Overexpression of Lipase Gene from *Alcaligenes* sp. JG3 and its Activity toward Hydrolysis Reaction

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Received: May 8, 2019
Accepted: July 18, 2019

DOI: 10.22146/ijc.45663

Abstract: *Bacterial lipase holds an important role as a new source for many industrial catalysts. The investigation and understanding of the lipase-encoding gene become apparent as the key step for generating high-quality lipase as biocatalyst for many chemical reactions. In this study, bacterial lipase from* Alcaligenes *sp. JG3 was produced via overexpression gene method. This specific lipase was successfully overexpressed using pQE-30 vector and E. coli M15[pREP4] as host, producing His-tagged protein sized 46 kDa and was able to hydrolyze triacylglycerol from olive oil with the calculated unit activity and specific activity of 0.012 U and 1.175 U/mg respectively. The in silico investigation towards lipase JG3 revealed that it was categorized as ABC transporter protein as opposed to the conventional hydrolase family. Lastly, amino acid sequences SGSGKTT from lipase JG3 was highly conserved sequences and was predicted as the ATP-binding site but the catalytic triad of serine, histidine, and aspartate has not been solved yet.*

Keywords: Alcaligenes*; lipase gene; enzyme activity; a transporter protein*

■ INTRODUCTION

Lipases (EC 3.1.1.3) are lipolytic enzymes that are able to perform as a biocatalyst for hydrolysis reaction of triacylglycerol to glycerol and free fatty acid [1]. Not only hydrolysis reaction, but a wide range of chemical reactions could also be assisted by lipase, namely acidolysis, transesterification, esterification, and aminolysis, which make lipase vastly used in various fields of industry. Even to this day, lipase is placed third behind protease and amylase for the most demanded enzyme for industry [2-3]. A more beneficial tendency on lipase study is involving microbial lipase as it has many advantages over lipase from plants and animals, such as; easy to grow, relatively low cost and has higher stability [4]. Looking at these properties, many explorations of microbial lipase have been reported, for example the cold-active lipase from *Candida albicans* [5], organic solvent-tolerant lipase from *Bacillus licheniformis* [6] and thermostable lipase from *Geotrichum candidum* [7].

The mechanism of chemical reaction catalyzed by lipase involves the specific active site that is highly conserved as pentapeptide Sm-X-Nu-X-Sm, where Sm (small residue) is usually glycine, X could be any residue and Nu (nucleophilic residue) is generally serine [8]. Although the active site of lipase usually remains the same, the homology and protein length of bacterial lipase may vary. Many reported that the protein size of bacterial lipase ranges from 20–60 kDa as determined by electrophoresis SDS-PAGE [3,9-10]. In Indonesia, a certain soil bacterium, *Alcaligenes* sp. JG3 was confirmed to have an extracellular lipase activity toward hydrolysis of olive oil which was up to 5.61 U/mg with the presence of organic solvent *n*-hexane [11-12]. For this bacterium, a particular name has been proposed based on the phylogenetic analysis and the morphological and biochemical tests which is *Alcaligenes javaensis* JG3 [13-14].

An attempt to understand the *Alcaligenes* sp. JG3's lipase (defined as Lip.JG3) have been carried out including investigation of its characteristic and activity as a catalyst, mapping its nucleotide and amino acid sequences and also cloning of the gene [11,15-17]. In this report, overexpression of Lip.JG3 has been carried out

successfully. By doing so, purified Lip.JG3 could be obtained and was able to be put on hydrolysis reaction in order to confirm the gene sequence ability as a lipase enzyme. The overexpressed Lip.JG3 were meant for possible further studies such as kinetics of lipase-catalyzed reaction determination and stability of over expressed Lip.JG3 investigation in order to be the new source of lipase for producing biodiesel or flavoring agent.

■ EXPERIMENTAL SECTION

Materials

Alcaligenes sp. JG3 bacterium samples were a collection of Laboratorium Penelitian dan Pengujian Terpadu UGM, originally isolated from the root of *Zea mays* in the agricultural land of Purwokerto, Central Java, Indonesia [18]. Primer forward Fjg3 (5'-ATGACCGAGC TGACTGTAG-3'), Fexp (5'-GGATCCACCGAGCTGA CTGTAGAC-3') and reverse Rjg3 (5'-TCAGGAGGGGT AAATCCAC-3'), Rexp (5'-AAGCTTGGAGGGGTAAA TCCACAG-3'), agarose, proteinase-K, ethidium bromide, DNA marker, nuclease-free water, Quick Miniprep Plasmid Kit (Invitrogen), QIAexpresionist Kit Type IV (Qiagen), BamHI and HindIII restriction kit (Promega), $MgCl_2$, NaCl, Sodium dodecyl sulfate (SDS), isopropanol, ethanol, tris base (Merck), Triton X-100, Na-EDTA (Sigma), TAE buffer, loading buffer (Vivantis), extra virgin olive oil (Bertolli), Pierce BCA protein assay kit and pre-stained protein marker (Thermo Fisher Scientific), Go taq green PCR mix, SOC medium, LB medium, nutrient agar, nutrient broth (NB), *n*-hexane, acetic acid, ammonium persulfate, tetramethylethylenediamine (TEMED), Coomassie blue, acrylamide, bis-acrylamide, glycine, phenolphthalein, and also antibiotic ampicillin and kanamycin. All the chemicals used in this study were of pro-analysis laboratory grade.

Instrumentation

The instruments used in this study were Thermal Cycler (Bio-Rad), SDS and Gel electrophoresis (Bio-Rad), Gel Documentation System (Bio-Rad), UV lamp (Bio-Rad), Autoclave (Hirayama HL 36 AE), Vortex (Barnstead), Water Bath Incubator (OSK Seiwa Reiko), Centrifuge (Sorvall Biofuge), UV-Visible Spectrophotometer (Shimadzu Probe), Shaking Incubator Chamber, Ultrasonic Cell Crusher (SJIA-250W), MEGA 7.0 software and bioinformatics online servers e.g. MUSCLE, PSIPRED and I-TASSER.

Procedure

DNA isolation and cloning of the lipase gene

DNA isolation, amplification gene and cloning of Lip.JG3 conducted in this study were derived from the previous research [17].

Construction of lipase gene DNA recombinant onto pQE expression vector

Fifty ng of isolate pGEM-T/Lip.JG3 was amplified using primer Fexp and Rexp by the following conditions: pre-denaturation for 5 min at 95 °C, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 30 sec and post-extension at 72 °C for 10 min. For constructing the pQE-lipase JG3 DNA recombinant, both lipase JG3 DNA and pQE vector needed to undergo restriction reaction to provide the complement nucleotides sites. A set of reactions was prepared (Table 1) and then incubated in a water bath at

Table 1. Composition mixtures for restriction reaction

Component	Volume (µL)	Component	Volume (µL)
Ultrapure water	0.8	Ultrapure water	14.8
Buffer E	2	Buffer E	2
Acetylated BSA	0.2	Acetylated BSA	0.2
Lip.JG3 DNA	15	pQE vector	1
Gently mixed by pipetting		Gently mixed by pipetting	
BamHI	1	BamHI	1
HindIII	1	HindIII	1
Gently mix by pipetting (final volume 20 µL)		Gently mix by pipetting (final volume 20 µL)	

37 °C for 4 h. After the reaction was finished, both lipase JG3 DNA and pQE vector were analyzed with electrophoresis agarose 1.5% and purified using Pure Link™ Quick Gel Extraction Kit. The ligation reaction was performed with a mixture of 15 and 5 μL of restricted lipase JG3 and pQE vector respectively, 8 μL 2xRapid Ligation Buffer and 2 μL T4 DNA ligase and then incubated overnight at 4 °C to generate pQE-lipase JG3 DNA recombinant.

Overexpression of lipase protein

Before the Lip.JG3 could be overexpressed, the recombinant DNA needed to be cloned into a compatible host. The gene transformation was carried out via a heat-shock procedure. Ten μL of the ligation mix was put on the sterile 1.5 mL microtube and 50 μL of the thawed *E. coli* M15[pREP4] aliquot was added to the tube. The mixture was then mixed gently and kept on ice for 20 min. After that, the tube was transferred to a 42 °C water bath for 90 sec and immediately returned to the ice for another 2 min. To the mixture, 940 μL SOC medium was added and incubated at 37 °C for 1.5 h with shaking. The *E. coli* aliquot was plated on LB-agar medium containing ampicillin 100 μg/mL and kanamycin 25 μg/mL incubated overnight at 37 °C.

For lipase protein overexpression, a single colony from LB-agar plate was picked, transferred into 5 mL LB-broth containing antibiotic and incubated overnight at 37 °C. Five hundred μL of overnight cultures then inoculated into 200 mL LB-broth and incubated with shaking until the OD_{600} was 0.5–0.7. After the desired OD_{600} was reached, IPTG was added to the cultures (to a final concentration of 1 mM) to induce expression and incubated at 37 °C with shaking for another 5 h. The cells were harvested by centrifugation (6000 rpm for 15 min) and washed twice using PBS buffer to remove all the medium. The harvested cells were then resuspended using 2 mL PBS buffer and sonicated for 4 min using 2 sec bursts and 1 sec cooling down between each burst to break down the cell. The lysate was centrifuged for 5 min at 6000 rpm and the supernatant was collected as crude protein extract.

Since the expressed lipase protein was tagged with 6xHistidine protein, it can be purified using Ni-NTA matrices. Five hundred μL Ni-NTA agarose slurry was loaded into purification column and 500 μL crude protein extract was transferred into the column, then the mixture was centrifuged at 2000 rpm for minutes (this process was repeated until all the crude extract was loaded into the column). The lysate-Ni-NTA mixture was washed three times using 400 μL PBS buffer containing 20 μM imidazole and then the lipase JG3 protein was eluted using 200 μL PBS buffer containing 250 μM imidazole. Each flow-through was collected separately for further analysis.

Concentration quantification of each flow-through was performed using bicinchoninic acid (BCA) protein assay. For the standard curve, a series of BSA (Bovine Serum Albumin) was made with the concentration ranging from 0–2000 μg/mL. As for the sample, 50 μL of each flow-through was mixed with 1 mL working reagent and then incubated for 30 min at 37 °C and then the absorbance of each sample was measured at wavelength 562 nm. The protein concentration can be calculated as the slope of the standard curve equation. For qualitative analysis, each sample was examined using electrophoresis SDS-PAGE (10% polyacrylamide) at 100 volts and visualized with Coomassie blue.

Determination of lipase activity via a hydrolysis reaction

Determination of lipase activity was conducted using the titrimetric method by measuring the free fatty acid formed from hydrolysis reaction. One gram of olive oil was diluted to a final volume of 10 mL using *n*-hexane. Five hundred μL of purified lipase was added to the mixture and incubated for 5 h on shaker incubator (37 °C, 150 rpm). After the reaction was completed, the polar phase was separated and diluted with the addition of 10 mL ethanol and 2–3 drops of PP indicator was added to the solution. The free fatty acid was titrated with NaOH 0.0125 M (standardized by oxalic acid). As a control reaction, the same mixture composition was used without lipase enzyme. The lipase activity can be calculated by the following equations:

$$\text{Unit activity}\,(\text{U}) = \frac{(\text{V NaOH sample} - \text{V NaOH standard})\,\text{M NaOH}}{\text{time of reaction}} \quad (1)$$

$$\text{Specific activity}\ (\text{U/mg}) = \frac{\text{unit activity}}{\text{enzyme mass}} \quad (2)$$

Analysis of lipase protein structure and function

In silico approximation was used to analyze the homology, structure, and function of Lip.JG3 [15]. Alignment of multiple lipase sequences was performed using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/), the distribution of secondary structure of Lip.JG3 was carried out using PSIPRED server (http://bioinf. cs.ucl.ac.uk/psipred/) and prediction of 3D for Lip.JG3 was conducted using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

■ RESULTS AND DISCUSSION

Construction of DNA Recombinant

Based on the restriction map and restriction enzyme list of the vector multi-cloning sites, a pair of expression primer was designed by adding the chosen enzyme restriction sites (G-GAT and A-AGC that would be cleavaged by BamHI and HindIII restriction enzymes respectively) to the prior primer pair (Fjg3 and Rjg3). Cutting the pQE-30 vector plasmid using this restriction enzyme, two sticky-end sites were created making the recombination DNA prone to be successful. The appearance of the cleavaged plasmid (only one band appeared) was somewhat different compared to the initial plasmid (three bands appeared) as shown in Fig. 1(a) and (b). This phenomenon occurred due to the three typical conformations of plasmid that no longer exist after being cleavaged by restriction enzymes. The isolated DNA of pGEM-T/Lip.JG3 were amplified using primer Fexp and Rexp resulting ~1100 bp amplicon as can be seen in Fig. 1(c). To this amplicon, the restriction procedure was also performed creating the compliment of the cleavaged plasmid.

Overexpression of Lipase JG3

Prior to overexpression, the cut pQE-30 and Lip.JG3 were ligated and transformed into the host cell of *E. coli* [M15pREP4]. This pQE-30 vector has many features like consisting of strong T5 promoters, containing two strong transcriptional terminators (t_0) to prevent read-through transcription and ensure the stability of the expressed protein and providing multiple cloning sites and 6xHis-tag coding sequence to ease the purification of the protein target. Since the T5 promoter can initiate a high transcription rate, the existence of high-level repressor becomes crucial to regulate the expression. Thus, any *E. coli* host strain containing the repressor [pREP4] shall be used for the production of recombinant protein.

The transformed *E.coli* containing recombinant of pQE30/Lip.JG3 was inoculated on LB-agar medium in the presence of ampicillin antibiotic (Fig. 2(a)). DNA recombinant was isolated and cut using the previously restricted enzyme to check the availability of pQE-30/Lip.JG3. The enzyme restriction produced two bands of DNA sized ~3 and ~1.1 kb representing the pQE-30 vector and Lip.JG3 gene (Fig. 2(b)). Having confirmed the presence of pQE-30/Lip.JG3 in the host, a single colony of *E. coli* was rejuvenated until OD_{600} was 0.5–0.7 before induced by the addition of IPTG. Analysis of crude and purified lipase using SDS-PAGE is presented in Fig. 3. The lipase JG3 protein appeared as a single band at ~46 kDa. Many comparable molecular weight from bacterial lipases have been reported, for instance, lipase from *B. stearothermophilus*, *T. atroviride* LipB, *Acinetobacter* sp. AU07, *B. thermoleovorans* ID-1, *Cohnella* sp. A01, which were 67.0 kDa, 57.0 kDa, 45.0 kDa, 34.0 kDa, 29.5 kDa, respectively, as determined using SDS-PAGE electrophoresis [19-21].

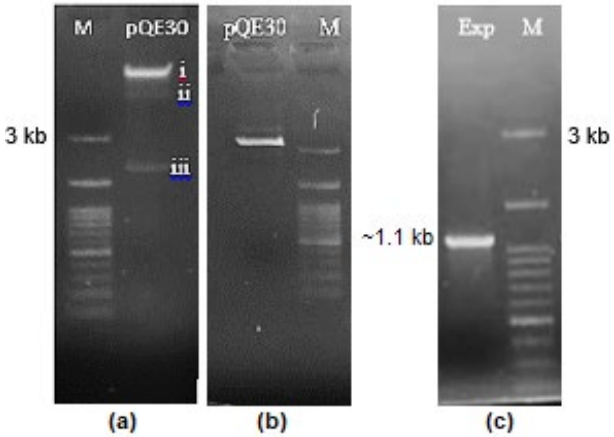

Fig 1. Electrophoresis visualization of (a) uncut pQE-30 vector, symbol i, ii and iii indicating three plasmid conformation state of relaxed, linear and supercoiled, (b) cut pQE-30 vector, (c) amplified Lip.JG3 using expression primer

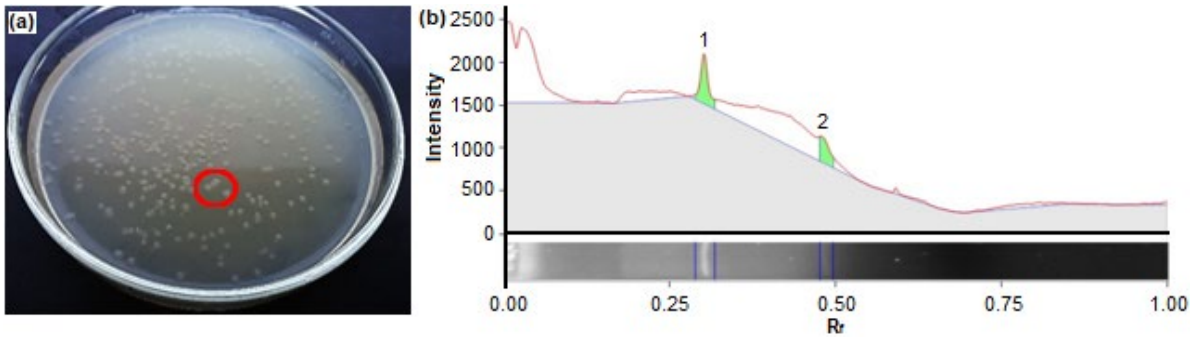

Fig 2. (a) Representative of the transformed *E. coli* colonies containing DNA recombinant of pQE-30/Lip.JG3 (circled in red) and (b) Visualization of BamHI and HindIII restriction using Gel Documentation System; peak 1 was the pQE-30 vector and peak 2 was Lip.JG3 gene

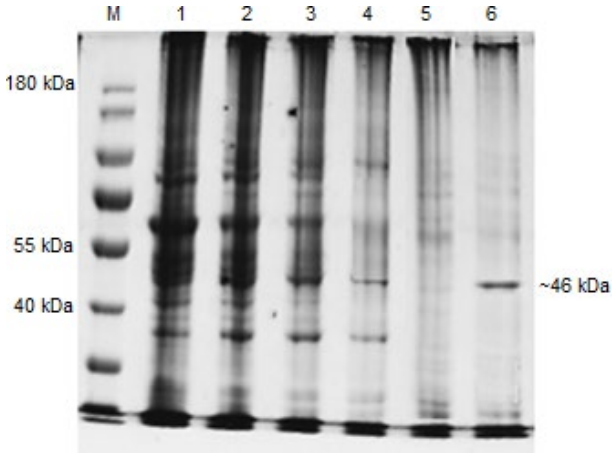

Fig 3. Visualization of SDS-PAGE analysis. Lane M: protein marker, 1: crude extract protein, 2: flow through from initial binding, 3: first wash, 4: second wash, 5: third wash and 6: purified Lip.JG3 protein from the Ni-NTA resin column

Lipase JG3 Activity on Hydrolysis Reaction

Determination of Lip.JG3 unit activity and specific activity were carried out via hydrolysis reaction toward extra virgin olive oil to produce a free fatty acid. Extra virgin olive oil was chosen instead of regular olive oil due to the high percentage of unsaturated long-chained ester in it and lipase favors such substrate [22]. The purification and activity of Lip.JG3 is summarized in Table 2. Both of the unit activity and specific activity for crude extract and second washed protein was lower than purified Lip.JG3 due to the presence of many other proteins that might interfere with the hydrolysis reaction although the amount of the protein itself was higher. On another report, a similar pattern of hydrolysis activity from purified lipase had higher activity than the crude extract from *Bacillus amyloliquefaciens* PS35 [23].

The specific activity of purified Lip.JG3 was lower than the previous study which was up to 5.61 U/mg [11]. This phenomenon might occur due to the incubation time of the previous study that was much longer (12 h) resulting in a more significant amount of lipase. Besides that, overexpression of Lip.JG3 involved washing steps that may reduce the amount of purified lipase, hence the lower the specific activity. Nevertheless, Lip.JG3 was proven to be able to catalyze the hydrolysis reaction and therefore can be put into consideration as the new source of microbial lipase. To increase the activity of His-tagged expressed enzyme, one may remove the histidine sequences. By doing so, the activity of lipase without histidines was able to increase up to 1.58 fold on *p*NPP assay [24].

Table 2. Summary of the purification of lipase and enzyme activity

Sample	Protein concentration (mg/mL)	Protein used on hydrolysis reaction (mg)	Unit activity (U)	Specific activity (U/mg)
Crude extract	2.173	1.086	0.005	0.005
Second washed protein	0.215	0.043	0.005	0.116
Purified Lip.JG3	0.051	0.010	0.012	1.175

Prediction of Lipase JG3 3D Structure

Structural knowledge of biomolecules is a vital key to comprehend the function and mechanism of action, because the different structure of one protein to another may lead to the differentiation of act. Bioinformatics is a recent approach to predict the structure and function of a protein [25]. Amino acids vary in their ability to form various secondary structure elements. This secondary structure can be used to enhance the multiple sequences alignment between proteins as it gives more accurate information than the simple sequence that is sometimes unalignable [26]. Fig. 4 shows the distribution of Lip.JG3 secondary structure that possessed 41.67, 25.89 and 32.44% of coil, strand and helix conformation, respectively.

I-TASSER, an automated protein structure, and function were used to predict the 3D model of Lip.JG3 as shown in Fig. 5(a). I-TASSER generates full-length atomic structural models from multiple threading alignments and iterative structural assembly simulations followed by atomic-level structure refinement [27]. Using multiple protein-protein network and structure comparison, I-TASSER deducted Lip.JG3 to have the most similar function with template protein of Fe^{3+} ions import ATP-binding protein FbpC since it showed a high score in C-Score (0.89). Although this template protein (PDB Hit: 3fvqA) acts as a transport protein, it is classified into hydrolase superfamily since ABC Transporter Protein is able to hydrolyze ATP to power up

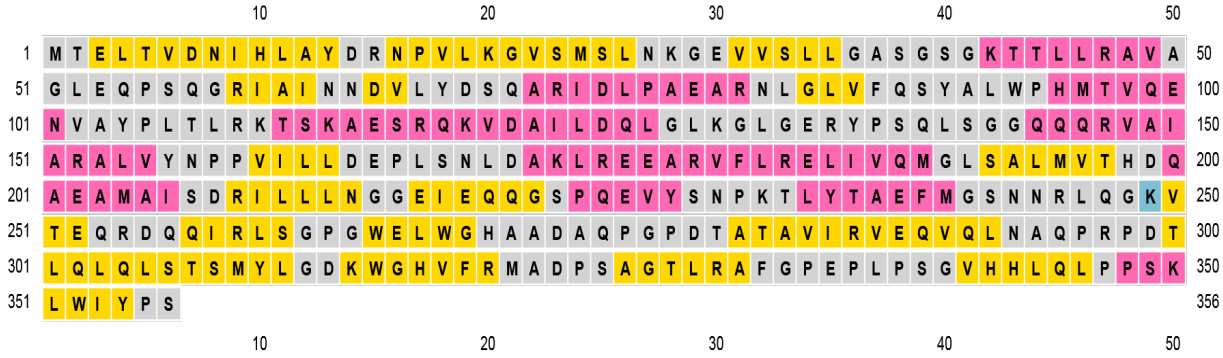

Fig 4. Distribution of secondary structure conformation for Lip.JG3, where the conformation of sheet, helix and coil are represented by the color of yellow, pink and grey respectively

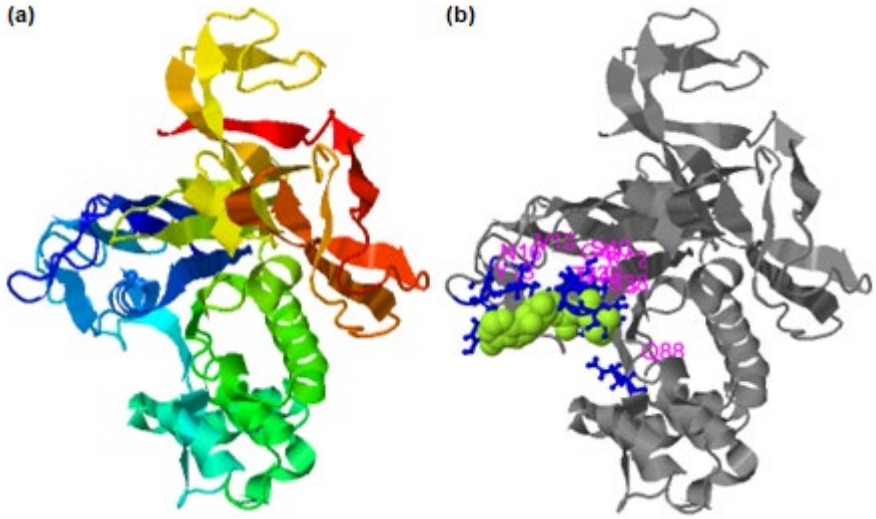

Fig 5. (a) Lip.JG3 3D structure prediction; blue to red color implies N- to C- termini (b) Predicted interaction of Lip.JG3 with ligand ATP, green and blue color represent ligand amino acid binding sites based on the template of PDB Hit: 3fvq

its act [28-29]. On the other hand, Lip.JG3's 3D model has the highest similarity with ABC-ATPase (PDB Hit: 1OXT) based on TM-align structural alignment program to match the model of all structures in the PDB library with TM score up to 0.939 [30].

Homology of Lipase JG3 Protein

Based on the similarity of sequence, structure, and function, lipases may be classified into different groups. There are eight known classes of microbial lipase based on the conserved amino acids sequence and biochemistry properties. Lip.JG3 consists of 357 amino acids and share 98% similarity with lipase from *Alcaligenes faecalis* subsp. *faecalis* NCIB 8687 but the nucleotide sequence share not as high similarity, due to the fact that one amino acid is encoded by more than one possible codon. That is why the nucleotide sequence of the lipase gene is varied although the conserved sequence is still able to be observed [31].

As stated above, Lip.JG3 has the characteristic of protein that comes from an ABC Transporter family. To validate that statement, homology analysis was performed towards other ABC Transporter lipases such as *Serratia marcescence* and *Alcaligenes faecalis* and is shown in Fig. 6. Based on the homology analysis of the three lipases, they share the conserved sequences of Walker-A (LLGASGSGKTT) and Walker-B (VILLDEP) motif and also ABC signature nucleotides (LSGGQQQRVAIA) underlined in red.

Active Site of Lipase JG3

Generally, lipases from hydrolase family have the catalytic triad of serine, histidine, and aspartate [32]. Since Lip.JG3 was suggested as ABC Transporter Protein, it might be difficult to pinpoint the catalytic triad precisely without Molecular Dynamic (MD) simulation. However, previous studies reported that lipase from *Serratia marcescens* has a loop or helical lid which is responsible for the lipase activation. The presence of this lid over the lipase's active site contribute lipase activity in the interphase of water-oil, when the lid is open lipase active site may bind substrate and convert it into desirable product [33-34].

Fig 6. Alignment of Lip.JG3 amino acid sequence against lipase/ABC Transporter from *Serratia marcecense* (NCBI ID: WP_025304186.1) and *Alcaligenes faecalis* (NCBI ID: HCA17200.1)

Many suggestions have been proposed to solve the mechanism of ABC protein to hydrolyze the appropriate substrate. The Walker-B motif, specifically the glutamic acid residue is predicted as the active site as it directly binds into the phosphodiester bond resulting hydrolysis reaction [15]. Residues of T^{43}, Q^{88}, $D1^{64}$, and E^{165} from Lip.JG3 amino acid sequence was predicted to have the ability to bind Mg^{2+}, as well as from another ABC Transporter protein. The presence of Mg^{2+} enhances the binding ability of phosphate residue on ATP. While the residues of Y^{13}, N^{16}, V^{18}, S^{38}, G^{39}, S^{40}, G^{41}, K^{42}, T^{43}, T^{44}, and Q^{88} are predicted to be the binding site for Ca^{2+} and the activity of crude extract lipase from *Alcaligenes* sp. JG3 is enhanced by 1.38 times with the addition of Ca^{2+} [11]. This type of metal ion bond implies that the act of this enzyme is supported by metal ion cofactors [35].

The classification of ABC transporter for Lip.JG3 might be highlighted due to the possibility of the protein secretion via ABC exporter pathway [36]. Although the amino acid sequences of Lip.JG3 responsible for ATP-binding was able to be defined and it is also able to catalyze hydrolysis reaction, the true catalytic sequences responsible to bind with the triacylglycerol has yet to be confirmed. Further simulation of the interaction between Lip.JG3 and oil substrate is still needed to be investigated.

■ CONCLUSION

The overexpression of lipase gene from *Alcaligenes* sp. JG3 was successfully carried out with *E. coli* M15[pREP4] as host, producing lipase protein sized 46 kDa. The Lip.Jg3 was able to hydrolyze olive oil with calculated unit activity and specific activity of 0.012 U and 1.175 U/mg respectively. From 3D protein structure analyses, Lip.JG3 belonged to the ABC transporter protein superfamily, indicating that it might be secreted via the ABC pathway.

■ ACKNOWLEDGMENTS

This research was fully funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia with the contract number 2033/UN1/DITLIT/DIT-LIT/LT/2018. Surajiman, Istini and Tri Purwanti are acknowledged for laboratory technical assistance.

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