BL21 (DE3) by heat shock method. Result of transformation grown in LB medium containing amphicillin and chloroamphenicol, IPTG and X-Gal. The media is used to select colonies of bacteria that grow, where the colonies were able to grow in media containing amphicillin and chloramphenicol indicates a bacterial colony, result of transformation. Selection of the result transformation colonies of bacteria used blue-white selection [14, 15], the selection is done by looking at the color of the growing colony . In this case it can be stated that the colony white grows is colonies that carry DNA inserts, while colonies blue is colonies were only carrying empty vector and do not carry DNA insertions. Blue-white selection results can be seen in Figure 1B.

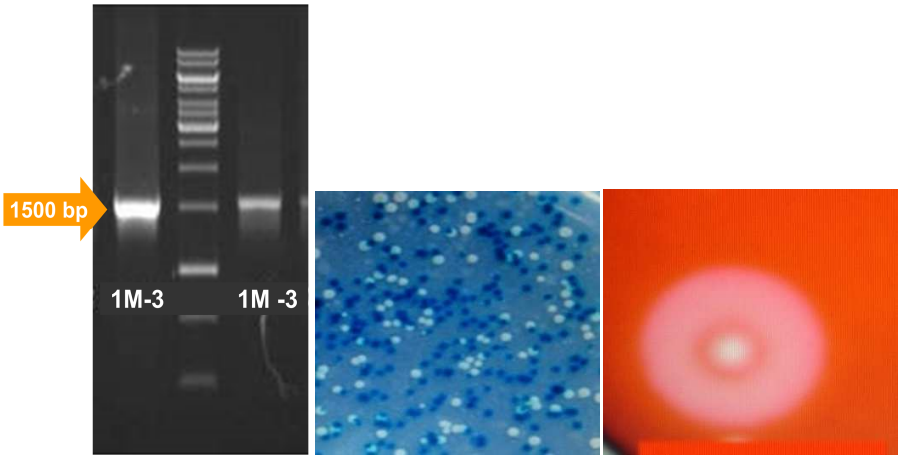

Figure 1, Visualization amplification of isolates 1M3 with primer Cel-F and Cel-R and a temperature of annealing at 69.4 °C, obtained the product about 1500 bp (A). Blue-white selection results of transformation using plasmid pGEM-T vector in a solid LB media containing 100 mg / µl amphicillin and added 100 ug / µl XGAL. chloroamphenicol, 100 mM IPTG and 50 mg agar plate (B) and Establishment of clear zone (C)

3.3. Colony PCR

Proof against result of transformation carries a recombinant plasmid (plasmid pGEM-T) which have successfully inserted an target DNA using PCR colony. Single colony which will be used as a template for amplification in the PCR-colony. Primer used in PCR-colony is T7SP6. Visualization the result of amplification of bacterial transformation carrying recombinant plasmid (pGEM-T),the results are shown on Figure 2 that the transformation of codes 1-7 and 12-13 provide PCR product ± 2000 bp , whereas the inserted DNA 1500 bp and 500 bp T7SP6 . This shows that the DNA insert ligation into pGEM-T vector and the vector transformation into E. coli BL21 (DE3) successfully conducted. Result of transformation with code 8 to 11 and 14-16 provide ± 500 bp, PCR product is only a product of the PCR primer T7SP6, this indicates that the colonies did not bring DNA insert, this proves unsuccessful ligation process is done. PCR products were sequenced colonies ± 2000 bp in both directions (F and R) to determine the sequence of transformation positive

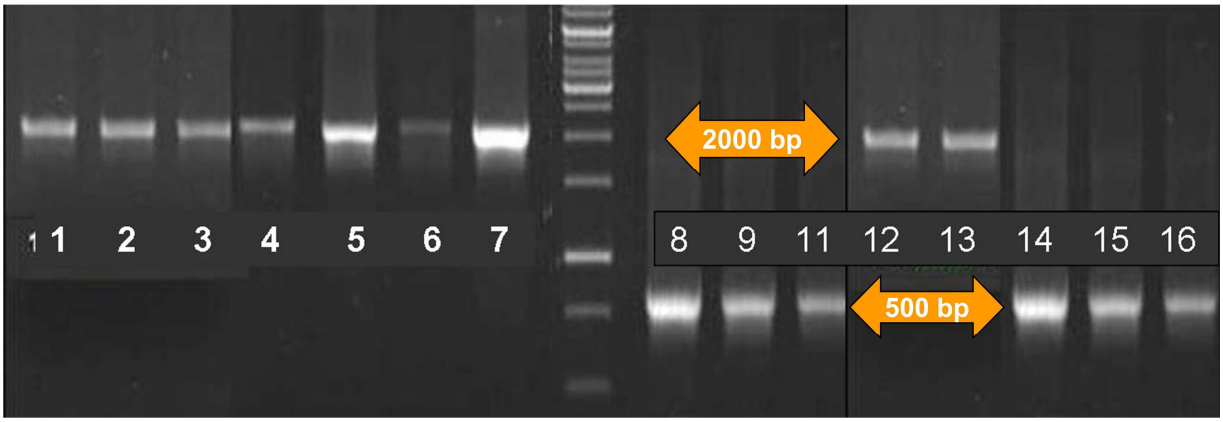

Figure 2. Visualization of amplification the product PCR-colony of E. coli recombinant with DNA inserts provide 2000 ± bp PCR product that carries recombinant plasmid pGEM-T as a positive transformation

3.4 Open Reading Frame Sequence Endo Beta -1,4-Glukanase

Results of analyzed using the program Vecscreen (Vector Contamination Screening), to separate the target DNA, and the vector plasmid DNA sequences that participate translated. The identification using VecScreen, sequences consisting 1500 bp. Results of the ORF sequences consisting of 1500 bp obtained 500 amino acids [16] (Figure 3)

5'3' Frame

et K R S I S I F I T C L L I T V L T **Met** G G L Q A S P A S A S G T K T P A A K N G Q L S I K
G T Q L V N R D G K A V Q L K G I S S H G V R W Y G D F V N K D S L K W L R D D W G I T V F R A A
Met Y T A D G G Y I D N P S V K N K V H E A V E A A K E L G I Y V I I D W H I L N D G Y P N Q H K E K
A K E F F K E **Met** S S L C G N T P N V I Y E I A N E P N G D V N W K R D I K P Y A E E V I S V I R K N D
P D N I I I V G T G T W S Q D V N D A A D D Q L K D A N V **Met** Y A L H F Y A G T H G Q S L R D K A N
Y A L S K G A P I F V T E W G T S D A S G N G A V F L D Q S R E W L N Y L D S K N I S W V N W N L S D
K Q E T S S A L K P G A S K T G G W P L T D L T A S G T F V R E N I L G N K D T T K E R P E T P A Q D N
P A Q E N G I S V Q Y K A G D G G V N S N Q I R P Q L H I K N N G N A T V D L K D V T A R Y W Y N A
K N K G Q N F D C D Y A Q I G C G N L T H K F V T L H K P K Q G A D T Y L E L G F K T G T L S P G A S
T G N I Q L R L H N D D W S N Y A Q S D D Y S F F Q S N T F K T T K K I T L Y H Q G K T I W L G T Q P

Figure 3. The amino acid residues of beta 1,4 -glukanase Endo gene consists of 500 amino acids

3.5. Phylogenetic tree Gen Endo beta 1,4-Glucanases from Bacillus sp RP1M-3

Phylogenetic trees for gene endo-1,4-β-Glucanases constructed of several genes endo-β-1,4-Glucanases derived from the genus Bacillus. Phylogenetic tree of endo-β-1,4-Glucanases shows that Egl protein from Bacillus sp. RP 1M-3 form groups with endo-1,4-β-Glucanases of Bacillus megaterium (AGW99981.1) with similarity 99.4%, Bacillus megaterium (ADI82821-1) with 98.8% similarity and Uncultured Bacterium (AGW99972.1) with a similarity of 98%, and based on this it can be concluded that the gene with a size of 500 amino acids, which has isolated of Bacillus sp 1M RP-3 are genes endo-1,4-β-Glucanases which express the protein Egl. Based on the results of BLAST GenBank database, which has isolated gene from Bacillus sp. RP 1-M3 includes genes endo-β1,4-Glucanases with 99.4% similarity with genes endo-β1,4-Glucanases from Bacillus megaterium (AGW99981.1) at the amino acid level

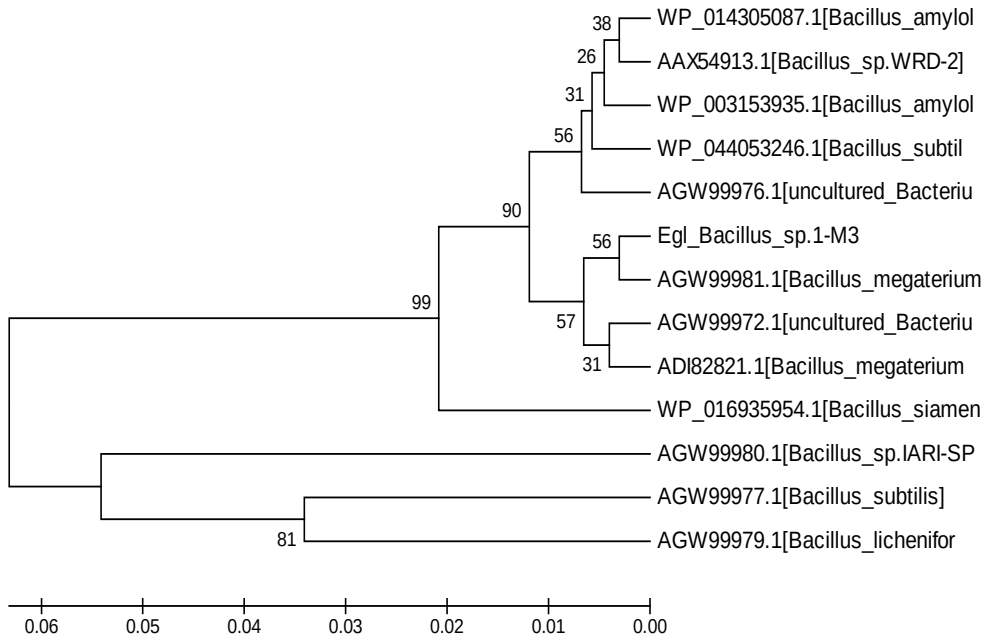

Figure 6. Phylogenetic tree gene endo β-1,4-Glucanases enzyme family GH5 Bacillus sp. RP.1M-3 clustered in Bacillus megaterium (AGW9981.1)

CONCLUSION

Bacillus sp RP 1 M-3 were isolated from a hot spring has a gene encoding the enzyme endo-β-1,4-Glucanases with a size of 1500 bp, produces a protein of 500 amino acid residues. Protein enzyme endo-β-1,4-Glucanases (Egl), these have a very high homology, 100% in gene amino acid level with endo-β-1,4-Glucanases from Bacillus megaterium (AGW9981.1)

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