Three Dimensional Structural Modelling of Lipase Encoding Gene from Soil Bacterium *Alcaligenes* sp. JG3 Using Automated Protein Homology Analysis

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Abstract: *Bacterial lipases have significant potential to be used as the biocatalyst for many chemical reactions. In this study, a novel gene encoding lipase was isolated from an* Alcaligenes *sp. JG3. A pair of designed primer has successfully isolated 1 kb (LipJG3) that shares 98% identity towards lipase from* Alcaligenes faecalis *during sequence analysis. By using* in silico *tools, LipJG3 was related to the transporter protein sequences. Three highly conserved regions consisting of EASGSKT, VILLD, and LSGGQQQRVAIA were found. These regions were known as ATP-binding signature at Walker-A and Walker-B motifs and the S signature of ABC transporter family respectively. In addition, the 3-D structure of LipJG3 has been suggested but the role of the catalytic triad residues have been not fully understood.*

Keywords: *alcaligenes; lipase gene; LipJG3; ABC transporter protein*

■ INTRODUCTION

Lipases (EC 3.1.1.3) are a group of hydrolase enzymes that break down the carboxyl ester bonds of triacylglycerols to fatty acids and glycerol under water-lipid interface. The hydrolytic property of lipases allows them to be used in several chemical reactions, including transesterification, acylation, alcoholysis, and resolution of racemates [1-2]. As a biocatalyst, the boundless industrial applications of lipases are apparent in beverages, food dressing, dairy, pharmaceuticals, cosmetics, paper and leather productions [3].

In general, the genes encoding lipase enzyme are highly variable in the length and homology of encoding sequences. Nevertheless, they share the similar signature motif of a pentapeptide region, Gly-X-Ser-X-Gly, the three-dimensional structural fold, α/β-hydrolase fold, and machinery catalytic residues, Ser, His, Asp or Glu [4]. The serine residue is usually located as a part of GXSXG region between the fifth β strand and the following α helix, forming a nucleophilic elbow with a sharp γ-like turn. However, the location of the other catalytic residues in the protein fold seems to vary [5].

Microbial lipases are further advantageous and more stable than lipases delivered from animals and plants. Furthermore, microbial lipases have the high yield potential, diverse properties available and ease of genetic manipulation [6-7]. Therefore, new exploration of microbial lipase is indispensable. In previous studies, an extracellular lipase from bacterial strain JG3 revealed an activity to catalyze the hydrolysis of triacylglycerol from olive oil [8]. However, the taxonomy status of this bacterium is not clear. The fragment of the lipase gene (0.4 kb) was amplified using degenerate PCR technique, resulting in a 98% similarity towards a lipase gene from *A. faecalis* [9]. Accordingly, this strain JG3 is considered as a member of *Alcaligenes* genus.

In this present investigation, we report the sequence analysis and characterization of the lipase gene from bacterial strain JG3 (LipJG3). The enzyme is expected to be successfully sequenced prior to gene cloning and genetic manipulation, possibly useful for catalyzing biodiesel production and biochemical reactions.

■ EXPERIMENTAL SECTION

Materials

The lipase producing bacterium strain JG3 collected from Purwokerto area, Central Java, was used

as the genomic DNA source. The PCR primers used in this study were synthesized at IDT DNA, Singapore. All analytical and molecular biology grade of chemicals used in this study were commercially available; Nutrient Agar (NA) and Nutrient Broth (NB) (Sigma Aldrich), DNA ladder (Vivantis), Go Taq Green (Promega), nuclease-free water (NFW) (Gibco), ExoSAP IT kit (USB), PureLinkTM Quick Gel Extraction kit (Life Technologies), and BigDye X-terminator Kit (Applied Biosystem).

Instrumentation

The instruments used in this study were the vortex machine (Thermolyne, UK), bio centrifuge (Sorvall, UK), UV-Vis Spectrophotometer (Shimadzu, Japan), Thermal Cycler (Bio Rad), Gel Electrophoresis (Bio Rad), UV transiluminaor (Bio Rad), and DNA Sequencer (Applied Biosystem 3500 Genetic Analyzer), MEGA 7.0 software, and bioinformatics online servers.

Procedure

Bacterial identification based on 16S rRNA sequence

A single colony of bacterial culture from the solid NA medium was taken and mixed well with 50 μL NFW. The mixture was incubated at 96 °C for 10 min and then centrifuged at 12,500 rpm for 3 min. The amplification of 16S rRNA gene was conducted as previously described by a previous study [10]. The universal primers used were 27F (AGAGTTTGATCMTG GCTCAG) and 1642R (CGGYTACCTTGTTACGAC). The 25 μL PCR mix contained 12.5 μL Go Taq Green, 1 μL bacterial DNA, 10 pmol (1 μL) primers, and 9.5 μL NFW. The condition for thermal cycling were as follows: initial denaturation at 95 °C for 4 min, followed by 30 cycles, including denaturation step at 95 °C for 50 sec, annealing step at 55 °C for 50 sec, elongation step at 72 °C for 2 min, and one last cycle of final elongation at 72 °C for 7 min. A single DNA fragment observed on the electrophoresis gel agarose was purified by ExoSAP IT kit prior to DNA sequencing. A cladogram with neighbor-joining algorithm was built up using MEGA 7.0 software [11].
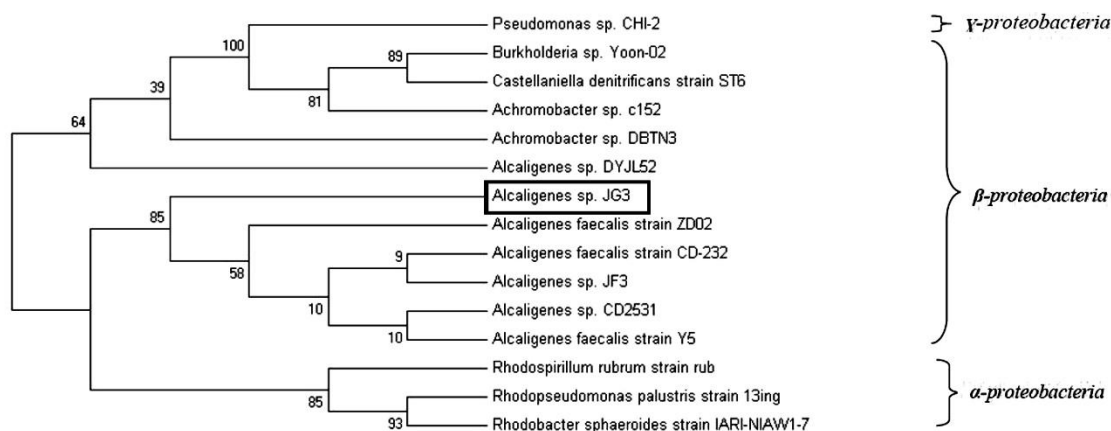
Isolation of lipase gene

Prior to lipase gene amplification, the bacterial cells were harvested by centrifugation at 12,500 rpm and washed with PBS (phosphate buffer saline) solution. A modified method according to a preceding study [12] was employed to isolate genomic DNA from bacterial strain JG3. Fifty ng of isolated DNA was taken as the template for PCR amplification. The lipase gene was amplified using primers, DF1 (ATGACCGAGCTGACT GTAG) and DR1 (AGGAGGGGTAAATCCACAG). The PCR conditions used were initial denaturation at 95 °C for 5 min, followed by 35 cycles, including denaturation step at 95 °C for 1 min, annealing step at 57 °C for 30 sec, elongation step at 72 °C for 1 min, along with one last cycle of final elongation at 72 °C for 5 min. The amplified gene was examined on 1.5% agarose gel, then extracted and purified by PureLinkTM Quick Gel Extraction kit.

DNA sequencing and sequence analysis

The lipase gene was sequenced using the aforementioned primers using an Applied Biosystem 3500 Genetic Analyzer and BigDye X-terminator Kit. Consecutively, the sequence was analyzed using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with other sequences in the GenBank, before translation into an amino acid sequence using the ExPAsy tool (http://web.expasy.org/translate/) to predict the possible open reading frame (ORF). The similar lipase sequences from the same bacterial genus of the JG3 bacterial sample retrieved from GenBank database were aligned using ClustalW.

Computer-aided modelling of 3D structure of LipJG3

To identify the conserved regions in the LipJG3 sequence, homology analysis using ConSeq server (http://consurf.tau.ac.il/) was carried out. The aligned homologous sequence were retrieved from UNIREF90 database. The MSA (Multiple Sequence Alignment) using MAFFT available in the server was performed by employing default parameter with Bayesian method to calculate the Conservative scores. Moreover, the secondary

Fig 1. Position of *Alcaligenes* sp. JG3 based on 16S rRNA gene sequence relative to other proteobacteria in a cladogram generated by MEGA 7.0. Numbers at nodes indicate bootstrap values expressed as a percentage of 1,000 replicates
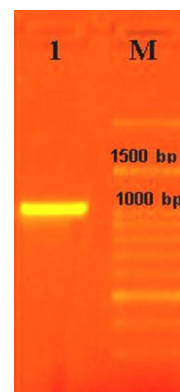
and tertiary structures of LipJG3 were aligned using SWISS-MODEL [13] and Phyre2 server (http://www.sbg.bio.ic.ac.uk/Phyre2) [14]. The chosen 3D model was analyzed and validated using MolProbity (http://molprobity.biochem.duke.edu/) [15]. The results were visualized using the UCSF Chimera version 1.11.2 [16].

■ RESULTS AND DISCUSSION

Currently, knowledge on lipases produced by genus *Alcaligenes* is very limited, obtained mainly from bacterial samples isolated from tropical area in Indonesia. The strain used in this study showed a considerable lipolytic activity [8] and is possibly a good source of new bacterial lipase [17]. On the other hand, recombinant technology through DNA cloning has led to far-reaching applications in industries, agriculture and environmental activities [18]. Hence, the characterization of DNA encoding lipase from strain JG3 is our point of interest. The present study reports the molecular characterization of *Alcaligenes* sp. JG3 lipase including its predicted 3D structure.

Identification of Lipase-Producing Bacteria

Strain JG3 is a gram-negative bacterium with a size of 0.7–1.0 × 0.5–2.6 μm of rod or coccobacilli shape [19]. In this study, about 1500 bp of 16S rRNA gene of strain JG3 has been successfully amplified to identify its genotypic character. BLAST results showed that strain

Fig 2. Gel electrophoresis analysis of PCR products using designed primers, DF1 and DR1. Lane 1,~1 kb PCR product; M: 100-bp DNA ladder (marker)

JG3 has the highest identity with *Alcaligenes faecalis*, corresponding to a 96% homologous significant value. This percentage (96%) similarity showed us the strain JG3 belongs to different species of *Alcaligenes* genus [20-21]. From the phylogenetic tree (Fig. 1.), a neighbor-joining algorithm showed that strain JG3 belongs to the group of *Betaproteobacteria* and its ancestry is closely related to the *A. faecalis* species. This means that the strain JG3 is within the outer group of *Alphaproteobacteria* (represented by genus *Rhodobacter*, *Rhodospirillum*, and *Rhodopseudomonas*) and *Gamma proteobacteria* (genus *Pseudomonas*). Accordingly, the strain JG3 was successfully confirmed as *Alcaligenes* sp. JG3.

Table 1. Alignment statistic between LipJG3 and lipase from *A. faecalis*

Score	Expect	Method	Identities	Positives	Gaps
655 bits(1690)	0.0	Compositional matrix adjust.	325/332(98%)	327/332(98%)	0/332(0%)

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Query    1 MSLNKGEVVSLLEASGSGKTTLLRAVAGLEQPSQGRIAINNDVLYDSQARIDLPAEARNL    60
            MSLNKGEVVSLL ASGSGKTTLLRAVAGLEQPSQGRIAINNDVLYDSQARIDLPAEARNL
Sbjct   24 MSLNKGEVVSLLGASGSGKTTLLRAVAGLEQPSQGRIAINNDVLYDSQARIDLPAEARNL    83

Query   61 GLVFQSYALWPHMTVQENVAYPLTLRKTSKAESRQKVDAILDQLGLKGLGERYPSQLSGG   120
            GLVFQSYALWPHMTVQENVAYPLTLRKT KAESRQKVDAILDQLGLKGLGERYPSQLSGG
Sbjct   84 GLVFQSYALWPHMTVQENVAYPLTLRKTPKAESRQKVDAILDQLGLKGLGERYPSQLSGG   143

Query  121 QQQRVAIARALVYNPPVILPDEPLSNLDAKLREEARVFLRELIVQMGLSALMVTHDQAEA   180
            QQQRVAIARALVYNPPVIL DEPLSNLDAKLREEARVFLRELIVQMGLSALMVTHDQAEA
Sbjct  144 QQQRVAIARALVYNPPVILLDEPLSNLDAKLREEARVFLRELIVQMGLSALMVTHDQAEA   203

Query  181 MAISDRILLLNGGEIGEQGSPQEVYSNPLTLYTAEFMGSNNRLQGKVTEQRDQQIRLSGP   240
            MAISDRILLLNGGEI +QG+PQEVYSNP TLYTAEFMGSNNRLQGKVTEQRDQQIRLSGP
Sbjct  204 MAISDRILLLNGGEIEQQGTPQEVYSNPKTLYTAEFMGSNNRLQGKVTEQRDQQIRLSGP   263

Query  241 GWELWGHAAAPLNAGQQATAVIRVEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMAD   300
            GWELWGHAAAPLNAGQQATAVIRVEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMAD
Sbjct  264 GWELWGHAAAPLNAGQQATAVIRVEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMAD   323

Query  301 PSAGTLRAFGPEPLPSGVHHLQLPPSKLWIYP 332
            PSAGTLRAFGPEPLPSGVHHLQLPPSKLWIYP
Sbjct  324 PSAGTLRAFGPEPLPSGVHHLQLPPSKLWIYP 355
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PCR Amplification of Lipase Gene

The DF1 and DR1 primer pair designed according to the reference sequence, lipase DNA sequence of *A. faecalis* subsp. *faecalis* NCIB 8687 (WP_003801168.1) successfully amplified a single, 1 kb fragment (Fig. 2.) of the genomic DNA of *Alcaligenes* sp. JG3. The purified amplified DNA was sent for sequencing. Based on BLASTn analysis, new gene sequence named as LipJG3 (KY750692), showed 91% identity with *A. faecalis* strain ZD02. The sequence, however, was also 73–75% similar to a lipase from the genus *Enterobacter*. During ClustalW alignment, LipJG3 showed high identity towards the *Alcaligenes* lipases deposited in GenBank, especially the *A. faecalis* MOR02 with a 96% similarity. Whereas, LipJG3 shared ~87% identity with the reference sequence.

The Deduced Amino Acid Sequence

The 1 kb amplified LipJG3 gene corresponded to 333 amino acid (aa) of the mature sequence. The output of BLASTp program showed significant identity both biologically and statistically to lipases of *A. faecalis* (98%)

with the query cover and E- value of 99% and zero, respectively (Table 1). For others, it was similar to lipase from *Kerstersia aviorum* by 71%, *Achromobacter* genus by 70%, while with *Buttiauxella* and *Cedecea* genus shared 69% similarity. This lipase sequence showed high similarity within lipases from *Alcaligenes* genus. However, it is showing varying percentages among the sequences mainly at the nucleotide level, although their amino acid sequences are quite similar to each other. This signifies possible variability of codon usage in several amino acid residues, in view of the fact that a high variability among microbial lipase gene is frequently observed [22-23].

A member of certain lipase gene family is often involved in varying metabolic pathways such as for fatty acid uptake or lipid digestion. The complexity of its mechanism of action as hydrolyze enzyme in different family class still remains questions for further understanding [24]. In order to classify LipJG3, an MSA was carried out by ConSeq server [25]. The first attempt was to find the biologically important protein residues (Fig. 3).
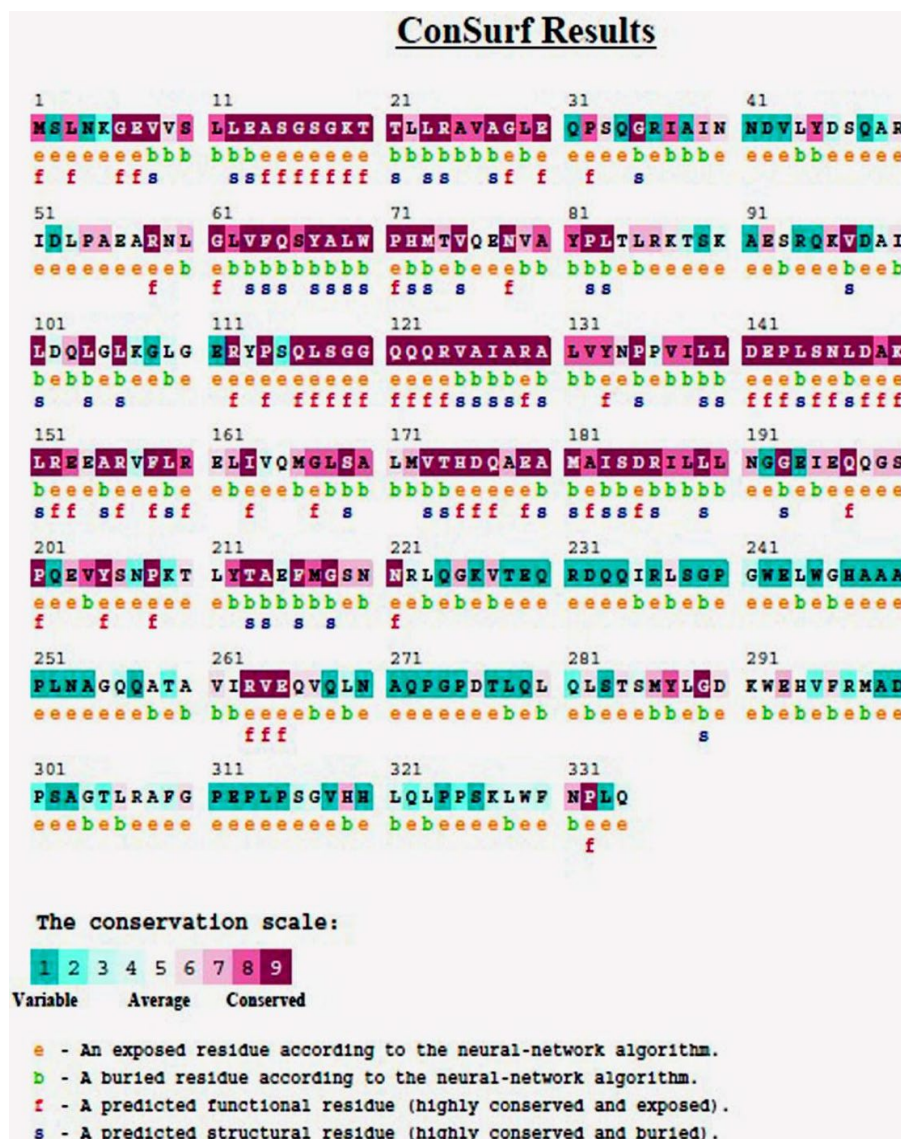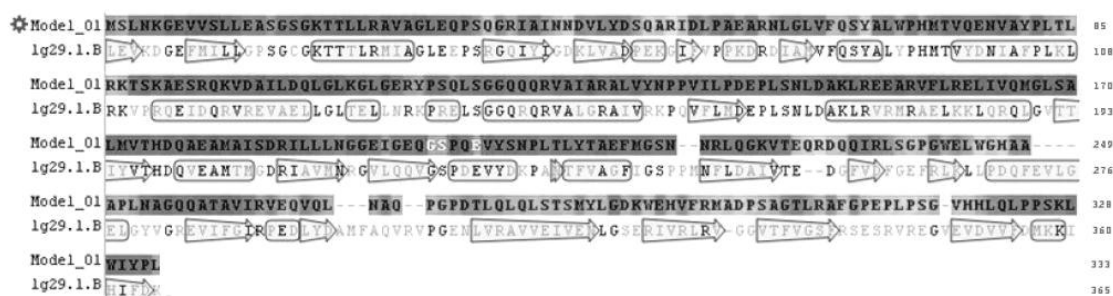
Fig 3. MSA output by Conseq server

Interestingly, the GXSXG motif, a well-known conserved region should exist in every lipase gene sequence. However, it is not found in the LipJG3 sequence observed on the MSA analysis result, indicating the uniqueness of this sequence. The LipJG3 showed several distinctive conserved sequences for common lipases including EASGSGKT and QLSGGQQQRVAIARA (Fig. 3.). These two conserved regions are well-known to form a part of the transporter protein sequence. Furthermore, the MSA output described that the residues present in the protein's N and C termini were highly exposed and could be interacting with the aqueous environment to form a
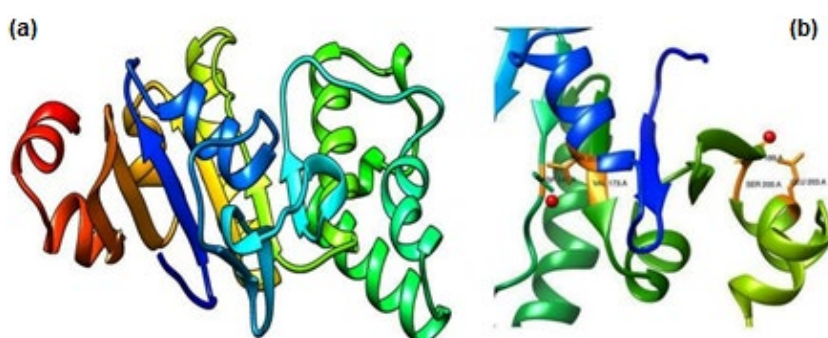
hydrogen bonding network that contributes to the stabilization of the three-dimensional structure [10].

The second attempt in LipJG3 homology analysis was to predict its structural model. The structure and function information on proteins are important for understanding their mechanism in the body. From the secondary structure prediction, LipJG3 contains 7 α-helices and 20 β-strands (Fig. 4.) and resembles an alpha-beta (α-β) protein fold, but its folding structure is different to that of ordinary lipases. Generally, lipases contain mostly eight strands of β-sheet with the second strand sited antiparallel and the third to eighth parallel

Fig 4. Secondary structure alignment between model (LipJG3) and template (PDB ID: 1g29.1) by SWISS-MODEL. Right arrow for β-strand, rounded rectangle for α-helix structures. White characters in grey boxes (G199, S200, E203) for residues in contact with ligand Mg^{2+}

Fig 5. (a) LipJG3 3D Structure based on d1g2912 template derived from Phyre2. (b) Close-up of residues (highlighted in orange shade) from SWISS-MODEL, which interact directly with Mg^{2+} ions. Original model is visualized in rainbow cartoon, where blue to red color runs from the N to the C termini

β-strands are connected by α-helices [5]. However, there are some modifications of lipase structures due to their variance in sequence length and conservation [26].

LipJG3 Structural Prediction and Validation

Apparently, LipJG3 belongs to the new lipase family or other protein classes. The highest identity of template-based modeling by Phyre2 was a 49% hit for the fold of P-loop containing nucleoside triphosphate hydrolase (family of ABC transporter ATPase) of *Thermococcus litoralis* (d1g2912) with 100% confidence. Based on this result, the 3D structure of LipJG3 was chosen as shown in Fig. 5(a). Moreover, its validity was also analyzed using MolProbity.

Two validation processes were performed. The first was the Asn/Gln/His residue flipping correction. The result showed that correction was not needed, hence indicating that all Asn, Gln, and His residues from LipJG3 were oriented correctly. The second process involved analyses on all atom contacts and geometry. The output

of this validation analysis is shown in Table 2. It showed that the structure of JG3's lipase has serious steric clashes and poor sidechain rotamers, while Ramachandran and Cβ deviation values were acceptable for this resolution. However, CαBLAM (Cα Based Low-Resolution Annotation Method) parameter displayed good percentage. Based on these results, the proposed 3D structure of LipJG3 is acceptable despite its low resolution, as the crystallographic structure of the similar protein, in this case, a lipase that belongs to NTPase hydrolase family, has yet to be solved.

LipJG3's Important Residues

The alignment result between LipJG3 and other lipases that belongs to the same group, it possesses similar sequence motifs, EASGSKT and LSGGQQQRV AIA as ATP- and GTP-binding site Walker-A (Fig. 6.) and an ABC transporter family signature respectively [27-28] as well as VILLD as ATP-binding signatures at Walker-B motif [29]. The same characteristics were also
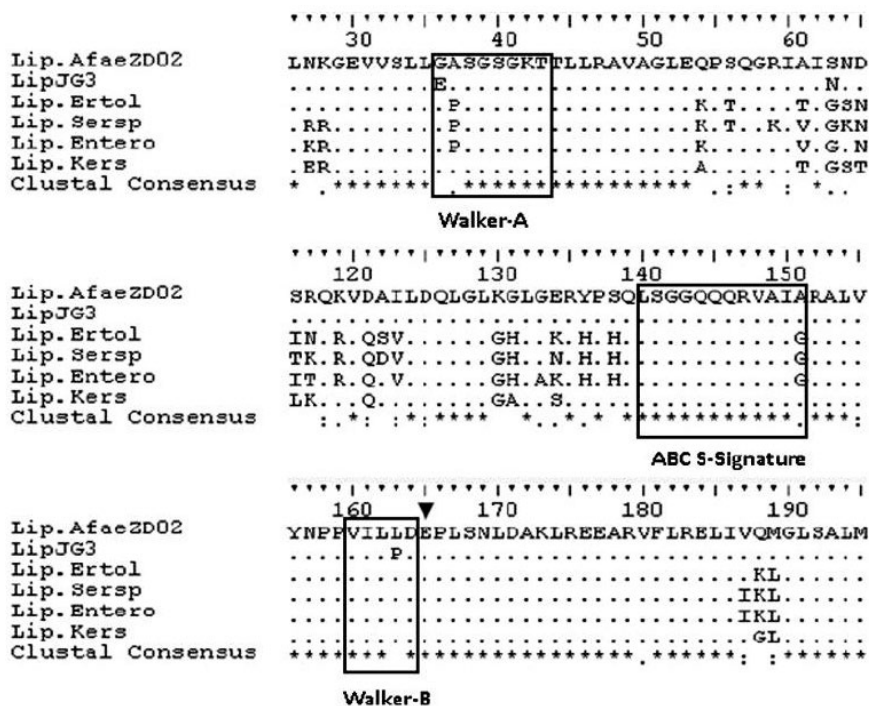
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Table 2. MolProbity result summary and criterion chart for LipJG3 structure

All-Atom Contacts	Clashscore, all atoms:	42.19		7th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	5	2.78%	Goal: < 0.3%
	Favored rotamers	167	92.78%	Goal: > 98%
	Ramachandran outliers	3	1.40%	Goal: < 0.05%
	Ramachandran favored	206	95.81%	Goal: > 98%
	MolProbity score	2.73		35th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations > 0.25Å	1	0.50%	Goal: 0
	Bad bonds	2/1692	0.12%	Goal: 0%
	Bad angles	38/2292	1.66%	Goal: < 0.1%
Peptide Omegas	Cis Proline	0/11	0.00%	Expected: ≤ 1 per chain, or ≤ 5%
Low-resolution Criteria	CaBLAM outliers	2	0.93%	Goal: < 1.0%
	CA Geometry outliers	3	1.40%	Goal: < 0.5%

In the column results, the left column gives the raw count, right column gives the percentage.

*100th percentile is the best among structures if comparable resolution; 0th percentile is the worst.

*MolProbity score combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

Fig 6. Partial sequence alignment of the amino acid sequence of LipJG3 against lipases from *Alcaligenes faecalis* (WP_060185877.1), *Erwinia toletana* (WP_017803199.1), *Serratia* sp. (WP_017893896.1), *Enterobacteriaceae* (WP_032660170.1)*, and Kerstersia gyiorum* (WP_068375151.1). Stars (*) indicate amino acid identity across all sequences. Black boxes represents ATP- and GTP-binding site Walker-A and Walker-B motifs as well as ABC transporter family signature**.** The position of the putative catalytic base is displayed by black triangle above the sequences

found in previous works, lipases from *Serratia marcescens* [27] *and Pseudomonas fluorescens* [30].

At least, there are two possible reasons underlined the classification of LipJG3. First, LipJG3 was involved in modification of nucleic acid, which are analogous to ABC transporters proteins. Consequently, this non-transporters ABC proteins have an ATP-binding fold and spatial conserved residues that might be important for ATPase activity. The second reason is its possibility secreted by ABC exporter pathway thus it brings the small unit characters of ABC transporter protein [31]. Additionally, the production of extracellular lipase from ABC pathway is more efficient than general secretion pathway [32].

One specific characteristic of any enzyme is its signature catalytic site used for the binding of a specific substrate. Generally, a lipase that belongs to the true lipase family has catalytic triad signature residues: a nucleophilic residue (cysteine, serine, or aspartate), a catalytic acid residue (aspartate or glutamate), and a histidine [33]. Since LipJG3 does not belong to the true lipase family, it is difficult to ascertain the catalytic triad, Ser (S), His (H), and Asp (D). Thus, it is also challenging to explain its role as a hydrolase enzyme. In the case of the ABC transporter protein itself, a previous study [34] proposed that a conserved glutamic acid residue (E) located next to the aspartic acid residue (D) of the Walker-B motif (Fig. 6) is directly responsible for the hydrolytic activity of the β-γ phospodiester bond. On the other hand, there are two Mg^{2+} ions (Fig. 5(b)) networking with the conserved region in the 3D model. One ion interacts with the catalytic triad of T^{20}, D^{143}, and V^{173} residues, while another interacts with the G^{199}, S^{200}, and E^{203} residues. Mg^{2+} ion was recognized as the cofactor enzyme for hydrolyzing ATP in ABC transport protein [35-36]. Thus, it might be possible for the two catalytic triads involved in hydrolyzing reactions of LipJG3. However, there are no reports explaining in detail about the catalytic site residues of lipases that belong to the ABC transport protein. In spite of this, gene expression and enhanced secretion of several lipases from this group have been reported [27,30,32]. Taking into consideration all of the evidence, LipJG3 could be used to hydrolyze triacylglycerides as these are the natural substrates of the enzyme, and convert them into biodiesel.

■ CONCLUSION

The novel bacterial lipase from *Alcaligenes* sp. JG3 (LipJG3) was characterized. It shared a 98% similarity in amino acid sequence level towards a lipase from *A. faecalis*. Due to its possibility to be secreted by the ABC pathway, LipJG3 brings the same nucleotide binding domains of ATPase. Thus, our results suggest that LipJG3 and its homolog proteins belong to the ABC transporter protein superfamily.

■ ACKNOWLEDGMENTS

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