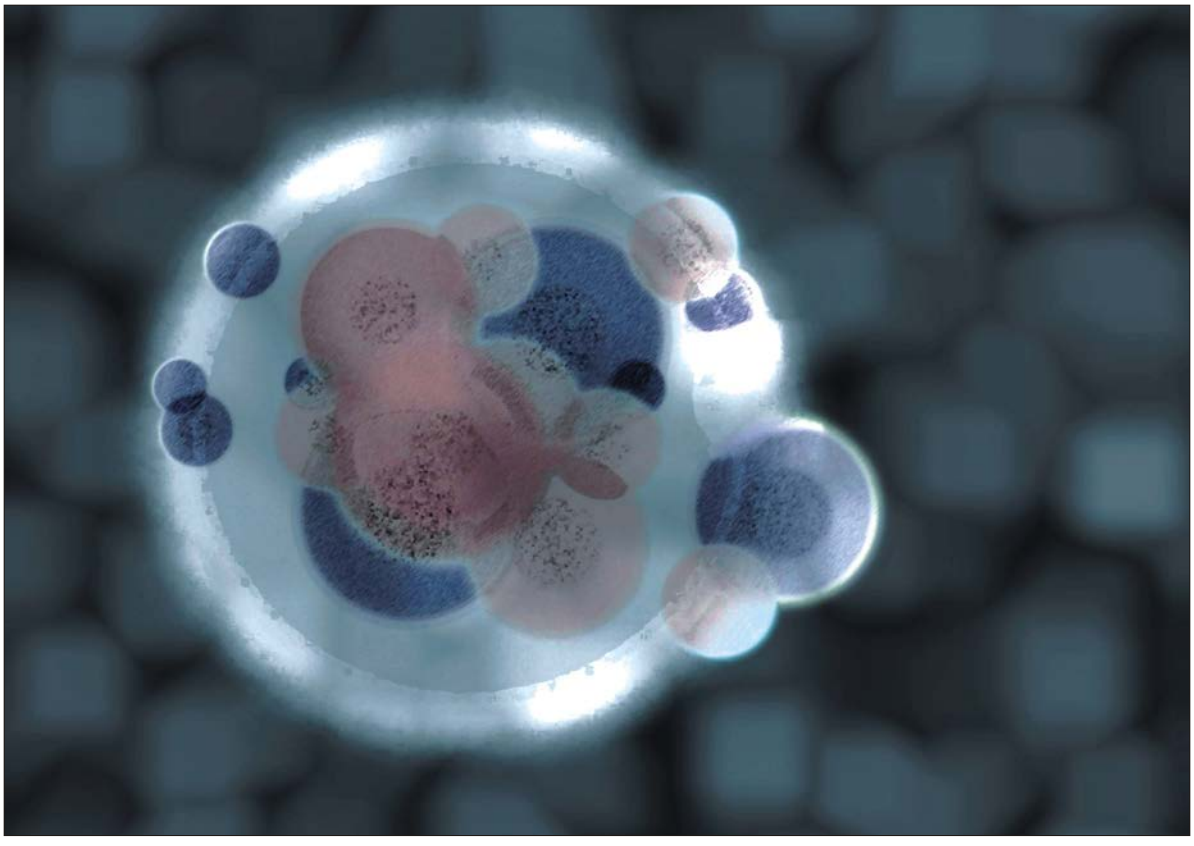

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Research Article
Characterization of Putative Lipase Gene from *Alcaligenes* sp. JG3 Bacterium via Cloning

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Abstract

Background and Objective: *Alcaligenes* sp. JG3 is an endemic bacterium from Indonesia, isolated from cultivated cornfield of Central Java. This bacterium is able to produce lipase with fairly high activity. This study aimed to determine the complete sequence of the lipase gene using cloning method. **Materials and Methods:** The primer pair used in this study (primer forward 5'-ATGACCGAGCTGACTGTAG-3' and reverse 5'-TCAGGAGGGGTAAATCCAC-3') were designed based on lipase gene from *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 and the gene was cloned using pGEM-T Easy Vector. The nucleotide sequence was analyzed using online software such as CLUSTAL Omega, BLASTp, ExPASy and Conseq Server. **Results:** The complete lipase JG3 gene sequence consists of 357 amino acids from ORF to the stop codon. Lipase JG3 had high similarity to other lipase from genus *Alcaligenaceae* which was up to 99% and also possessed some characteristic of ABC transporter protein. **Conclusion:** The designed primer pair based on lipase gene from *Alcaligenes faecalis* were able to amplify 1071 bp lipase gene of *Alcaligenes* sp. JG3 and the gene was successfully cloned into *Escherichia coli*.

Key words: *Alcaligenes* sp. JG3, lipase, transporter gene, sequencing, cloning

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are hydrolases that act on ester bonds in carboxylic esters[1]. Lipases frequently used to catalyze the hydrolysis of fatty acid esters in an aqueous environment[2]. Although being widely produced by plants, animals and microorganisms, lipases from microorganisms, such as bacterium and fungus, are the most used in industries and biotechnology development such as food industry, pharmaceuticals, biofuels production and detergent degradation[3]. This tendency is mostly because bacterial lipases are easier to be manipulated genetically and environmentally which is very beneficial for research or mass production[4].

In the more molecular perspective, lipases are serine hydrolases containing the consensus sequence G-X1-S-X2-G as the catalytic moiety, where G-glycine, S-serine, X1-histidine and X2-glutamic or aspartic acid[5]. Many bacterial lipases that have been investigated keen on to form catalytic triad of Ser, Asp and His as a functional lipases[6]. There are lipases gene from diverse bacteria that have been successfully cloned and characterized[7-9]. As in Indonesia, there was a specific bacterium cultivated from cornfield in Central Java whose lipase activity had been investigated which was up to 25 U mL^{-1}. Only later it was known that the bacterium stated before belongs to genus *Alcaligenes*, which is *Alcaligenes* sp. JG3 having shared similarity with *Alcaligenes aquatilis* and *Alcaligenes faecalis* up to 96% from 16s rRNA analysis[10,11].

Although the activity of the lipase enzyme has been clarified, there is no investigation yet regarding of the nucleotide sequence of the particular lipase gene. By doing so, the yield, activity and even stability of microbial lipase can be enhanced using such technique such as heterologous or homologous expression, screening of stronger promoter and codon optimization[12].

Sequencing bacterial DNA or gene of interest in order to know its nucleotide sequence can be achieved directly from PCR amplification. However, there are some advantages to clone the DNA itself to appropriate plasmid vector (cloning) before continuing on sequencing. They are; to get accurate sequence of few and last nucleotides if the primer was used to amplify it beforehand, if the bacterial DNA replication have proof reading activity so less chance of error, the ease of generating DNA fragment array/library, easier to multiply whenever needed and If the DNA fragment is a Coding Sequences (CDS), it can be further studied using expression vector[13,14]. Therefore, this study aimed to determine the nucleotide sequence of lipase gene from *Alcaligenes* sp. JG3 via cloning method.

MATERIALS AND METHODS

Materials and instrumentation: *Alcaligenes* sp. JG3 bacterium samples were collection of Laboratorium Penelitian dan Pengujian Terpadu UGM, originally isolated from Purwokerto, Central Java, Indonesia. Primer forward (5'-ATGACCGAGCT GACTGTAG-3') and reverse (5'TCAGGAGG GGTAAATCCAC-3') agarose, proteinase-K, ethidium bromide, DNA marker, nuclease free water, Quick Miniprep Plasmid Kit (Invitrogen), $MgCl_2$, NaCl, Sodium dodecyl sulfate (SDS), isopropanol, ethanol, tris base (Merck), triton X-100, Na-EDTA (Sigma), TAE buffer, loading buffer (Vivantis), Go taq green PCR mix, pGEM-T Vector System, IPTG, X-Gal, *E. coli* JM109 High Efficiency Competent Cells (Promega), SOC medium, LB medium, nutrient agar, nutrient broth (NB) and sterile aquadest (LPPT) and also antibiotic ampicillin. All the chemicals used in this study were of pro-analysis laboratory grade. The instruments used in this study were Bio Rad Thermalcycle PCR, Bio Rad [Wide Minisub[R] cell GT] electrophoresis, Bio-Rad UV lamp, Nuaire laminar flow, Olympus light microscope, Hirayama HL 36 AE autoclave, Barnstead vortex, OSK Seiwa Reiko water bathincubator, Sorvall Biofuge centrifugator and Shimadzu Probe UV-Visible Spectrophotometer.

Experimental procedures

DNA isolation: For the DNA isolation, some fresh and visible colonies of *Alcaligenes* sp. JG3 from NB media were treated according to the previous study[15]. Eight milliliter fresh NB cultured bacteria was centrifuged at 4000 rpm, 4°C for 10 min. The pellet was then homogenized using 5 mL of Phosphate Buffer Saline (PBS), pH 7.4 solution and centrifuged as previous manner. The pellet obtained was lysed using 5 mL lysis buffer (0.32 M sucrose, 10 mM tris-HCl of pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) and was centrifuged and the pellet was washed twice using 3 mL washing buffer (0.075 M NaCl, 0.025 M EDTA). The pellet was then resuspended with 500 µL resuspend solution (10 mM tris-HCl pH 8.0, 2 mM EDTA) and mixed with 1, 7 µL SDS 10% and 20 µL proteinase-K (10 mg mL^{-1}) for 1 h incubation at 65°C. After that, 500 µL NaCl (5 M) was added to the mixture and centrifuged by the same manner. Polar layer of the mixture then transferred to a new micro tube with addition of isopropanol 1:1 (v/v) until the DNA thread appeared by gently inverting the tube. The DNA pellet was obtained by centrifugation and diluted using 50 µL buffer TAE (10 mM Tris, 1 mM EDTA pH 7.5). To calculate the concentration of the isolated DNA, the absorbances at 260 nm were measured using UV spectrophotometer.

Primer design: The forward (5'-ATGACCGAGCTGACTGTAG-3') and reverse (5'-TCAGGAGGGGTAAATCCAC-3') primers were designed based on lipase gene from *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 bacterium that already stored in gene bank with accession number WP_003801170.1 and were analyzed using online software Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

DNA amplification: A mixture containing 12.5 µL Go Taq Green PCR mix, 20 pmol of forward and reverse primers respectively, 1 µg isolated DNA and nuclease free water to a final volume of 25 µL were added to the PCR tube. The PCR process were performed under conditions: Initial denaturation at 95°C for 5 min, followed by 35 cycles consist of denaturation at 95°C, annealing at 52°C and extension at 72°C for 1 min each and for the post-extension was at 72°C for 5 min. For the visualization of the PCR product, electrophoresis analysis was used. Five microliter of PCR product was loaded into 1.5% agarose gel with 1×TAE running buffer. Electrophoresis agarose analysis was performed under 50 voltage for 1 h. The size of the DNA was estimated using DNA marker.

Ligation: For a positive control, a mixture of 5 µL 2×Rapid Ligation Buffer, 1 L pGEM-T Easy Vector, 2 µL control insert DNA and 1 µL T4 DNA ligase were added to 0.5 mL tube. Whereas for standard reaction, the control insert DNA was replaced by 3 µL PCR product. Nuclease free water was added to both mixture to a final volume of 10 µL and was mixed by pipetting until it become homogenous. The mixtures then were incubated overnight at 4°C.

Gene transformation: Two microliter of ligation reactions were put in 1.5 mL tube on ice and then 50 µL of *E. coli* JM109 High Efficiency Competent Cells were added. The mixtures then were left on ice for 20 min. After 20 min, the mixtures were heat-shocked for 50 sec in a water bath at exactly 42°C and immediately returned the tube to ice for 2 min. To the mixtures, 950 µL SOC medium were added and incubated for 1.5 h at 37°C with shaking. The transformation occur in culture and then diluted with ratio 1:10 using SOC medium and plated to the LB medium containing X-Gal/IPTG/ampicillin (200 µL each plate). The transformed cells may be harvested after overnight incubation at 37°C (white colonies generally contain DNA insert).

DNA sequencing and homology analysis: Sequencing DNA was performed at 1st Base Laboratory, Singapore. The homology of the DNA sequence was analyzed using CLUSTALOMEGA (http://www.ebi.ac.uk/Tools/msa/clustalomega/), Conseq Server (http://consurf.tau.ac.il/) and sBLASTps (http://blast.ncbi.nlm.nih.gov/Blast.cgi) online softwares. The determination of the Open Reading Frame (ORF) was conducted using ExPASy translate tool (https://web.expasy.org/translate/) online software.

RESULTS AND DISCUSSION

The lipase gene sequence from *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 was chosen as the reference on making primer because it comes from the same genus (hence shares high similarity) and already available in gene bank. Using the very beginning sequence containing ORF and end sequence from *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 as the primer pair, it was hoped to cover the complete nucleotide sequence. The primer pair was analyzed using online software PrimerBlast because of its ability to overcome the specificity problem of the target[16].

Amplification of DNA using primer pair forward and reverse produced one specific band as shown in Fig. 1a. The amplicons size was very close to the reference lipase (approximately 1100 bp based on Fig. 1a) which indicating the primer pair was very specific. Although a single clear band was produced from PCR amplification, cloning was still conducted

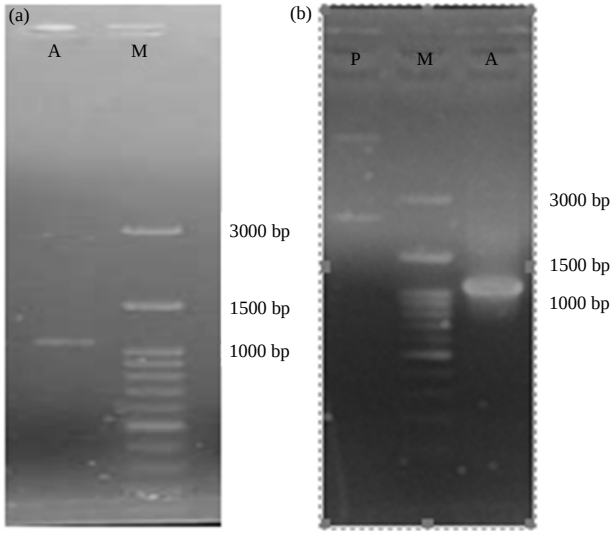

Fig. 1(a-b): (a) Electrophoresis of PCR amplification from DNA isolate JG3 (A) and DNA marker (M) and (b) Electrophoresis of isolate plasmid transformed (P), PCR amplification of isolate plasmid using primer forward and reverse (A) and DNA marker (M)

```
1    ATGACCGAGC TGACTGTAGA CAATATCCAC CTGGCCTACG ACCGCAACCC CGTCCTGAAA
61   GGCGTCTCCA TGAGCCTGAA CAAAGGTGAA GTGGTTTCCC TGCTGGGTGC CTCGGGCAGC
121  GGCAAAACCA CCTTGCTACG CGCCGTTGCC GGGCTGGAAC AACCATCCCA GGGCCGCATC
181  GCCATCAATA ATGACGTGCT GTACGACTCG CAGGCCCGTA TCGACCTGCC CGCTGAAGCC
241  CGCAATCTGG GCCTGGTGTT CCAGTCCTAC GCCTTGTGGC CACACATGAC GGTGCAGGAG
301  AACGTCGCTT ACCCCTTGAC GCTGCGCAAG ACATCCAAAG CGGAAAGCCG CCAGAAAGTG
361  GACGCCATTC TGGATCAGCT GGGCTTGAAA GGTCTGGGCG AACGCTACCC CAGCCAGCTG
421  TCTGGCGGGC AACAACAACG TGTCGCCATT GCGCGTGCTC TGGTCTATAA CCCGCCTGTG
481  ATCTTGCTGG ACGAGCCGCT GTCCAACCTG GATGCCAAGC TGCGCGAAGA AGCCCGCGTC
541  TTCCTGCGCG AGCTGATTGT GCAGATGGGT TTGTCGGCTT TGATGGTGAC CCATGACCAG
601  GCGGAAGCCA TGGCGATTTC GGACCGAATC TTGCTGCTGA ACGGTGGTGA AATCGAGCAG
661  CAAGGTAGCC CGCAAGAGGT CTACAGCAAT CCCAAGACGC TGTACACCGC CGAGTTCATG
721  GGCTCGAACA ACCGTCTGCA AGGCAAGGTG ACGGAACAAC GTGACCAGCA AATTCGCCTA
781  AGTGGGCCCG GCTGGGAATT GTGGGGCCAT GCCGCCGCTC CCCTGAACGC TGGCCAGCAA
841  GCCACCGCCG TGATTCGTGT CGAACAAGTG CAGTTGAATG CACAACCCGG CCCCGATACG
900  CTGCAACTAC AACTGTCCAC CAGCATGTAC CTGGGCGACA AGTGGGGGCA CGTTTTCCGC
961  ATGGCCGACC CGTCTGCCGG CACCTTGCGT GCTTTCGGGC CCGAGCCCCT GCCCAGTGGT
1021 GTGCATCACC TGCAATTGCC ACCCTCCAAA CTGTGGATTT ACCCCTCCTG A
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Fig. 2: Complete nucleotides sequence of lipase JG3

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ref      MTELTVDNIHLAYDRNPVLKGLSMSLNKGEVVSLLGASGSGKTTLLRAVAGLEQPSQGRI
JG3      MTELTVDNIHLAYDRNPVLKGVSMSLNKGEVVSLLGASGSGKTTLLRAVAGLEQPSQGRI
         ********************:****************************************

ref      AINENVLYDSQNRIDLPAEARNLGLVFQSYALWPHMTVQDNVAYPLTLRKTSKAQSRQRV
JG3      AINNDVLYDSQARIDLPAEARNLGLVFQSYALWPHMTVQENVAYPLTLRKTSKAESRQKV
         ***::****** ***************************:**************:***:*

ref      DAILDQLGLKGLGERYPSQLSGGQQQRVAIARALIYNPPVILLDEPLSNLDAKLREEARV
JG3      DAILDQLGLKGLGERYPSQLSGGQQQRVAIARALVYNPPVILLDEPLSNLDAKLREEARV
         ************************************:************************

ref      FLRELIVKMGLSALMVTHDQAEAMAISDRILLLNGGEIEQQGTPQEVYSNPKTLYTAEFM
JG3      FLRELIVQMGLSALMVTHDQAEAMAISDRILLLNGGEIEQQGSPQEVYSNPKTLYTAEFM
         *******:************************************:****************

ref      GSNNRLQGKVTEQRDQQTRLSGPGWELWGHAAAPLSAGQHATAVIRVEQVQLNAQPGPDT
JG3      GSNNRLQGKVTEQRDQQIRLSGPGWELWGHAAAPLNAGQQATAVIRVEQVQLNAQPGPDT
         **************** ****************.***:**********************

ref      LPLQLSTSMYLGDKWEHVFRMADPTAGTLRAFGPEPLPSGVHHLQLPPSKLWIYPS
JG3      LQLQLSTSMYLGDKWGHVFRMADPSAGTLRAFGPEPLPSGVHHLQLPPSKLWIYPS
         * ************ ********:******************************
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Fig. 3: Alignment of lipase JG3 and lipase reference from *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 (reference)

prior to the sequencing. It is to maintain the product size that can be reduced during sequencing due to lack of concentration. The total 1071 bp matched perfectly with the size of the band and with the total nucleotides of the lipase reference which is 1071 bp as shown in Fig. 2 and 3.

To get the overall comprehensive nucleotide sequence, cloning of lipase gene toward vector pGEM-T and *E. coli* as host was done. The successfully cloning protocol were indicated by the appearance of the blue and white colonies. Another indicator used to determine the success of cloning is transformation efficiency that can be measured in Colony Forming Unit (CFU) per µg of DNA used. The calculated transformation efficiency for control positive and lipase JG3 was 1.51×10^5 and 1.54×10^3 CFU ng^{-1}, respectively. The low

Table 1: Output BLASTp analysis

Description	Query cover (%)	Identity (%)
ABC transporter ATP-binding protein (*Alcaligenes faecalis*)	100	99
ABC transporter ATP-binding protein (*Alcaligenes faecalis*)	100	99
ABC transporter ATP-binding protein (*Alcaligenes faecalis*)	100	99
ABC transporter ATP-binding protein (*Alcaligenes faecalis*)	100	97
Spermidine/putrescine ABC transporter ATP-binding protein PotA (*Alcaligenes faecalis*)	100	96
Lipase partial (*Alcaligenes* sp. JG3)	93	97
ABC transporter ATP-binding protein (*Kerstersia gyiorum*)	100	72
ABC transporter ATP-binding protein (*Achromobacter xylosoxidans*)	99	70
Lipase (*Achromobacter* sp. KAs 3-5)	99	70

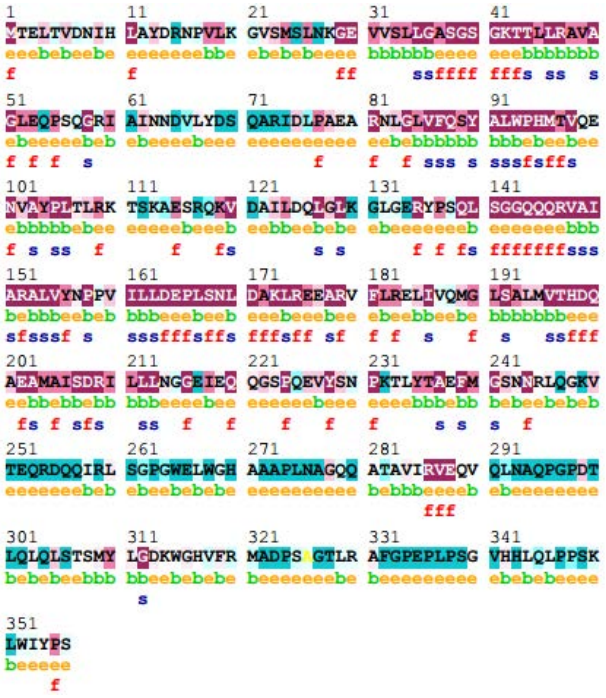

Fig. 4: Identification of functional and structurally important residues from lipase JG3 using
Conseq server (e: Exposed residue, b: Buried residue, f: Predicted functional residue, s: Predicted structural residue)

value for the lipase JG3 transformation efficiency might be due to the inadequate optimization of the DNA concentration used in cloning process. Nevertheless, it is not a major problem since the sequencing protocol could be forged ahead and resulted in satisfying sequence. The plasmid containing lipase gene then was isolated and amplified as can be seen in Fig. 1b. This amplified DNA from the transformed gene was the DNA that was being sequence in order to determine the complete nucleotide sequence.

The lipase gene obtained from DNA sequencing encoded 357 amino acids (1071 bp) and share such a high similarity towards some lipase genes from other *Alcaligenes bacterium*. Amino acids alignment of lipase JG3 with lipase reference shared high similarity (95.51%) but without the presence of the pentapeptide GXSXG .To support this fact, a further analysis using BLASTp was conducted as presented in Table 1. Although the amino acid shares high similarity to other lipase from *Alcaligenes* bacteria, the results from BLASTp seems to contradict it. The BLASTp analysis of lipase JG3 did not match the expected result to be the lipase protein. Top six output from the analysis suggested the protein JG3 here is an ABC transporter protein. Only with the ranked 7 and 10 showed that this particular protein is lipase, but the value of the percent identity is not as convincing as from the top six output.

Analysis of predicted functional and conserved motif using Conseq server (Fig. 4) shows four possible active sites of lipase JG3 which are SGSGKTT, SGGQQQR, DEPLSNL and VTHDQ. Both of the edges of lipase JG3 are exposed to the aqueous environment, making it able to form hydrogen bond which help to stabilize the 3D conformation of the protein[17]. Although it is commonly known that lipases have G-X-S-X-G as conserved motif pentapeptide, not all bacterium lipases do. Lipase from *Bacillus cereus* and esterase from *Geobacillus thermodenitrificans* T2 possess sequence of GDSL as the conserve motif, where the serine active site is located near the N-terminus[18,19]. Furthermore, a subgroup of this GDSL family was classified as the SGNH hydrolase family, consist four conserved amino acid residues of Ser, Gly, Asn and His in four blocks[20]. Another example is lipase from *Bacillus licheniformis* from India whose conserved sequence are ITITGCGNDL and NLYN[17]. These indicate that lipase or hydrolase family enzyme does not always necessarily follow the pentapeptide motif of G-X-S-X-G to be able to have the activity.

The simplest explanation for this phenomenon is by looking the secretion mechanism of lipase enzyme. There are three major secretion pathways of lipase in Gram-negative bacteria; via two-step mechanism involving stopover in the periplasm, through inner and outer membrane simultaneously and ATP-binding cassette (ABC) exporter pathway[21]. This last pathway is generally followed by the C-terminal signal lipase[22]. There is a bacterial lipase from *Serratia marcescens* that also

belong to the ABC protein family due to its lipase is able to bind ATP[23]. Therefore, the lipase JG3 is potentially followed the ABC pathway so that it has the characteristic and classified as ABC protein from BLASTp analysis. And the complete sequence of the lipase JG3 gained in this study shall complement the initial work of the fragment characterization from the lipase gene of *Alcaligenes* sp. JG3[24]. This study only limited on nucleotide sequence determination based on the comparison towards available data on the gene bank and not for the activity of the enzyme towards lipase catalyzed reaction yet. Thus, further examination of the lipase JG3 activity (for example in hydrolysis or esterification) is strongly needed further, using such technique as overexpression prior to it.

CONCLUSION

It is concluded that using the designed primer pair from lipase of *Alcaligenes faecalis* sub sp. *faecalis* NCIB 8687 and via cloning it with *E. coli* as host, the putative lipase gene sequence from *Alcaligenes* sp. JG3 has been adequately determined, encoding 1071 bp nucleotides (357 amino acids). Although the similarity toward other lipase gene from genus *Alcaligenes* are doubtlessly high, it also carries ABC transporter protein characteristics.

SIGNIFICANCE STATEMENT

This study determined the fully possible nucleotide sequence of putative lipase gene from *Alcaligenes* sp. JG3 via cloning method which have never been investigated before. Lipase gene obtained from this research does not follow the traditional lipase which have pentapeptides Gly, Asp, Ser, Asp and Gly as the conserved region which make it interesting and have the possible different activity from other lipases. So, the findings from this particular study might be useful for many lipase gene characterization in the future to be used as comparison.

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