Report

International Journal of Integrative Biology
A journal for biology beyond borders

IJIB
ISSN 0973-8363

Diversity of gene encoding thermostable Lipase from compost based on metagenome analysis

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Submitted: 1 Apr. 2015; Revised: 13 May 2015; Accepted: 27 Jun. 2015

Abstract
Five lipase gene have been cloned from domestic compost through metagenomic approach. Based on the sequence of the genes, all of the the clones show highly homolog to the lipase from *Pseudomonas* genus. The highest homology appeared to the *Pseudomonas stutzeri* gene with the homology varies from 96-100%. In addition, all clones showed similar motif to some conserved region of lipase family I.1. Based on the homology analysis, the clones are suspected to *Pseudomonas stutzeri* lipase family I.1 group. Two clones showed significant differences among the others, namely LK 3 and LK 5. LK 3 contains deletion of 31 amino acid residues in the N-terminal region resulting on lacks of the signal peptide region. While LK 5 showed a few amino acid substitution at around Asp^{255} as catalytic residue, in addition, Asp^{255} was replaced by Thr^{255}. Further analysis on the superimposed of 3D structure prediction between LK 5 with 1ex9 as template showed that the catalytic residues (Asp^{255}) and the surrounding region exhibited difference orientation when replaced by Thr^{255} and other amino acid substitution in LK 5 clone. Based on the above result suggested that amino acid substitution on LK 5 changed the conformation of the active center at the enzyme.

Keywords: Diversity; Lipase; Compost; Metagenome.

INTRODUCTION

Lipase is one of the group of hydrolase enzymes that function to catalyze hydrolysis of triglycerides become fatty acids and glycerol. In addition, lipases also catalyze other reactions such as esterification, interesterification, transesterification and separation of racemic mixtures (Sharma *et al*., 2001; Houde *et al.*, 2004; Hasan *et al*., 2006; Treichel *et al.*, 2010). The ability of lipase in fairly extensive biotransformation reaction make lipase widely applied in various fields of industry such as food, detergents, pharmaceuticals, textiles, leather, cosmetics, paper and organic synthesis (Jaeger and Reetz, 1998; Sharma *et al*., 2001; Houde *et al*, 2004, Hasan *et al* ., 2006).

Bacterial lipase are grouped into eight family, where family I, a *true* lipase group, is the largest group containing 6 subfamily. Sub-family I.1, I.2, I.3 are group of enzymes that produced by *Pseudomonas sp* (Arpigny and Jaeger, 1999). These lipase families are widely used in industry, especially for the production of chiral chemical compounds which used as the basic for the synthesis of pharmaceuticals, pesticides and insecticides (Theil, 1995). Lipases have characteristic folding pattern known as α/β hydrolase with a mixed central containing the catalytic residues. The catalytic center of the lipase consist of three catalytic residues: nucleophilic residue (serine, cysteine or aspartate), catalytic acid residue (aspartate or glutamate) and histidine residue. The nucleophilic serine residue usually appears in the conserved pentapeptide Gly-X-Ser-X-Gly (Jaeger *et al*., 1994; Arpigny and Jaeger, 1999).

Some researches were carried out to isolate lipase through cultivation such as from thermostable lipase-producing microorganism isolated from hot spring at around West Java (Widhiastuty *et al*., 2009; Febriani *et al*., 2010; Febriani *et al*., 2013), compost of thermogenic phase (Madayanti *et al*., 2008), and

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halophilic lipase isolated from the Mud Crater of Bleduk Kuwu, Central Java (Parwata *et al*., 2014; Asy'ari *et al*., 2014). Another method is also developed to obtain lipase by isolating microbial genomes directly from nature or environment without cultivation, known as metagenome (Handelsman, 2004). This method has been successfully performed for other enzymes (Suhartia *et al.*, 2014).

Compost is an artificial geothermal environment and consists of organic materials from domestic waste. During the composting process, the organic components are biochemically decomposed by mesophilic and thermophilic bacteria under aerobic conditions, where thermostable enzymes such as lipase plays an important role in this stage (Ishii and Takii, 2003; Madayanti *et al*., 2008). Research on microorganisms in various types of compost have been carried out and shows the diversity of microorganisms and enzymes produced in each phase of composting (Tiquia, 2002; Tiago *et al*., 2004; Ohnishi *et al.*, 2010; Hu *et al*., 2010; Partanen *et al*., 2010; Viera *et al.*, 2012; Safika *et al.*, 2013).

In this report, we present the diversity of genes encoded lipase cloned directly from traditional domestic compost.

MATERIALS AND METHODS

Materials

The microbial samples were collected from compost at TPS Sabuga, ITB, Bandung, Indonesia. The sampling was carried out for some stage during thermogenic phase. The primer used for PCR were FxlipS2 (5'ATGAACAAGAACAAAACCTTGCTCGCC 3') and RxLips2 (5'TCAGAGCCCCGCGTTCTTCAA 3').

Composting and sampling

Compost sample was taken from composting at TPS Sabuga, ITB, Bandung with the distance at around 1 km from the Biochemistry Laboratory. The composting process used domestic waste as compost material with traditional process. 1-2 kg of compost at thermogenic phases (45-67 ^{o}C) were collected from about 30 cm of compost surface. Samples were immediately used for further analysis.

Preparation of sample

30 gram of the fresh sample was added into 270 ml of sterile distilled water and mixed by shaking for 30 min. The extract was filtered through 0.22-µm-pore-size cellulose membrane filter (Sartorius, Germany). The pellet in the membrane was resuspended in STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA) and then stored at -20 ^{o}C until used for DNA isolation.

Isolation of total DNA community from compost

Total DNA chromosome from samples were extracted using the Power soil DNA isolation kit (MO BIO Laboratories, Inc., California). The extraction was based on the manufacture instruction. The DNA was stored at -20 ^{o}C until use.

Amplification of thermostable lipase gene

The whole lipase genes was amplified by using a set of primers namely FxLips2 and RxLips2. PCR was performed by using Sso fast evagreen supermix. A total of 20 µL PCR reaction mixture (10 µL Ssofast, 2.5 pmol primer pairs, 2 µL of community DNA as template and ddH_2O) was used for amplification. The PCR was carried out with and initial denaturation step at temperature of 98 °C for 7 min, each cycle of denaturation at 98 ^{o}C for 30 sec, annealing at 55 ^{o}C for 30 sec, extension at 72 ^{o}C for 1 min for 35 cycle and final extension at 72 ^{o}C for 10 min. PCR product was then examined with ethidium bromide-stained agarose gel.

Cloning and Sequensing

pJET1.2/blunt cloning kit (Fermentas) was used for cloning of PCR product. The ligation reactions were performed according to the kit manual. Total of 20 µL reaction mixture (10 µL buffer reaction, 24 ng/µL PCR product, 1 µL DNA blunting enzyme; 50 ng/µL pJet1.2/blunt cloning vector; 1 µL T4 DNA ligase and water nuclease free was used for ligation. The ligation product were used to transform *Escherichia coli* TOP 10 (Invitrogen). All sequencing was carried out in an ABI PrismR 3100 Genetic Analyser (Applied Biosystems) by the Macrogen Sequensing Service (Korea).

Sequence analysis

Homology analysis were performed by aligning the DNA sequences with NCBI data using BLASTN program (Altschul *et al*., 1997). Prior to homology analysis, the sequences were exposed to ORF finder to determin the coding region. The coding region was then translated *insilico* based on BLASTP program. Comparison the sequence with the GenBank was performed with Clustal W software package and BioEdit version 7.2 (Hall, 1999). The potential signal peptide of the identified lipase genes were predicted using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP), meanwhile the predictions of transmembran regions was performed using TMHMM Server 2.0 program

(http://www.cbs.dtu.dk/services/TMHMM). The ProtParam tool was used to calculate theoretical parameters of the protein (Gasteiger *et al*., 2005). Phylogenetic analysis was carried out with the maximum likehood method using Jones-Taylor-Thornton (JTT) model with Mega version 6 software. Bootsrapping (10.000 replicates) was used to estimate the confidence level of phylognetic reconstructions (Tamura *et al*., 2013).

3D Stucture Modeling

Three dimensional structure modeling was performed by superposition of amino acid sequence with protein structures in the Protein Data Bank (PDB). The amino acid sequence of the sample were submitted to the Swiss-Model server (Rost *et al*., 2004). Comparative models were built from parent PDB detected by Psi-Pred and evaluated by ProQ program (http://www.sbs.su.se/~bjornw/ProQ/ProQ.cgi). Visualizing of 3-dimensional models was used VMD (visual molecular dynamic) program.

RESULTS AND DISCUSSION

Lipase Gene from compost

Total DNA chromosome from variations of thermogenic phase at 45, 56, 60.5, 65 and 67 °C was successfully isolated. The DNA was used as template to amplify lipase gene fragment by using FxlipS2 / RxlipS2 primers. The fragment at around 0.9 kb were successfully amplified (Fig. 1). The amplicons have been clone and analyzed. From 21 positive clones, 5 of them appeared to have high homology with lipase gene, namely LK 1, LK 2, LK 3, LK 4 and LK 5. The above clones have been sequenced and the data were deposited into GenBank with accession number of KP204883, KP204884, KP204885, KP204886 and KP204887.

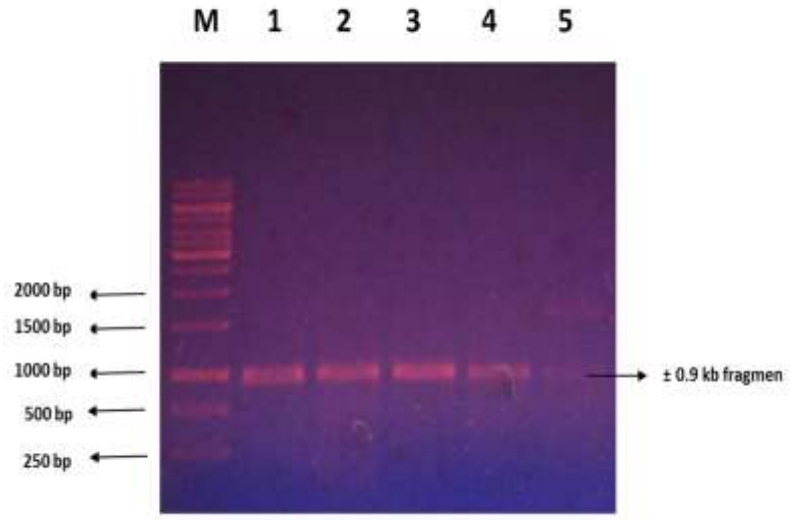

Figure 1: Electrophoregram of lipase genes from PCR product by using FxlipS2/RxlipS2 primer. M = marker DNA ladder; 1-5 = amplicons from each sampling point.

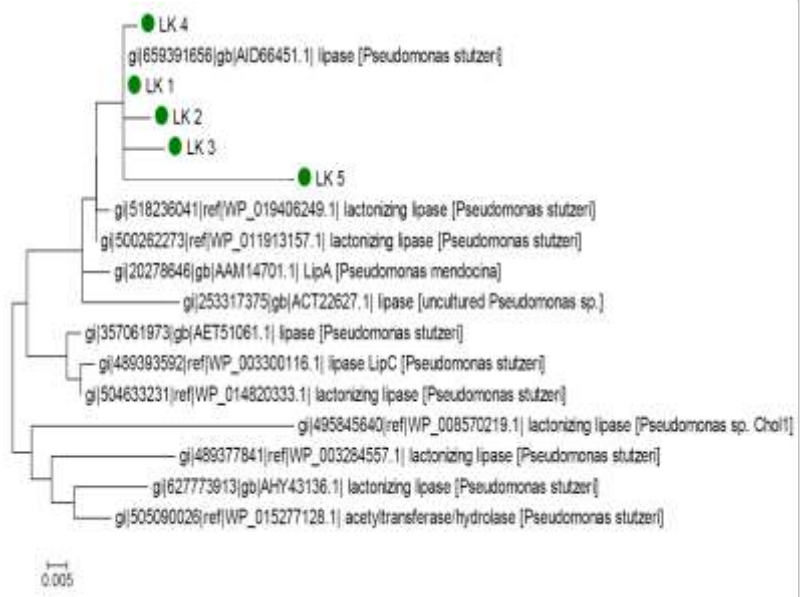

Figure 3: Phylogenetic tree of lipase from *P. stutzeri*. Green circles show of the fifth sample.

Sequences and Homology of the clones

Based on the sequence analysis of the genes, four clones (namely LK 1, LK 2, LK 4 and LK 5) have ORF at around 936 bp encoding 311 amino acids residues, while ORF of LK 3 only contains 843 bp encoding 280 amino acids residues. The sequence aligment to the NCBI data showed that all of the clones are closed homology to lipase from *Pseudomonas* genus with the highest homology to the lipase of *P. stutzeri* (AID66451.1). Further analysis by comparing the clones with the lipase of *P. stutzeri* showed that the clones show varies homology from 96-100 % (Table 1 *[Supplementary data]*), while variation among the sample are from 88-99 % (Table 2 *[Supplementary data]*). Amino acid homology of the clone to family I.1 lipase showed that all clones contain conserved motif of family I.1 including tetrapeptida, pentapeptida, catalytic triad and

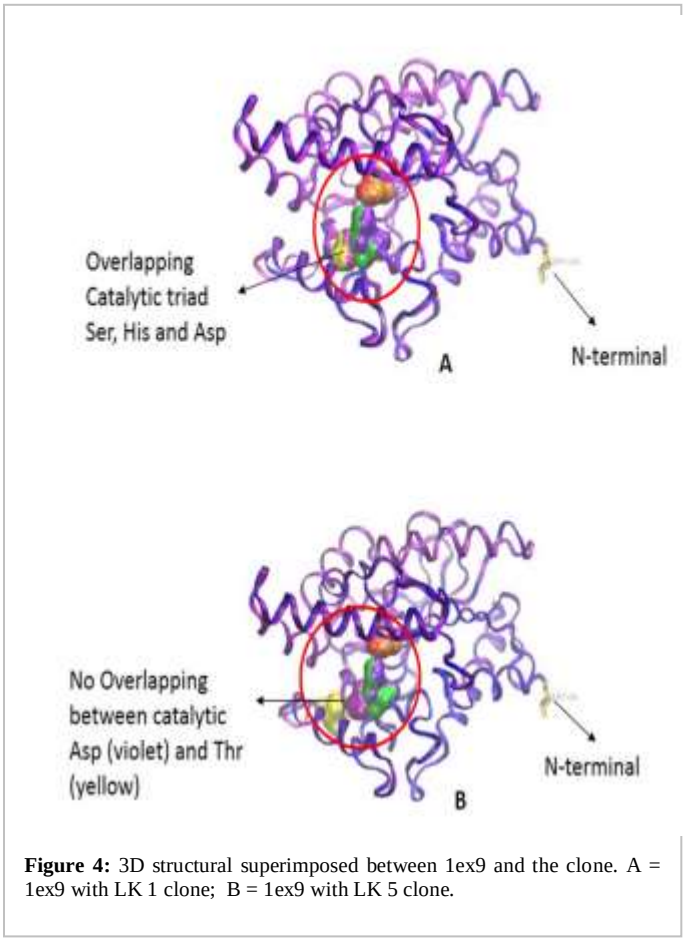

Figure 4: 3D structural superimposed between 1ex9 and the clone. A = 1ex9 with LK 1 clone; B = 1ex9 with LK 5 clone.

the conserved residues for the folding protein (Fig. 2 *[Supplementary data]*).

Tetrapeptide residues (Fig. 2 indicated by C) is conserved in the bacterial family I.1 lipase with Gly-Hyd-X-Gly (Hyd = Met, Leu, Val) that forming an oxyanion hole. The oxyanion hole has an important role to stabilize the tetrahedral intermediate conformation formed during the acylation reaction step. The stability of intermediate is formed by the amide group of the main chain Met^{43} residue as the second residue in tetrapeptida motif with His^{110} (Jaeger *et al.*, 1999; Nardini *et al*., 2000). The oxyanion hole motif was also stabilized by hydrogen bond between His^{41} and Arg^{83} (Fig. 2 indicated by circle ○). The last residues are also consereved in the family I.1 lipase (Nardini *et al.*, 2000). The similarity is also founded in the pentapeptide residues (Gly-X-Ser-X-Gly) that is conserved in all lipases with serine as nucleophilic residue (Fig. 2 indicated by D). The position of nucleophilic residue at the surface of the molecule is easy to form hydrogen bond with the side chain of His and other side of Asp as catalytic residues. Both residues are conserved in all of lipases including the clones (Fig. 2 indicated by star (☆)). The other conserved residues are Asp^{240} and Asp^{279} that play role bond calcium ion to form calcium bridges (Fig. 2 indicated by triangle △). Calcium brigde contributes to keep the conformation His^{277} at the correct position in the active site (Nardini *et al.*, 2000). Meanwhile two Cys residues (Cys^{211} and Cys^{261}) (indicated by regtangle in Fig. 2 indicated by regtangle □) are important in forming disulfide bonds (Arpigny and Jaeger, 1999; Nardini *et al.*, 2000). Based on amino acid sequence homology suggested that the five clones are member of family I.1 lipase group.

The sequences have also been compared with the data on Lipase Engineering Database (LED) which classifying lipase based on the amino acid sequence that is involved in the formation of oxyanion hole (Pleiss *et al.*, 2000; Fischer and Pleiss, 2003). The result showed that all clone are belong to GX class, abH15.02 superfamily with *P. stutzeri* as specific organism.

However there are clones, namely LK 3 and LK 5, are the most differences compared to the data from the *Pseudomonas* lipase in Gen Bank. LK 3 appeared 31 amino acids deletion (from amino acid number 10-40) at N-terminal region resulting the lipase that lack of signal peptide and transmembran domain (Table 3 *[Supplementary data]*; Fig. 2 indicated by A) which have important role in the mechanism of secretion of family I.1 lipaseusing Type II pathway (Jaeger *et al.*, 1999). Lipase without signal peptide was isolated from *P. fluoresecens* C9 derived from raw milk (Dieckelman, 1998) and *P. protegen* LipA Pf-5, however the last gene contains transmembran domain (Zha *et al.*, 2014). Meanwhile on LK 5 and other 3 clones produce propeptida containing 311 amino acid residues with 24-residues for signal peptide. The signal peptide is composed of positively charged amino acids at the N-terminal region (MNKNK), followed by hydrophobic residues (Fig. 2 indicated by B). The existing of signal peptide is important in the process of secretion (Zha *et al.*, 2014). Protein without the signal peptide is hardly secreted into extracytoplasma and remain as intracellular protein.

Meanwhile LK 5 showed 11 amino acid substitution in the conserved C-terminal region (Fig. 2 indicated by E). The substitution caused Asp^{255} as the catalytic residue replaced by Thr, meanwhile Cys^{261} which is involved on disulfide bond formation, was replaced by Ala. Substitution of Asp^{255} to Gln on lipase at *P. glumae* exhibited a half activity of the enzyme (Noble *et al.*, 1993). However substitution of Asp^{255} → Thr is still unclear yet. Mutations in Cys residues involved in disulfide bond formation has also been studied at *P. aeruginosa* lipase in which mutation of Cys → Ser showed more sensitive to heat denaturation and proteolytic degradation, suggesting that disulfide bonds are formed to stabilize the molecule (Jaeger *et al.*, 1999; Nardini *et al.*, 2000). In addition few amino acid substitution occur in the samples compared to that the *P. stutzeri* (Table 4 *[Supplementary data]*). Further analysis by

construction of phylogenetic tree showed that all clones are cluster in one branch (Fig. 3).

3D-Structure Prediction of Lipase

3D structure prediction of lipases were constructed based on Swiss Model (Rost *et al*., 2004). Lipase of *P. aeruginosa* was used as template. The analysis of structure prediction using Psi-Pred program showed less difference between the LK 3 and other clones, although the clone is loss of 31 amino acid residues. Based on the above analysis the overall structure of the five clones are less differences.

Four clones (LK 1, LK 2, Lk 4 and LK 5) have catalytic residues consisting of Ser^{109}, Asp^{255} and His^{277}, while LK 3 has catalytic residue at position Ser^{78}, Asp^{224} and His^{246}. In this active site, lipases contain hydrophobic pocket that play role in substrate recognition by the major residue Gly^{45}, Phe^{46}, Tyr^{54}, Trp^{55}, Leu^{278}, Val^{281} and Phe^{284}. Whereas in LK 3 hydrophobic residues that contribute to the pockets are at position Gly^{14}, Phe^{15}, Tyr^{23}, Trp^{24}, Leu^{247}, Val^{250} and Phe^{253}. The above result is in line with the lip C *P. stutzeri* (Maraite *et al*., 2013). In addition, based on the model of molecular dynamics simulations in the presence of water and tetrahydrofuran (THF) showed two lids cover the active site (Maraite *et al*., 2013). The lids showed at the same position for 4 clones, meanwhile for LK 3, the lid showed at different position.

The most difference was shown by LK 5 where the clone has 11 amino acid substitution at around catalytic aspartate residue. Further analysis to probe more detail concerning on the conformational change at around catalytic residues, two clones LK1 and LK 5 were superimposed to structure of 1ex9. The results showed that LK1 has overlapping catalytic triad position (Fig. 4A), meanwhile LK 5 showed difference orientation at position of aspartate catalytic residue and the surrounding region when replaced by threonine and other amino acid substitution (Fig. 4B). This result suggested that amino acid substitution on LK 5 changes the conformation of the active center at the enzyme.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

We thanks to Directorate General of Higher Education, Department of National Education for funding this research through Doctoral Dissertation Grant 2014 (Research Grant Number 227/UN26/8/PL/2014) to Nurhasana.

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