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Cloning and Characterization of Lipase Gene from a Local Isolate of *Pseudoxanthomonas* sp.

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Lipase gene from *Pseudoxanthomonas* sp. was cloned through *in vitro* amplification from total chromosomal DNA. The gene was sequenced and characterized, coding for 312 amino acid residues. Homological analysis showed that the gene has 98% similarity to lipolytic gene from *Uncultured Pseudomonas* sp (GenBank No. AKA58891.1). Further analysis appeared that the sequences showed similar unique motifs of lipase sub-family I.1, such as pentapeptide (GHSHG) motif, tetrapeptide (GMLG) motif, and catalytic triad. In additional, 3D structure analysis based on crystal structure of *Pseudomonas aeruginose* (PDB ID 1ex9) showed that both structure of lipases are similar except on the conformation of catalytic residue of His^{277} showing to shift more far away compared to that the control.

Keywords: *P. seudoxanthomonas* sp., Thermostable lipase, Cloning, Sequencing, Characterization.

Lipase is an enzyme that catalyzes hydrolysis of triacylglycerol into fatty acids and glycerol on the interface between water and organic solvent (Lotti *et al*., 2007). Lipases also show many catalytic activities besides hydrolisation such as esterification, trans-esterification, inter-esterification, acidolysis, aminolysis, alcoholysis and racemic resolution (Houde *et al*., 2004; Salihu and Alam, 2014, Sharma *et al*., 2001; and Brilliantoro *et al*., 2015). Since the enzymes show wide range activities, lipases are widely used in various fields of industries, such as food, detergents, cosmetic, biomedicine, biopolymers, biosurfactan, biodesel, and pharmaceutical industries (Jaeger and Eggert, 2002; Gupta *et al*., 2004; Houde *et al*., 2004; Salihu and Alam, 2014). Most of lipases used in industries are isolated from microorganism, since lipases from bacteria shown and an activity in wide range of temperature and pH (Akhmaloka *et al*., 2006).

Thermostability is desirable property for commercial lipase since enzymatic reaction at a high temperature could increase conversion process and the solubility of the substrats, reducing contamination and lowering the viscosity of the medium (Leow *et al*., 2004). Therefore, exploration of lipase from other sources have still extensively been carried out (Akhmaloka *et al*., 2006).

Some of thermophilic microorganisms have been isolated from compost (Madayanti *et al*., 2008; Nurhasanah *et al*., 2015; Syihab *et al*., 2015), hot spring and other sources (Akhmaloka *et al*., 2006; Syihab *et al*., 2015). Some of the isolated showed lipolytic activity (Widhiastuty *et al*., 2009; Febriani *et al*., 2013; Nurhasanah *et al*., 2017).

In previous paper (Syihab *et al*., 2015), we reported five isolates of lipolytic thermophiles

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with alcohol tolerance. In this paper cloning and characterization of one of the lipase will be described.

MATERIALS AND METHODS

Chemicals

Common chemicals with pro analysis grade were purchased from Merck (Germany) and Sigma-Aldrich (USA). Bacterial growth nutrients, such as yeast extract, tryptophan were obtained from Bio Basic (Canada). Biochemical reagents such as dNTPs, PCR buffer, *Taq* DNA Polymerase were purchased from Fermentas (USA) and Kapa Biosystems (USA). Oligonucleotides (primers) were obtained from Macrogen (South Korea) and Integrated DNA Technologies (Singapore). Purification of PCR product used GeneJET Gel Extraction Kit (Thermo Scientific). The cloning was performed by using *pJET1.2/blunt* vector and T4 DNA ligase purchased from Promega (USA). Restriction enzymes were purchased from Thermo Scientific (USA).

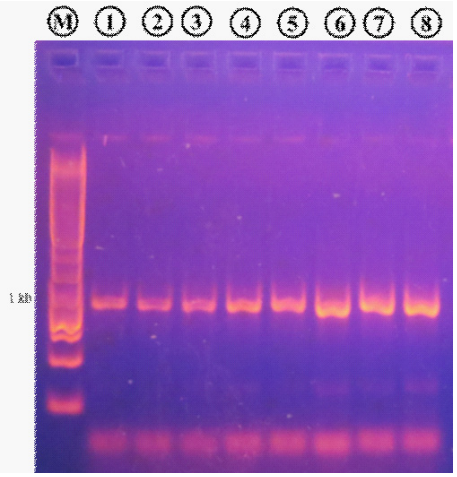

Fig. 1. Amplicon of PCR amplifications of lipase genes. Assigned lane numbers are as follow M = marker DNA ladder 1 kb; 1 =Annealing temperature of 50^{O}C; 2 = for 49.2^{O}C, 3 =for 47.9^{O}C; 4= for 46.2^{O}C; 5 =for 43.9^{O}C; 6 =for 41.9 ^{O}C; 7= for 40.6 ^{O}C and 8= for 40 ^{O}C.

Strain and Medium

Thermostable bacteria were obtained from our culture collections in the Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung. The culture of *Pseudoxanthomonas taiwanensis* (AL17) was incubated in shaker incubator at 55^{o}C with aeration rate at 150 rpm. *Escherichia coli* TOP 10 was used as host for gene cloning. The isolate was cultivated using modified media of Luria Berthani composed of 0.1% CaCl2.H2O, 0.5% yeast extract, 0,5% lab lemco/meat extract and 0,1% NaCl.

Isolation of Chromosomal DNA

Chromosomal DNA was isolated by using modified method of Zhou *et al*. (1996). The collected DNA pellet was separated from the supernatant and dried, subsequently followed by resuspension with 50µL ddH2O and stored at 4^{o}C. The DNA was used for PCR.

PCR and Sub-Cloning of Lipase Gene

Cloning of lipase gene in each bacterial isolate was started by *in vitro* amplification of the gene by PCR technique using a pair of specific primers xFLipS2 (5'-ATGAACAAGAACAAAACCTTGCTCGCC 3') and xRLipS2 (5' TCAGAGCCCCGCGTTCTTCAA-3') (Asy'ari *et al*., 2014). A typical PCR mixture (50 ìL

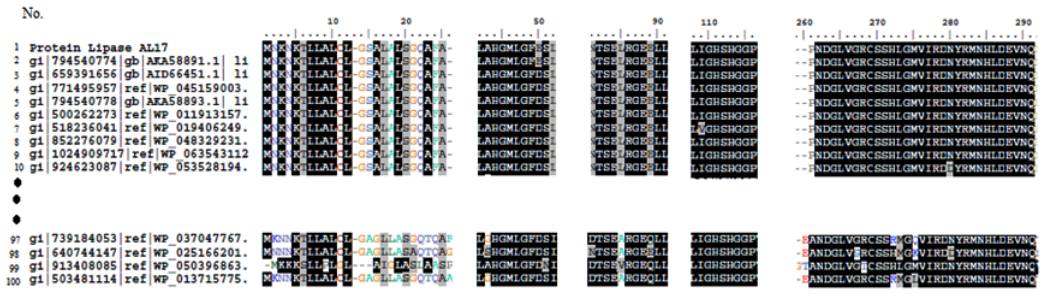

Fig. 2. Conserved region of lipase covers of GMLG (tetrapeptide), GHSHG (pentapeptide), oxyanion and catalytic triad in 9 highest lipase sequences. A description of the figure is as follows: A. position of signal peptide; B. tetrapeptide motif; C. pentapeptida motif; • catalityc triad; Δ Asp residue; • Arg residue

in volume) was prepared by mixing 5 ìL of PCR buffer 10´, 2.5 mM MgCl2, 250 ìM of deoxynucleosid-e triphosphate, 0.25 ìM of each primers, and 1.25 U of *Taq* DNA polymerase. PCR was conducted by the following protocol: an initial denaturation was set at 98°C for 4 min followed by 35 cycles of denaturation (@30 s at 98°C), an annealing was programmed at 55°C for 30 s, while an extension and final extension were carried out at the same temperature at 72°C for 1 min and 5 min, respectively. The product of PCR was verified by electrophoresis technique using submerged horizontal electrophoresis gel for 45 min at 70 volts. The purification of PCR product was carried out by GeneJET Gel Extraction Kit. Finally, purified DNA was re-suspended to 50 ìL buffers (10 mM Tris-HCl, pH 8.5). The purified DNA solution was stored at -20°C.

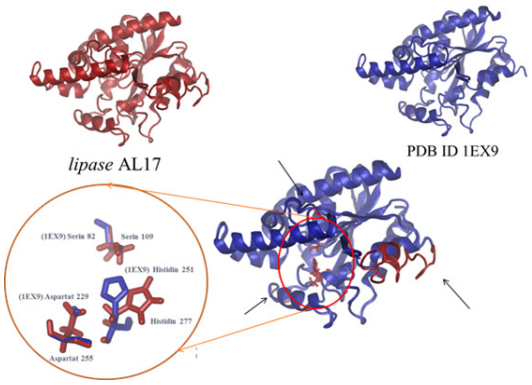

Fig. 3. Crystal structure comparison between Lip AL17 and I EX 9. A. Model Structure of Lip AL17; B. Crystal Structure of I EX 9; C. Superimposed between Lip AL17 and I EX 9, His^{277} Lip AL17 showed to shift more far away compared to that the control (I EX 9)

Construction of recombinant plasmid was carried out by ligating the PCR products with *pJET1.2/blunt* plasmid. The competent cell of *E. coli* Top 10 was prepared following the standar method. Transformation of *E. coli* was conducted by heat shock method. 100 mL of the transformed cells were spreaded on LB agar (LBA) media containing 100 mg/mL of ampicillin, 15 mg/mL of tetracycline and incubated at 37°C for overnight. Plasmid isolation from the transformed cell was carried out by the alkaline lysis method. Finally to verify the recombinant plasmid containing DNA insert, the plasmid was digested with *Bgl* II and visualized by agarose gel electrophoresis.

Sequencing of Lipase Gene

Lipase genes were sequenced based on Dideoxy- Sanger dye terminator method at Firstbase, Malaysia. The sequences were validated by analyzing the electrophoregram data using Sequence scanner 2 (Applied Biosystems). In order to combine the partial gene sequences into a full gene, we used DNA Baser Sequence Assembler v3 program (Heracle BioSoft).

Sequence Analysis

Deduced amino acid sequences of lipase were performed by *in silico* translation using Bioedit software and online server of ExPASy-Translate tool available at http:// web.expasy.org/translate/. Homologycal analysis were carried out using NCBI-Blastp analysis program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A hundred of high homologous sequences were used to generate phylogenetic profile using MEGA 6 software based on the Neighbor-Joining clustering method. Amino acid composition and alignment analysis were performed using "Amino Acid Composition" and

Table 1. Homological result of Lip AL17 to 10 highest homolog. All of ten best homolog are lipases

No.	Description	Total score	Ident	Accession
1.	lipase [uncultured Pseudomonas sp.]	624	98%	AKA58891.1
2.	lipase [Pseudomonas stutzeri]	624	98%	AID66451.1
3.	lactonizing lipase [Pseudomonas stutzeri]	622	98%	WP_045159003.1
4.	lipase [uncultured Pseudomonas sp.]	622	98%	AKA58893.1
5.	lactonizing lipase [Pseudomonas stutzeri]	620	98%	WP_011913157.1
6.	lactonizing lipase [Pseudomonas stutzeri]	619	97%	WP_019406249.1
7.	lactonizing lipase [Pseudomonas stutzeri]	618	97%	WP_048329231.1
8.	lipase [Pseudomonas stutzeri]	617	97%	WP_063543112.1
9.	lipase [Pseudomonas stutzeri]	616	97%	WP_053528194.1
10.	LipA [Pseudomonas mendocina]	613	96%	AAM14701.1

"ClustalW Multiple Alignment" programs which are integrated in the Bioedit software.

The analysis of lipase gene sequences was carried out by evaluating contents of GC, GCAT,and GC-AT of codon usage. The last two analyses were conducted with Bioedit-software,while the third analysis was calculated with an online program available at http:// www.bioinformatics.org/sms2/codon_usage.html. The analysis of *codon usage* was performed on the 1st, 2nd and 3rd base position. All analyzes above were not only performed on the isolate lipase sequences but also to other lipase genes from the other microorganism.

RESULT AND DISCUSSION

Cloning and Sequencing of Lipase Genes

Lipase genes have been successfully amplified from *Pseudoxanthomonas* sp (Figure 1) local strains by *in vitro* amplification. The gene, namely *LipAL17* codes for 312 amino acids with length of 936 bp. The lipase gene was cloned into *E. coli* Top 10 and deposited to the GenBank database with accession number of ID 1918703, respectively.

Homological of the Lipase

Homological analysis of deduced amino acid sequences showed that the gene appeared highly similarities to several lipases (Table 1), such as lipases from *Uncultured Pseudomonas* sp AKA 588891.1 and AKA 58893.1 and the *Pseudomonas stutzeri* AID 66451.1 and WPO 45159003.1 with percent identity of 98%.

From 100 best homology sequences, the gene showed similar sequence with other lipases containing same conserved regions, such as GGGX, GXSXG (pentapeptide), oxyanion and catalytic triad (Ser, Asp, and His) (Figure 2). The presence of these conserved regions in the gene suggesting that the lipase is belonged to a member of family I.1. Lipase from sub-family I.1 have molecular masses in the range of 30-32 kDa and display a higher sequence similarity to the *Pseudomonas aeruginosa* (Jaeger and Eggert, 2002). The subfamilies I.1 also share important structural features. A part from the residues forming the catalytic triad, two aspartic residues involved in the Ca^{2+}-binding site (Nardini *et al*., 2000). Two cysteine residues forming a disulphide bridge are conserved in a majority of the sequence (Nardini *et al*, 2000). Residues involved in the formation of both the Ca^{2+}-binding site and the disulphide bridge are located in the vicinity of the catalytic His and Asp residues (Jaeger *et al*., 1999) and believed to be important role on the stabilization of the active center of the enzymes.

For further characterization 3D structure of the lipase was constructed in *silico* based on 3D structure of the *P. aeruginosa* (POB ID I EX 9). The superimposed result showed that 3D structure of LipAL17 and I EX 9 are similar (Figure 3) except on the conformational of His^{277} in the active center of the enzyme that is believed to have an impact on the activity of the enzyme (Nurhasanah *et al*., 2015; Nurhasanah *et al*., 2017)

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