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Research Article
Characterization of Lipase Gene Fragment from *Alcaligenes* sp. JG3 Bacterium

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Abstract

Objective: Degenerate PCR technique has been successfully used to identify a lipase gene fragment of *Alcaligenes* sp. JG3. Primers were designed based on lipase genes of bacterium *Azospirillum* sp. B510 and *Alcaligenes faecalis* using PrimerBlast software. **Methodology:** Sequence analysis of the fragment with a size of 0.4 kb amplified using the forward primer (AlF4) 5′-GTCTACAGCAATCCCAAGAC-3′ and reversed primer (AlR4): 5′-GGAGGGGTAAATCCACAGTT-3′ represented a 394 bp nucleotide sequence which has been submitted to NCBI GenBank with Accession No. of KP872319. **Results:** The obtained DNA sequence was confirmed as part of lipase gene as it shared 98% amino acid and 88.07% nucleotide similarity with lipase gene of *Alcaligenes faecalis*, that covered approximately 27% the gene. **Conclusion:** The designed primers based on lipase gene of *Alcaligenes faecalis* were able to amplify 394 bp lipase gene fragment of bacterial strain *Alcaligenes* sp. JG3.

Key words: *Alcaligenes* sp. JG3, lipase gene, degenerate PCR

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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

Lipase (EC 3.1.1.3) is an enzyme catalyzing hydrolysis as well as trans-esterification of triacylglycerol. The lipase-catalysed hydrolysis reaction could be easily reversed in non-aqueous media or in media with a very low water content, in ester synthesis or transesterification reactions[1]. The applications of lipases are widely ranged starting from oleochemical, detergent, organic industries, leather industry, environmental management, cosmetics and perfume industry, biomedical applications and biosensors[2].

Lipases are widely available in nature, as being produced by plants, animals and microorganisms. Nevertheless, lipases from microorganisms, such as bacterium and fungus, are the most used in industries and biotechnology[3]. It is because lipases from microorganisms have high stability in organic solvent, need no cofactor, highly specific substrate and enantioselective[4]. Some of the bacteria producing lipase are from genus *Bacilus*, *Pseudomonas* and *Staphylococcus* such as *Bacillus pumilus*, *Psyhrobacter* sp. and *Geobacillus thermodenitrificans*[5-8].

Indonesia is a country with great biodiversity, including the type and variety of microbes. Many local strains of bacteria Indonesia that have been characterized produce a particular enzyme. One of the original strain isolated from Indonesia is *Alcaligenes* sp. JG3. These bacteria were isolated from corn roots, sized about 1.5 µm, Gram-negative, have curved morphology with rounded end and have flagella as moving apparatus[9]. Strain *Alcaligenes* sp. JG3 was previously biochemically identified as *Azosprillum* sp., before polyphasic approach was applied to confirm its identity[10]. Cell extracts of these bacteria showed activity against triacylglycerol hydrolysis high enough showing these bacteria have the potential to produce lipase[11].

Lipase-based technology development is mostly done by lipase produced by microorganisms or cloning other organism's lipase genes in bacterium like *E. coli*. This approach, in addition is due to the ease of microbial lipase production compared to lipase production from animals or plants. Also, cloning the genes into microorganism leads to the possibility of genetic engineering to produce superior lipases. To perform genetic engineering, characterization of information regarding the gene encoding lipase absolutely necessary. One way to characterize a gene is by using PCR technique. The PCR to amplify gene was reported not only for prokaryotic cell[12] but also eukaryotic cell[13]. The PCR technique has also been reported to be successfully used for characterization, cloning and overexpression of lipase gene of various microbes. The conserved sequence of amino acid lipase having long been known, GXSXG, has been successfully used as a basis for designing PCR primers used for amplification of thermostable lipase gene in *Geobacillus thermoleovorans*[14,15]. It was previously reported that designing degenerate primers with multiple approaches amino acid sequence alignment of lipases and esterases has been successfully used to identify cold active lipase gene from bacteria from Antarctic sea water[16].

This study was aimed to characterize the sequence of genes encoding lipase of *Alcaligenes*, which is expected to be used for cloning and genetic engineering of lipase. This study is part of an effort in exploration of Indonesia's biodiversity. The approach used was technical PCR with primers designed based on the sequence of the lipase genes of other *Azospirilium* and *Alcaligenes* species, which have been previously published in the GenBank.

MATERIALS AND METHODS

Material: *Alcaligenes* sp. JG3 bacterial samples were collected from Microbiology Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia, which were grown in both nutrient agar and nutrient broth media. The PCR primers were synthesized at 1st Base Malaysia. Chemicals used were agarose (Invitrogen), $MgCl_2$ (Merck), triton X-100 (Sigma), NaCl (Merck), Na-EDTA (Sigma), Sodium Dodecsyl Sulfate (SDS) (Merck), proteinase-K (Invitrogen), isopropanol (Merck), etanol (Merck) are all in analytical grade. The TAE buffer, loading buffer (Vivantis), ethidium bromide (EtBr) (Invitrogen), DNA marker (Invitrogen), tris base (Merck) were used in electrophoresis.

Experimental procedure

PCR primer design: Primer pairs used in this study were designed based on the complete sequence of lipase genes of *Azospirillum* sp. B510 (Accession No. NC_013857) and *Alcaligenes faecalis* subsp. *faecalis* NCIB 8687 bacterium (Accesson No. NZ_AKMR01000005) using PrimerBlast online software. Key parameters used in designing primer included target fragment size of 300-600 bp, primer length of 18-22 base, GC content of 50-60% and Tm of 53-60°C.

Culture development: All studies related with the cultivation of the bacteria were performed aseptically in a laminar. Bacteria were retrieved from a single colony of old media, which was then sub-cultured on new media. Isolates of *Alcaligenes* sp. JG3 of an old medium was transferred to NA medium (nutrient agar) using aseptic loopful and grown back by incubation for 24 h at 37°C. Pure isolates of bacteria that have been sub-cultured on NA medium were then inoculated

in liquid medium Nutrient Broth (NB). After that, the isolates were incubated in a shaker incubator for 24-48 h at 37°C.

DNA isolation: Isolation of genomic DNA of the bacteria was carried out using the modified version of DNA extraction method previously introduced by Zakary[17]. Five milliliters of bacterial culture in NB liquid medium was centrifuged at 4000 rpm for 10 min at 4°C. Supernatant was separated and the pellet was re-suspended in 5 mL of PBS. The obtained suspension was then centrifuged at 4000 rpm for 10 min at 4°C. The pellet was again re-suspended with 5 mL of lysis buffer (0.32 M sucrose, 10 mM tris-HCl pH 7.5, 5 mM $MgCl_2$ and triton X-100 1%) and then centrifuged similarly at 4000 rpm. The obtained suspension was washed with 3 mL of washing buffer (0.075 M NaCl, 0.025 M EDTA) and then centrifuged. The washing step was repeated twice. The washed pellet was re-suspended once more in 10 mM tris HCl buffer pH 8 and 2 mM EDTA 500 mL, then 1.7 mL of 10% SDS and 20 mL proteinase K were successively added into the suspension. The mixture was incubated at 65°C for 1 h before added with 500 mL 5 M NaCl. After 10 min centrifugation, the proteins present in the mixture were precipitated. Water layer was taken out and the DNA was visible after addition of isopropanol (volume 1:1). The DNA was isolated from the mixture by centrifugation for 10 min at 4000 rpm DNA. The pellet was dried at room temperature and then re-dissolved in 50 mL of tris-EDTA.

PCR amplification: A mixture containing an iLustra™ puReTaq Ready-To-Go PCR bead, 25 pmol of forward, 25 pmol of reverse primers and 1 µg isolated DNA were added into PCR tubes to perform PCR. 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 52°C and extension at 72°C for 1 min each. The post-extension step was at 72°C for 5 min. For visualization of the PCR product, electrophoresis analysis was performed. Ten microliters of each PCR product was loaded into 1.5% agarose gel with 1×TAE running buffer and 1 µL loading buffer. Electrophoresis agarose analysis was performed at 50 V for an hour. The size of the isolated DNA was estimated using DNA marker.

DNA sequencing and homology analysis: The isolated DNA fragments were obtained and sequenced at 1st Base Laboratory, Malaysia. The homology level of the nucleotide sequence of each fragment was analyzed using BLASTn online software, while the alignment of it with sequences of lipase genes previously used to design the primer was performed using Clustal Omega online software.

RESULTS

Two sequences of lipase from *Azospirillum* sp. B510 and *Alcaligenes faecalis* subsp. *faecalis* NCIB 8687 were used as template of primer design considering that both are taxonomically close to the targeted microbe (*Alcaligenes*). Table 1 and 2 show the candidate of primers pairs as the output of PrimerBlast design.

Among the designed primer, PCR amplification using three pairs of primers AzF3-AzR3, AzF5-AzR5 and AlF4-AlR4 resulted in single fragment as seen on the gel electrophoresis displayed in Fig. 1. The amplification product using AzF5-AzR5 and AlF4-AlR4 have sizes, which were approximately 0.4 kb, close to the expected result based on the sequence length of the lipase gene used as the template in primer designing, which were 359 and 390 bp, respectively.

Nucleotide sequences confirmed that the size of the obtained PCR products were 829, 410 and 394 bp amplified using primers AzF3-AzR3, AzF5-AzR5 and AlF4-AlR4, respectively. The complete sequence of each of three fragments is shown in Fig. 2.

Table 1: Output of the design using primer using lipase gene of *Azospirillum* sp. B510 as template

Primer sequence (5'->3')	Position of the primers*	Size amplification target* (bp)
AzF1 : CACCTACGCCTATGACCAG	285-303	619
AzR1 : CTTCAGGTCACGCAACAG	903-886	
AzF2 : CACCTACGCCTATGACCAG	286-304	403
AzR2 : CGATCCCTTGATGACCAG	903-886	
AzF3 : GGATCACCTATACCCTCGTC	563-582	341
AzR3 : CTTCAGGTCACGCAACAG	903-886	
AzF4 : CACCTACGCCTATGACCAG	285-303	355
AzR4 : GATGTTGTCGGATGGATG	639-622	
AzF5 : CACCTACGCCTATGACCAG	285-303	359
AzR5 : TCTCGATGTTGTCGGATG	643-626	

*Based on the lipase gene sequences of *Azospirillum* sp. B510

Table 2: Output of the design using primer using lipase gene of *Alcaligenes faecalis* subsp. *faecalis* NCIB 8687

Primer sequence (5'->3')	Position of the primers*	Size aplification target* (bp)
AlF1: CTGGTTTTCCAGTCCTATGC	253-272	446
AlR1: GTCTTGGGATTGCTGTAGAC	698-679	
AlF2: CAGGACAATGTGGCTTATCC	295-314	397
AlR2: GATTGCTGTAGACCTCTTGC	691-672	
AlF3: CTTTGATGGTGACCCATGAC	578-597	354
AlR3: GATACATGCTGGTGGATAGC	931-912	
AlF4: GTCTACAGCAATCCCAAGAC	679-698	390
AlR4: GGAGGGGTAAATCCACAGTT	1068-1049	
AlF5: CCTGGTTTTCCAGTCCTATG	252-271	346
AlR5: GTCATGGGTCACCATCAAAG	597-578	

*Based on the lipase gene sequences of *Alcaligenes faecalis* subsp. *faecalis* NCIB 8687

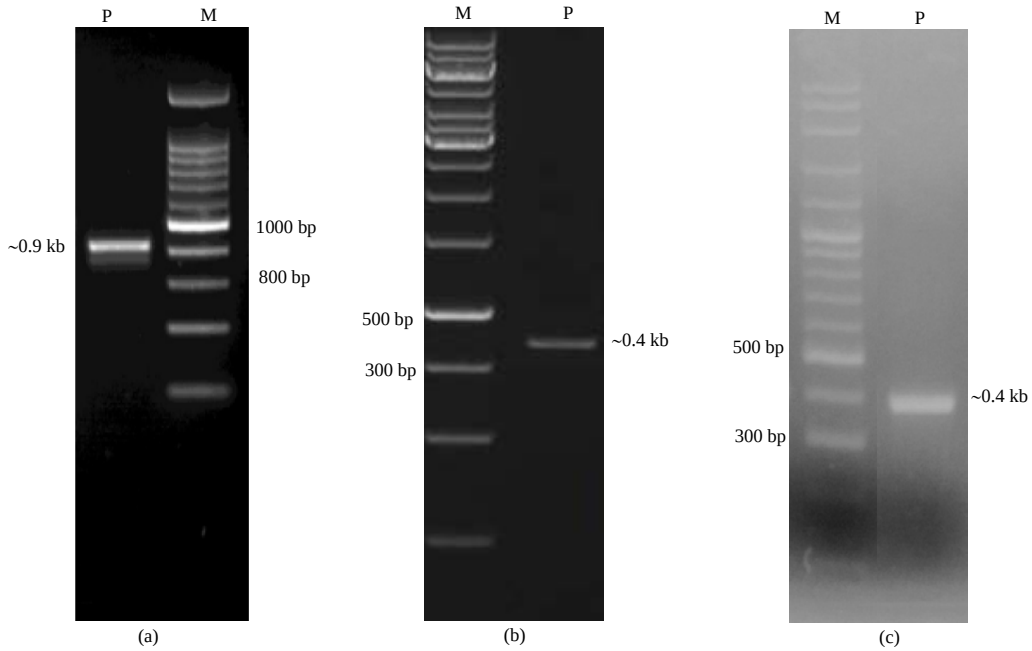

Fig. 1(a-c): Electrophoresis of PCR amplification of *Alcaligenes* sp. JG3 using primer (a) AzF3-AzR3, (b) AzF5-AzR5 and (c) AlF4-AlR4 show single PCR fragment, M: marker, P: PCR product

```
(a)
TTTTCAGGGG ACGGCAACAG GCTCGTACCT CAACATCAAC TTGGAGGATG 50
CCGGGGACAG CGGCCGTACG AGCGCTAGGG CCGAGCGACG TTATGACTTC 100
CGTTAGTAGG GGAACAATCC GTACAGTGCG CAAGCCCGGC CGGATTACAT 150
GGCCACCCGG ATTGGCGCCA CCAATAGCGG GGGCTCTACG TTGGGAACGG 200
AATCAGGCCG TCGTGCGTGC AGCATTTGGC GGGGTAGCGC GCATATGAAT 250
TACGAGTGCT GAACCAAATC AATCCGACAT ACTATGGGGA GAGCGTGATC 300
GGTGCACTGG AAGACATAAC GCAAGCCACG GTGGATGATT ATACCCGGGC 350
AGCCGGTCGC CCACAAACAT CTGGAAGCGG GGGTGATCAA AGATTTGTGC 400
ATAAAACAGG CGCCGGACGT CCGGAGCGAG TTTTTTGCAC GGATCTTTGA 450
TACACAGGAT TTCGAGCTCC CACTGGGCGA GCTGGCTGCC GGCCGTTAGA 500
ATCGGATCGC CGTGGACGGA TGGGGTAACC TGTCTTCAAA CACACCGATC 550
ACGTCCTCAC GATACCGTCC ACAGGGTTGA TGTCCTCCGA CCACTGCCAA 600
CTGATCTGCC ACGCCCTAGG GTTGGCCACT CCACGTCCAG CCAGAAGAGG 650
GCCAGATTGC CGTCGCCAGG CAAGAGCCGC CATAAAGACA TGGGCCCAAT 700
CAGCGTTCCA TCGGTAATCG CCCAGCATGG TCTATGACAG GAAGTCGCCG 750
TCTTAAACAA AGTCAGAGTC GGGCTATCCC CTGGCGCTTG TAGTAAGCGA 800
CGTAGACGGC GTGTGCAGTC GAGCATGCC    829
(b)
TCACCTACGC CTATCACCTA CGCCTATGGC CGAATGCGAG GCAGCTCGGA 50
CGATCTGTGA GATGTGCTGA TTTCACCCAG TTCGGCCCGT TGCACCGGCA 100
CATGGACGTC GCGGCGACCC TCGGCTGGAA GCTCGCCGGC AAGAAATCCC 150
GCTGACCAGC AGCAAGAAGA AAACCATGTA CTAAAACCCG TGTGTCGCAA 200
AAACCAGATG CACATCCTGC GTGGAGGTTT GCTGATGAAA CCTGCTGGCG 250
ATCGCTGCGG CCACTCTGAA GGCCTTCATG GAATTCTGTC AAAGGATCAC 300
CTGTGTGCCC TGCCTGAACG ACTGTGCCTT CGCCCCGTTT CAAGGGCAGT 350
TCTACTCACC GCTGCTTGGA CCCATCCTGG CATGAACATC CCATCCGACA 400
ACATCGAGAA 410
(c)
AGTTGTCTAC AGCAATCCCA AGACGCTGTA CACCGCCGAG TTCATGGGCT 50
CGAACAACCG TCTGCAAGGC AAGGTGACGG AACAACGTGA CCAGCAAATT 100
CGCCTAAGTG GGCCCGGCTG GGAATTGTGG GGCCATGCCG CCGCTCCCCT 150
GAACGCTGGC CAGCAAGCCA CCGCCGTGAT TCGTGTCGAA CAAGTGCAGT 200
TGAATGCACA ACCCGGCCCC GATACGCTGC AACTACAACT GTCCACCAGC 250
ATGTACCTGG GCGACAAGTG GGAGCACGTT TTCCGCATGG CCGACCCGTC 300
TGCCGGCACC TTGCGTGCTT TCGGGCCCGA GCCCCTGCCC AGTGGTGTGC 350
ATCACCTGCA ATTGCCACCC TCCAAACTGT GGTTTAACCC CCTC 394
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Fig. 2(a-c): Sequence of PCR amplification using primer, (a) AzF3-AzR3, (b) AzF5-AzR5 and (c) AlF4-AlR4

In order to confirm the identity of the sequence, Blast analysis was done involving homology search of all possible fragments gene deposited in genebank. Blastx method was used to search protein database using a translated nucleotide query. Fragment 829 bp did not show any specific information regarding with lipase as the highest similarity score was with a hypothetical protein of *Pseudomonas putida*. However, the percent identity was only confirmed at the level of 39% with query cover only 57% of the sequence. The 410 bp fragment shared high similarity with α/β hydrolase of *Pseudomonas mosselii*.

The Blastx analysis result of the 394 bp fragment is summarized in Table 3a and b. It was confirmed that the sequence is part of the lipase gene, since the highest score of the Blasx is lipase gen of *Alcaligenes faecalis* with percent identity of 98% and query cover up to 98%. The sequence was translated at reading frame +2 into 129 amino acid sequence that 127 of them matched with amino acid sequence of lipase of *Alcaligenes faecalis*. Only two amino acids of the sequence did not match leading to query cover 99%. The alignment between fragment 394 bp lipase *Alcaligenes* sp. JG3 to lipase gen of *Alcaligenes faecalis* shown at Fig. 3 support the Blast analysis and at the same time the position of the 394 fragment at the lipase gene.

DISCUSSION

Although, the conserved sequence of lipase's amino acid, the GXSXG, has long been known the variation of lipase gene sequence among microbes has also been reported. These lipase genes have low homology level making it hard to isolate them using PCR technique[18,19]. One possible way to characterize these genes is by using degenerate primers consisting of mixture of primers with all possibilities of codons of conserved amino acid of lipase. Another approach is designing the PCR primer based on sequence of lipase gene of closely related microbes. *Alcaligenes* sp. JG3, previously identified as *Azospirillum* sp. which has close relation to *Azospirillum* sp. while *Alcaligenes* sp. JG3 is taxonomically closed to *Alcaligenes faecalis*. Lipase gene of both microbes have been characterized. Meanwhile, software to be used to design primer has been intensely developed to become more sophisticated in terms of accuracy to find gene specific primers. PrimerBlast was chosen as the primer design software for its ability to overcome the specifity problem of PCR target product[20]. Both primer designing result in each five pairs of best prove the excellence of the PrimerBlast.

High accuracy on the product size and single distinct amplicon for both amplification may showthat the primer pairs used were specific for certain sequence of bacterium

Table 3a: Blastx analysis output of 394 bp fragment nucleotide sequence

Description	Query cover (%)	Identity (%)
Lipase (*Alcaligenes faecalis*)	98	98
Lipase (*Alcaligenes faecalis*)	98	95
Spermidine/putrescine ABC superfamily ATP binding cassette transporter, ABC protein (*Bordetella holmesii* 70147)	98	53
Lipase (*Bordetella hinzii*)	98	54
ABC transporter ATP-binding protein (*Bordetella holmesii*)	98	53
Lipase (*Cedecea neteri*)	98	55
Cell processes; transport of small molecules (*Achromobacter xylosoxidans*)	98	56
Lipase (*Achromobacter* sp. RTa)	98	57
ABC transporter, ATP-binding protein (*Cedecea davisae*)	98	54
Putative transporter (*Achromobacter xylosoxidans*)	98	56

Lipase: *Alcaligenes faecalis*, Sequence ID: ref|WP_035267901.1|Length: 356Number of Matches: 1

Table 3b: Alignment statistics for mach #1

Score		Expect	Method	Identities	Positives	Gaps	Frame
267 bits (683)		3e-86	Compositional matrix adjust.	127/129(98%)	127/129(98%)	0/129(0%)	+2
Query	5	VYSNPKTLYTAEFMGSNNRLQGKVTEQRDQQIRLSGPGWELWGHAAAPLNAGQQATAVIR 184					
		VYSNPKTLYTAEFMGSNNRLQGKVTEQRDQQIRLSGPGWELWGHAAAPLNAGQQATAVIR					
Sbjct	227	VYSNPKTLYTAEFMGSNNRLQGKVTEQRDQQIRLSGPGWELWGHAAAPLNAGQQATAVIR 286					
Query	185	VEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMADPSAGTLRAFGPEPLPSGVHHLQL 364					
		VEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMADPSAGTLRAFGPEPLPSGVHHLQL					
Sbjct	287	VEQVQLNAQPGPDTLQLQLSTSMYLGDKWEHVFRMADPSAGTLRAFGPEPLPSGVHHLQL 346					
Query	365	PPSKLWFNP 391					
		PPSKLW P					
Sbjct	347	PPSKLWIYP 355					

```
CLUSTAL 2.1 multiple sequence alignment
AlcF4R4 ------------------------------------------------------------
A_fecalis ATGACCGAGCTGACTGTAGACAACATTCATCTGGCCTACGACCGCAACCCGGTTCTGAAA 60

AlcF4R4 ------------------------------------------------------------
A_fecalis GGCCTGTCCATGAGCCTGAACAAAGGTGAGGTGGTTTCCCTGCTGGGGGCCTCGGGTAGT 120

AlcF4R4 ------------------------------------------------------------
A_fecalis GGCAAAACCACCTTGCTGCGTGCGGTTGCCGGGCTCGAACAGCCCTCCCAAGGGCGTATC 180

AlcF4R4 ------------------------------------------------------------
A_fecalis GCCATCAATGAGAACGTGCTGTACGACTCGCAAAACCGTATCGACCTGCCCGCCGAGGCG 240

AlcF4R4 ------------------------------------------------------------
A_fecalis CGCAATCTGGGCCTGGTTTTCCAGTCCTATGCCTTGTGGCCACACATGACCGTGCAGGAC 300

AlcF4R4 ------------------------------------------------------------
A_fecalis AATGTGGCTTATCCGCTCACGCTGCGCAAAACCTCCAAGGCGCAAAGCCGCCAAAGGGTG 360

AlcF4R4 ------------------------------------------------------------
A_fecalis GACGCCATTTTGGATCAATTGGGGTTGAAAGGCCTGGGCGAGCGCTACCCCAGCCAACTG 420

AlcF4R4 ------------------------------------------------------------
A_fecalis TCTGGAGGGCAACAGCAGCGTGTGGCCATTGCCCGTGCGCTGATTTACAACCCCCCGGTC 480

AlcF4R4 ------------------------------------------------------------
A_fecalis ATCTTGCTGGATGAGCCACTGTCCAACCTGGATGCCAAGCTGCGTGAAGAGGCCCGCGTG 540

AlcF4R4 ------------------------------------------------------------
A_fecalis TTCCTGCGCGAACTGATTGTGAAAATGGGCCTGTCCGCTTTGATGGTGACCCATGACCAG 600

AlcF4R4 ------------------------------------------------------------
A_fecalis GCGGAGGCCATGGCGATTTCGGACCGGATCCTGTTGCTGAACGGTGGCGAAATCGAGCAA 660

AlcF4R4 --------------AGTTGTCTACAGCAATCCCAAGACGCTGTACACCGCCGAGTTCATG 46
A_fecalis CAAGGCACCCCGCAAGAGGTCTACAGCAATCCCAAGACCCTGTACACCGCCGAGTTCATG 720
 **: ******************** **********

AlcF4R4 GGCTCGAACAACCGTCTGCAAGGCAAGGTGACGGAACAACGTGACCAGCAAATTCGCCTA 106
A_fecalis GGCTCGAACAATCGTCTGCAAGGCAAGGTTACCGAGCAACGCGATCAACAAACTCGGCTT 780
 ********** ********** ** **.***** ** **.**** *** **:

AlcF4R4 AGTGGGCCCGGCTGGGAATTGTGGGGCCATGCCGCCGCTCCCCTGAACGCTGGCCAGCAA 166
A_fecalis AGCGGCCCTGGTTGGGAGTTATGGGGCCATGCCGCCGCTCCCCTGAGCGCCGGCCAGCAC 840
 ** ** ** ** *****.**.**********.*** ********.

AlcF4R4 GCCACCGCCGTGATTCGTGTCGAACAAGTGCAGTTGAATGCACAACCCGGCCCCGATACG 226
A_fecalis GCCACCGCCGTGATTCGCGTCGAACAAGTGCAACTGAATGCGCAGCCCGGCCCCGATACG 900
 ********** **********. *******.**.**********

AlcF4R4 CTGCAACTACAACTGTCCACCAGCATGTACCTGGGCGACAAGTGGGAGCACGTTTTCCGC 286
A_fecalis CTGCCACTGCAGCTATCCACCAGCATGTATCTGGGCGACAAGTGGGAGCACGTTTTCCGC 960
 ****.***.**.**.********** ****************************

AlcF4R4 ATGGCCGACCCGTCTGCCGGCACCTTGCGTGCTTTCGGGCCCGAGCCCCTGCCCAGTGGT 346
A_fecalis ATGGCCGACCCGACTGCCGGCACACTGCGAGCTTTCGGACCTGAACCACTGCCCAGTGGC
1020
 **********:**********. ****:********.** **.**.**********

AlcF4R4 GTGCATCACCTGCAATTGCCACCCTCCAAACTGTGGTTTAACCCCCTC--- 394
A_fecalis GTGCATCATCTGCAATTGCCGCCCTCCAAACTGTGGATTTACCCCTCCTGA 1071
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Fig. 3: Alignment between fragment 394 bp lipase *Alcaligenes* sp. JG3 to lipase gen of *Alcaligenes faecalis*

from genus *Alcaligenes*. However, to confirm if the fragment is truly part of lipase gene, sequences of the fragments should be analyzed further. Meanwhile, the PCR amplification using AzF4-AzR4 resulted in a fragment with a size of approximately 0.9 kb, which was much higher than the expected result (341 bp). Previous study reported that the size of the PCR product, which does not fit is not always associated with the incorrect amplification. Therefore, it was also interesting to look into sequence of this PCR product. This unmatched size of DNA target could also be due to the fact that the variability of the sizes of the microbial lipase genes is very high.

Sequencing of PCR amplification product was performed to the purified PCR product without cloning prior to sequencing. The approach was chosen because clear single fragment was clearly observed on the gel electrophoresis and it was also aimed to check the purity of the PCR product. If the

PCR product is mixture of DNA fragments with similar size then the sequencing process could not result in a good sequence. The direct sequencing of the obtained PCR fragments successfully proved that each of the PCR product was associated with a single DNA fragment.

Blastx analysis of both 829 and 410 bp fragment did not match the expected lipase gene. Top 10 Blastx analysis output for the two sequence fragments are genes from bacteria of the genus *Pseudomonas*. The fact that bacteria of the genus *Pseudomonas* are also found in many agricultural land[21], location where the reported bacteria was isolated. Lipase is a type of hydrolases which mentioned before have very low homology in the DNA level, but the low value of percent identity (59%) with only 30% cover query between the obtained 410 bp DNA with the sequence of α/β hydrolase of *Pseudomonas mosselii* aborts this possibility of both 829 and 410 bp fragments as part of lipase gene.

The sequence of 394 bp fragment was convincing confirmed as part of lipase gene. Unfortunately, the obtained sequence of the lipase gene fragment does not cover GXSXG conserved sequence. Therefore, it could not be concluded if the gene follows the conserved or not. However, it seemed that amino acid sequence of lipase gene present in *Alcaligenes* sp. JG3 is quite similar to that in *Alcaligenes faecalis*, although both are isolated from very different places. *Alcaligenes faecalis* was isolated in Scotland[22], while *Alcaligenes* sp. JG3 is an Indonesian local strain[9]. The presence of lipase gene in *Alcaligenes* is not surprising, since previously the presence of glycerol metabolism genes in this bacteria, which is closely related to lipase[23] had been reported. Interestingly, among many *Alcaligenes* species, only lipase of *Alcaligenes faecalis* has high score, the second highest score of *Alcaligenes* lipase is lipase of *Alcaligenes* sp. DH1f with only 56% of percent identity. This fact confirm report on high variability among lipase gene on microbes reported earlier[18,19].

This percent identity is much lower comparing to percent identity in the amino acid sequence level. This reveals variability of codon usage among *Alcaligenes*. A much lower level of homology in nucleotide sequence could be expected for alignment analysis of the sequence with that of lipase gene of other *Alcaligenes* species, since the homology in the amino acid level was already confirmed very low. The data also confirmed that the correct sequencewas as expected by position of the primer as shown in Table 2. The obtained sequence covered 294 bp of the 1071 complate gen, which is equal to 27.45% of the gene.

The result was also re-confirmed the identity of strain JG3. In a previous study, this bacterial strain was regarded as *Azosperillum* sp. before the subsequent polyphasic study identified it as member of *Alcaligenes* sp.[10]. In fact, PCR amplification using primers designed based on the published lipase gene of *Azospirilium* did not come out with any significant information, while PCR amplification using primers design based on published lipase gene of *Alcaligenes* species decisively identified the fragment as lipase gene which highly homologous to lipase of *Alcaligene faecalis*. This supports the conclusion that the used bacterial strain is *Alcaligenes* sp. JG3. The sequence resulted in this study has been submitted to NCBI genebank with Accession No. of KP872319.

CONCLUSION

The designed primers based on lipase gene of *Alcaligenes faecalis* were able to amplify 394 bp lipase gene fragment of bacterial strain *Alcaligenes* sp. JG3. The identity of the gene fragment was confirmed by Blast analysis as part of bacterial lipase sharing up to 98% amino acid and 88.07% nucleotide similarity with lipase of *Alcaligenes faecalis*. This finding also reconfirm the identity of the bacterial as *Alcaligenes* sp. JG3 instead of *Azospirilium* sp. as claimed before.

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