MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.13, No.1, March 2019, p 9-15

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.13.1.2

Isolation of a Functional Gene Encoding Homologous Lysophospholipase from Indonesian Indigenous *Bacillus halodurans* CM1

SHANNI FERNANDA[1], ABINAWANTO[1], AND IS HELIANTI[2*]

[1]*Department of Biology, FMIPA Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia;*
[2]*Center for Bioindustrial Technology, Agency for Assessment and Application of Technology (Badan Pengkajian dan Penerapan Teknologi), Kawasan Puspiptek Jl. Raya Puspiptek Serpong, Tangerang Selatan, 15314, Indonesia*

Lipase is a biocatalyst widely used in industry, for example detergent, pharmaceutical, food, or oil purification. One of the most widely lipase used for oil purification is lysophospholipase. As much as 50% of industrial enzyme needs are supplied from microorganisms. However, enzyme productivity from wild type microbial strain is usually limited and not applicable in industry, so that genetic engineering is necessary. Cloning gene encoding for lysophospholipase from *Aspergillus niger* and *Cryptococcus neoformans* have been conducted, but has never been conducted from alkalothermophilic bacteria, such as *Bacillus halodurans*. *Bacillus halodurans* CM1 is an alkalothermophilic bacterial strain isolated previously that has many industrially potential enzymes. This study aimed to isolate one of the gene encoding lipase from *Bacillus halodurans* CM1 and cloned into *Escherichia coli* DH5α using the pGEM-T easy vector. The gene fragment encoding lysophospholipase obtained with size 783 base pairs and had 100% similarity with gene encoding lysophospholipase from *Bacillus halodurans* C-125 (No access GenBank: BA000004.3). *E. coli* harbouring the recombinant plasmid with the gene also showed activity on trybutiryn medium compared to negative control.

Key words: *Bacillus halodurans* CM1, cloning, lysophospholipase

Lipase adalah biokatalis yang banyak digunakan di industri, misalnya deterjen, farmasi, makanan, atau pemurnian minyak. Salah satu lipase yang paling banyak digunakan untuk pemurnian minyak adalah lysophospholipase. Sebanyak 50% kebutuhan enzim industri diperoleh dari mikroorganisme. Namun, produktivitas enzim dari mikroba galur liar biasanya terbatas dan tidak fisibel di industri, sehingga diperlukan rekayasa genetika. Kloning gen pengkode lysophospholipase dari *Aspergillus niger* dan *Cryptococcus neoformans* telah dilakukan, akan tetapi yang berasal dari bakteri alkalothermophilic, seperti *Bacillus halodurans*, belum pernah dilakukan. *Bacillus halodurans* CM1 adalah galur bakteri yang diisolasi sebelumnya yang memiliki banyak enzim yang potensial bagi industri. Penelitian ini bertujuan untuk mengisolasi gen lysophospholipase dari *Bacillus halodurans* CM1 dan dikloning ke *Escherichia coli* DH5α menggunakan vektor pGEM-T. Plasmid rekombinan disekuensing. Hasilnya didapat *open reading frame* (ORF) lysophospholipase berukuran 783 pasangan basa dan kemiripan 100% dengan gen pengkode lysophospholipase dari *Bacillus halodurans* C-125 (nomor akses GenBank: BA000004.3). Dari pengamatan zona bening di sekitar klon positif re *E. coli* kombinan, produk gen ini juga menunjukkan aktivitas pada medium Tributirin dibandingkan dengan kontrol negatif.

Kata kunci: *Bacillus halodurans* CM1, cloning, lisofosfolipase

Enzymes are widely used as biocatalyst in many aspect of daily life, such as detergents, medicines, food, and oil refining. As much as 50% or more of these needs come from microorganisms, because microbial enzyme is commonly easier to cultivate. Enzymes that are widely used in the oil refining industry are lipase and phospholipase (Borrelli and Trono 2015). During the last decade, the identification and production of phospholypase has attracted research interest because of its commercial application in various industries.

Phospholipase especially lysophospholipase can more easily hydrolyze the crude oil than lipase (Cesarini *et al.* 2015). It is capable of hydrolyzing both acyl groups of phospholipids to produce phosphoglycerates and fatty acids (Ramrakhiani and Chand 2011). It is being used to produce useful phospholipid derivatives, to reduce the cholesterol content of food, and to refine vegetable oils, especially in terms of crude oil degumming. Compared with traditional physical degumming methods, enzymatic degumming can greatly reduce the consumption of chemicals while producing very little wastewater. This leads to an economical, efficient, and stable green oil degumming process (Jiang *et al.* 2011a; Ramrakhiani and Chand 2011). The lysophospholipase has been also

*Corresponding author: Phone: +62-21-7560694; Email: is.helianti@bppt.go.id

applied in oil refining (Cesarini *et al.* 2015).

Substantial effort has been made to develop phospholipases, including three commercial phospholipases, for use in oil degumming. Phospholipases have been shown to reduce the phosphorus content of oil to 5 ppm. With some phospholipases, an additional bleaching step is needed after enzymatic degumming. In addition, the supply of phospholipases is generally limited and cannot meet the increasing market demand for phospholipase (Jiang *et al.* 2011b).

In addition, the use of enzymes in oil purification industry causes oil free from harmful chemicals. The use of enzyme in this application is better. However, the use of enzymes is limited because of the availability and price. Therefore, genetic engineering is required to produce high amounts of enzymes (Cesarini *et al.* 2015).

The genes encoding some of these phospholipase have been cloned and expressed, mainly in *Escherichia coli* systems (Chandrayan *et al.* 2008; Jiang *et al.* 2012). For example, Jiang *et al.* expressed the phospholypase B (PLB) from *Pseudomonas fluorescens* in *E. coli* BL21 and achieved a PLB activity of 176.2 U·mg-1 (Jiang *et al.*, 2012). When Chandrayan *et al.* introduced the gene encoding the PLB from *Pyrococcus furiosus* into *E. coli* BL21(DE3) (pLysS), this phospholypase was expressed as inclusion bodies and refolded using heat and denaturant treatment (Chandrayan *et al.* 2008). Lysophospholipase from *Aspergillus niger* has also been cloned and expressed in *Pichia pastoris* (Zhu 2007; Coe *et al.* (2003) as also cloned *lysophospholipase* gene from *Cryptococcus neoformans*.

However, cloning of gene encoding lysophospholipase from alkalotermophilic bacteria, such as *Bacillus halodurans* has never been conducted. *Bacillus halodurans* CM1 is bacterial strain of Badan Pengkajian dan Penerapan Teknologi Culture Collection (BPPTCC) isolated from hot spring sediment in Cimanggu, West Java. The bacteria have a similarity of 99% with 16S rRNA of *B. halodurans* C-125. It have many industrially potential enzymes (Ulfah *et al.* 2011). Previous research has shown that this bacteria have lipase enzymes (Aisyah *et al.* 2017), however, the study about their properties and respective gene have not been carried out. This study aimed to isolate one of the gene encoding lipase from *B. halodurans* CM1 and cloned into *E. coli* DH5α using the pGEM-T easy vector.

MATERIALS AND METHODS

Medium. Horikoshi medium was used for cultivation of *Bacillus halodurans* CM1. Luria Bertani (LB) medium contain ampicilin, X-Gal, and IPTG were used for cultivation of recombinant *E. coli* Dh5α.

Extraction Genomic DNA from *B. halodurans* CM1. Extraction of the genome DNA of *B. halodurans* CM1 performed using phenol-chloroform extraction method with modifications (Saito and Miura 1963). The result of extraction genome visualisation was observed in agarose 1% by electrophoresis.

Amplification of Fragment Gene Encoding *Lysophospholipase*. Forward primer 5'-ATGTGGAAATGGGAAGTTGCTGAGC-'3 dan reverse primer 5'-CTATGATAATTGCTGTTCGA TAAAAAAACAGG-'3 were designed based on sequences of genes encoding *lysophospholipase* from *B. halodurans* C-125 on the site http://www.genome.jp and used in amplification of gene terget. The amplification was performed using KAPA Extra Hot Start Taq DNA polymerase based on the protocol of KAPA (KAPA Biosystems 2017) under PCR condition 95 ºC 3 min, 95 ºC 30 sec, annealing 57 ºC 30 sec, extension 72 ºC 1 min, and continued to extra extension 72 ºC 10 min.

Transformation of Recombinant Plasmid pGEM-T easy to *Escherichia coli* DH5α Ligation of the PCR fragment into pGEM-T easy vector using T4 DNA ligase was carried out based on protocol of Promega (2015). Plasmid pGEM-T easy that contained *lysophospholipase* gene was transformed into competent cell *Escherichia coli* DH5α by heat shock methods (Hanahan 1983). Screening of transformant was perfomed by screening blue-white that used LB agar containing 100 µg mL^{-1} ampicillin, 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG), and 4% 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). The culture then was incubated over night at 37 ºC. The color of positive colonies that contains plasmid with gene encoding lysophospholipase are white, but the color of negative colonies are blue. Positive colonies were cultured in a liquid LB medium containing ampicillin for extraction plasmid DNA.

Extraction and Verification of Plasmid of Recobinant *Escherichia coli* DH5α. Extraction plasmid from positive colonies of recombinant *Escherichia coli* DH5α was performed by using the alkaline method (Sambrook and Russel 2001). The extracted plasmid was confirmed by digestion using

the enzyme *Eco*RI.

Sequencing and Analysis of DNA Sequences. The plasmid that has been confirmed by digestion was delivered to *First Base* for sequensing primer *forward* PUC M13 (-40) and primer *reverse* M13 (-20). The sequencing result is then analyzed with BioEdit and Sequence Scanner 2.0 (Applied Biosystems) software, then sequenced using CLUSTAL W program at http://www.genome.jp. Sequencing analysis of their relationships with genomic databases available on GenBank using the bioinformatics approach is the basic technique of the Local Search Alignment Tool (BLASTn) at http://www.ncbi.nlm.nih.gov/blast.cgi.

Qualitative Test of Gen Products on Lipid Media. The *Escherichia coli* DH5α sample containing the lysophospholipase gene from *Bacillus halodurans* CM1 was inoculated on the LB medium to produce ampicillin and incubated at 37 °C for 24 hours. The same procedure was also done to the negative control (blue colony). The culture of the sample was incubated using an incubator shaker at a rate of 150 rpm at 37 °C for overnight, then the culture was redistributed by diverting 1 mL into 7 mL LB with ampicillin. Refreshed cultures are allowed for ± 3 hours to reach various ODs between 0.7 and 0.8. OD measurements were performed using spectrophotometry at a wavelength of 600 nm. The OD value between the culture sample and the negative control is attempted to have the same measurement value. Cultures that have achieved these OD values, are re-inoculated by taking 2 mL into 50 mL LB with ampicillin, 2% tributyrin and 0.1 M IPTG; then incubated at the incubator shaker at 37 ºC, 150 rpm and overnight. A total of 1.5 µL sample cultures were spotted using micropipets into LB media containing Tributyrin (TBA) and 0.1 M IPTG for qualitative assay (Litthauer *et al.* 2010). Incubation is carried out at 37 °C for 24-72 hours. Lipolytic activity indicated by lypolitic index of recombinant *E. coli* DH5α was compared to the negative control.

RESULTS

Genomic DNA Extraction and PCR Amplification Result. Genomic DNA could be extracted from *B. halodurans* CM1, and the results were visualized o na 1% agarose gel, and DNA fragment more than 10.000 bp was detected (Fig 1A). The genome can be extracted well, and did not contain any contaminants. By using the designed primers, the specific bands lies between 750 bp and 1.000 bp were detected (Fig1B).

Sequencing and Analysis of DNA Sequences. After ligation of the PCR product into pGEM T easy vector, the white colonies grew on transformation plate were picked.There are 102 white colonies, however, only two clones used for futher analysesof extraction plasmid.The plasmid before (Fig 2A) and after verification using *Eco*RI restriction enzyme showed 2 bands at ±3009 bp and ± 789 bp (Fig 2B).The clones that has been confirmed by *Eco*RI digestion further used for sequencing. DNA sequencing result showed that both clones showed open reading frame of protein. Analyze of DNA sequence with BLAST showed gene has 100% similarity with gene encoding *lysophospholipase* from *Bacillus halodurans* C-125. It can be concluded, *lysophospholipase* gene had been isolated and cloned into plasmid pGEM-T easy (Fig 3).

Qualitative Test of Expression of Gen Products on Lipid Media. Inoculation of positive colonies on LB tributyrin, ampicillin, and IPTG agar media was carried out to determine the presence of lipase activity showed by clear zone. The addition of IPTG was done to induce T7 promoters in the vector so that the gene could be translated. The positive colonies growing on the medium showed a clear zone after 3 days incubation compared to negative control (Fig. 4). Therefore the gene encoding lysophospholipase homolog showed the true lipase activity against tributyrin. Tributyrin is one lipase substrate that can be used to measure lipolytic activity, even can be used also to measure the activity of phospholipase and lysophospholipase.

Alignment of the deduced amino acid with other lysophospholipase showed that, B. halodurans CM1 phospholypase has homology with other *Bacillus* phospholipase. For example with that of *Bacillus pseudocaliphilus* there was 57% homology, and with that of *B. thuringiensis* there was 43% homology (Fig 5).

DISCUSSION

Bacillus halodurans CM1 is very unique bacterial strain isolated previously from Indonesia hotspring (Ulfah *et al.* 2011). This bacterial strain is very potential in producing xylanase and the gene has been cloned (Helianti *et al.* 2018). Other than xylanase, protease, amylase, etc were also produced (Ulfah *et al.* 2011). Previous research has shown that this bacteria have lipase enzymes (Aisyah *et al.* 2017), however, the study about their properties and respective gene have not been carried out.

Further investigation showed that, lysophos-

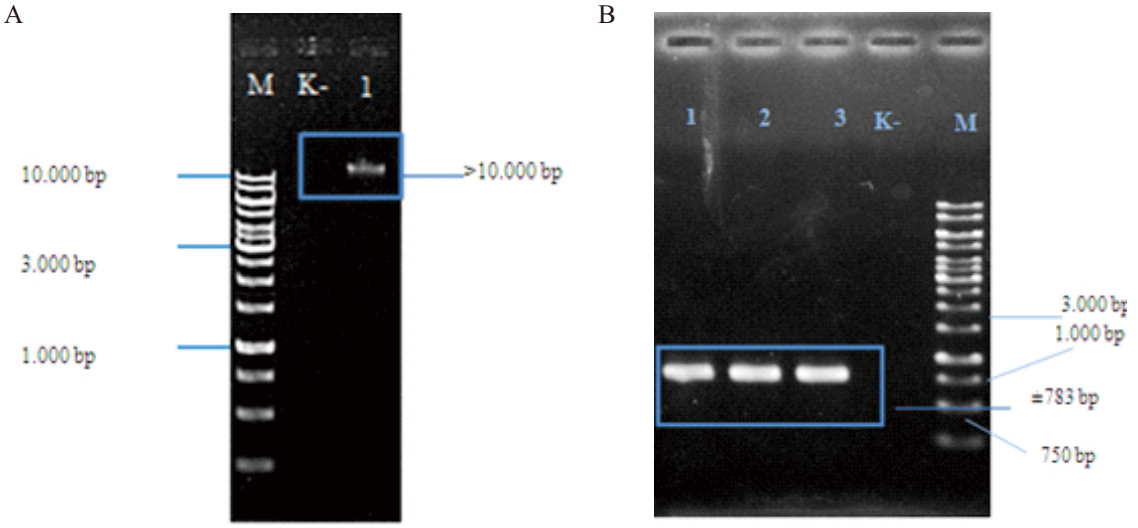

Fig 1 Genomic DNA extracted from*Bacillus halodurans* CM1 (A);and the PCR amplification of target gene (B).

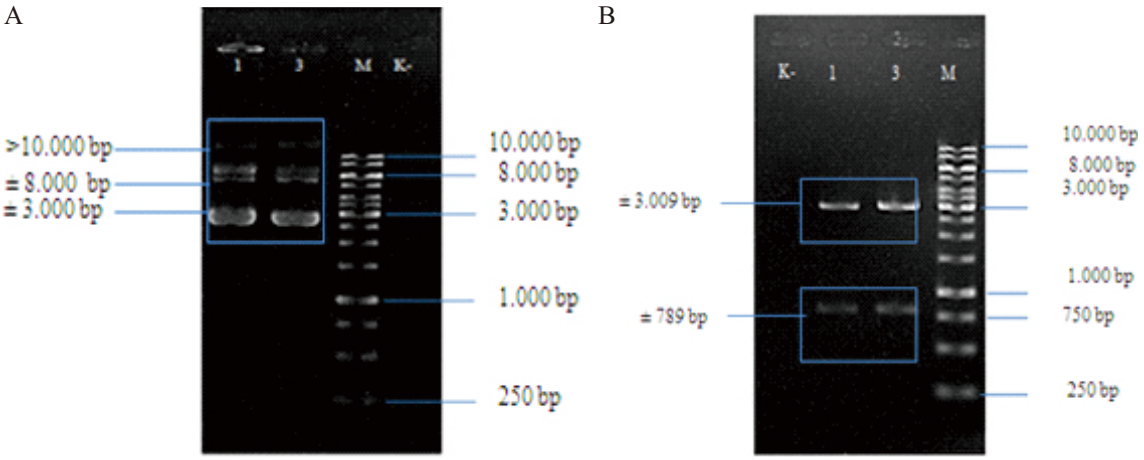

Fig 2 Histogram the number colony of *Azotobacter* sp. during 60-days incubation.

pholipase from *Aspergillus niger* has also been cloned and expressed in *Pichia pastoris* (Zhu 2007). The *lysophospholipase* gene from *Cryptococcus neoformans* has also been cloned (Coe *et al.* 2003). However, based on our further study none of this study related to the cloning of lysophospholipase gene from *B. halodurans*. Based on genomic information, *B. halodurans* C-125 has at least 3 kinds of putative lipase genes, namely: phospholipase/carboxylesterase, acetyl esterase, and lysophospholipase (Takami *et al.* 2000). However, we are not sure which gene from these putative that have matched our primer, and is this gene homologous with our bacterial strain's lysophospholipase. Therefore, we choose one of these lipase genes to be isolated using PCR approach.

In this study, pGEM T-easy vector was used, since this cloning is TA cloning vector that utitilize the PCR product by Taq polymerase that have A-cohesive end, have blue-white screening system, has T7 or SP6 promoter system, and gave very good result in gene isolation in many reports (Helianti *et al.* 2010; Helianti *et al.* 2018). Qualitative assay of lipase activity was confirmed by the clear zone around the colony. This result showed that this lypophospholipase gene product had true lipase, the same result was reported by Ramchuran et al 2006 and Sharma *et al.* 2018. However, lipolytic activity of lysophospholipase will be more optimal when on a specific substrate, such as agar medium containing lysolecithin or egg yolks (Merino *et al.* 1999). Using this DNA vector, the target gene could be expressed (Fig. 5). Compared to a negative control *E. coli* clone with plasmid harbouring

```
lysoPL C-125 ATGTGGAAATGGGAAGTTGCTGAGCCGCGTGGGGTGGTCGTCGTCATTCATGGGGCGGGAGAACACCAT
reverse      ATGTGGAAATGGGAAGTTGCTGAGCCGCGTGGGGTGGTCGTCGTCATTCATGGGGCGGGAGAACACCAT
forward      ATGTGGAAATGGGAAGTTGCTGAGCCGCGTGGGGTGGTCGTCGTCATTCATGGGGCGGGAGAACACCAT
             ************************************************************************
lysoPL C-125 GAACACCATGGGCGTTATCAATGGCTCGCAAAAAAGTTTAATAGCATCGGATTATCTGTAGTGATGGGT
reverse      GAACACCATGGGCGTTATCAATGGCTCGCAAAAAAGTTTAATAGCATCGGATTATCTGTAGTGATGGGT
forward      GAACACCATGGGCGTTATCAATGGCTCGCAAAAAAGTTTAATAGCATCGGATTATCTGTAGTGATGGGT
             ************************************************************************
lysoPL C-125 TTCCAACAGTACATTGATGTTGTCTTGGAATGGGTGGAAGCAGCTAAGTTGGAGCACGTGCCAATCTTC
reverse      TTCCAACAGTACATTGATGTTGTCTTGGAATGGGTGGAAGCAGCTAAGTTGGAGCACGTGCCAATCTTC
forward      TTCCAACAGTACATTGATGTTGTCTTGGAATGGGTGGAAGCAGCTAAGTTGGAGCACGTGCCAATCTTC
             ************************************************************************
lysoPL C-125 TGTTTGGCCACAGCATGGGCGGACTTGTAGCCGTTCGCACGATGATTGAAGGAGGCACATTGCCAGTGC
reverse      TGTTTGGCCACAGCATGGGCGGACTTGTAGCCGTTCGCACGATGATTGAAGGAGGCACATTGCCAGTGC
forward      TGTTTGGCCACAGCATGGGCGGACTTGTAGCCGTTCGCACGATGATTGAAGGAGGCACATTGCCAGTGC
             ************************************************************************
lysoPL C-125 GTGCTGTCATTCTTTCATCACCATGCTTTGATTTATATCAGTCACCTGGGAAAGGAAAAGAATTGGCTT
reverse      GTGCTGTCATTCTTTCATCACCATGCTTTGATTTATATCAGTCACCTGGGAAAGGAAAAGAATTGGCTT
forward      GTGCTGTCATTCTTTCATCACCATGCTTTGATTTATATCAGTCACCTGGGAAAGGAAAAGAATTGGCTT
             ************************************************************************
lysoPL C-125 CGAAAATGTTGCACCGAGTAACGCCTACTTTCTCGCATCATTCAGGCATTCGTTCCGATTTAGTTACTC
reverse      CGAAAATGTTGCACCGAGTAACGCCTACTTTCTCGCATCATTCAGGCATTCGTTCCGATTTAGTTACTC
forward      CGAAAATGTTGCACCGAGTAACGCCTACTTTCTCGCATCATTCAGGCATTCGTTCCGATTTAGTTACTC
             ************************************************************************
lysoPL C-125 GAAATGAAGAGATTCGTGAAGCCTACTTGAAGGATGAGCTTAGAGTAACAAAAGTGTCCACGAAATGGT
reverse      GAAATGAAGAGATTCGTGAAGCCTACTTGAAGGATGAGCTTAGAGTAACAAAAGTGTCCACGAAATGGT
forward      GAAATGAAGAGATTCGTGAAGCCTACTTGAAGGATGAGCTTAGAGTAACAAAAGTGTCCACGAAATGGT
             ************************************************************************
lysoPL C-125 ATTATGAGTTATCGAAGGCGATGCGAGATACCCGTCGTTATCCTGAAAAGTTCCCGAACGTACCATTGC
reverse      ATTATGAGTTATCGAAGGCGATGCGAGATACCCGTCGTTATCCTGAAAAGTTCCCGAACGTACCATTGC
forward      ATTATGAGTTATCGAAGGCGATGCGAGATACCCGTCGTTATCCTGAAAAGTTCCCGAACGTACCATTGC
             ************************************************************************
lysoPL C-125 TGTTATGCAGGCGGGAGAAGATTATATCACGGATAGAAAAGCGGCGTGGGAATGGTTTAATTCGGTTCA
reverse      TGTTATGCAGGCGGGAGAAGATTATATCACGGATAGAAAAGCGGCGTGGGAATGGTTTAATTCGGTTCA
forward      TGTTATGCAGGCGGGAGAAGATTATATCACGGATAGAAAAGCGGCGTGGGAATGGTTTAATTCGGTTCA
             ************************************************************************
lysoPL C-125 AGTAACGGAAAAGGCCTATAAAGAGTGGAATGGACTCTATCATGAAATTTTTAATGAGCCTGAGCGGGA
reverse      AGTAACGGAAAAGGCCTATAAAGAGTGGAATGGACTCTATCATGAAATTTTTAATGAGCCTGAGCGGGA
forward      AGTAACGGAAAAGGCCTATAAAGAGTGGAATGGACTCTATCATGAAATTTTTAATGAGCCTGAGCGGGA
             ************************************************************************
lysoPL C-125 GGCTGTGTTTCAATACACCTGTTTTTTTATCGAACAGCAATTATCATAA
reverse      GGCTGTGTTTCAATACACCTGTTTTTTTATCGAACAGCAATTATCATAG
forward      GGCTGTGTTTCAATACACCTGTTTTTTTATCGAACAGCAATTATCATAG
             ************************************************_
```

Fig 3 The nucleotidesequence of gene encoding lysophospholipase *Bacillus halodurans* CM1 compared to *B. halodurans*C-125.

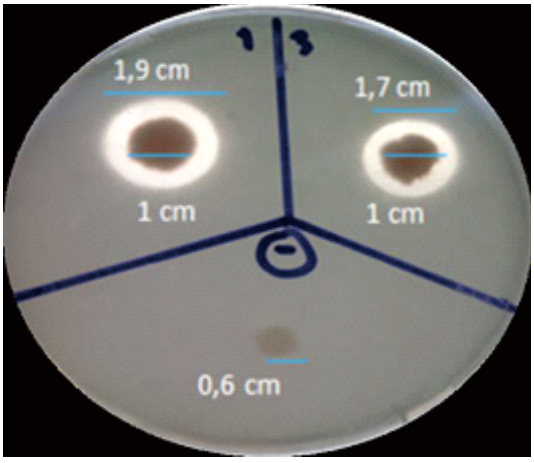

Fig 4 Clear zone around positive clones.

```
CM1_lysophospholipase              ----------MWKWEVAEPRGVVVVIHGAGEHHGRYQWLAKKFNSIGLSV
Bpseudalcaliphilus_phospholipa     ----------MWTYASKDARATIVLIHGAGEHHGRYEWLAQKWNEHGIHV
Alteribacillus_persepolensis       ---------MMKNWMCDRARGTVLIIHGAGEHHGRYEWVIQYLNQLRFHV
Bthuringiensis_Lysophospholipa     MKKSEMEESRMWNYEAEEAKAVIVIVHGAMEYHGRYEAVAEMWNHIGYHV
                                             * .:    .:.:::::*** *:****: : :  *     *

CM1_lysophospholipase              VMGDLPGQGRTRGKRGHIQSFQQYIDVVLEWVEAAKLEHVPIFLFGHSMG
Bpseudalcaliphilus_phospholipa     IMGDLPGQGKTRGKRGHINQFSQYIDAVQEWVDEAKKFEQPIFILGHSMG
Alteribacillus_persepolensis       VSGDLPGHGRTRGKRGHIDTFDQYINTVYEWYKEAASYELPVFLFGHSMG
Bthuringiensis_Lysophospholipa     VMGDLPSHGTTSRNRGHIDSFDEYIEEVKLWVKEARKYRLPIFLFGHSMG
                                   : ****.:* *  :****: *.:**: *  * . *   . *:*::*****

CM1_lysophospholipase              GLVAVRTMIEGGTLPVRAVILSSPCFDLYQSPGKGKELASKMLHRVTPTF
Bpseudalcaliphilus_phospholipa     GLVAIRYVMESKAKDIQGLLLSSPCLGLFRPIKTSKDLASKVLNRLTPTL
Alteribacillus_persepolensis       GLVAIRTLMEK-YMPIKGIILSSPCLGLYEYPSKAADVAAKMFHRIAPTF
Bthuringiensis_Lysophospholipa     GLIVIRMMQETKREDVDGIILSSPCLGVLAGPSAPLQAASKILNIIAPKL
                                   **:.:* : *     : .::*****:.:        : *:*::: ::*.:

CM1_lysophospholipase              SHHSGIRSDLVTRNEEIREAYLKDELRVTKVSTKWYYELSKAMRDTRRYP
Bpseudalcaliphilus_phospholipa     TVASGINSNHVTRDEQIRDQYVRDELRVTKVSVRWYQELHKNMHLATRYP
Alteribacillus_persepolensis       KAKSGIRASRVTRSPEARAAYEKDEFNVSVVTARWYQETLKAIKRSFFEA
Bthuringiensis_Lysophospholipa     QFATNLTVEMSTRNHEVRDAMENDSLFLRKVSVRWYSELTKSIEIAHKKI
                                      :.:  .  **. : *    .*.: :  *:.:** *  * :. :

CM1_lysophospholipase              EKFPNVPLLVMQAGEDYITDRKAAWEWFNSVQVTEKAYKEWNGLYHEIFN
Bpseudalcaliphilus_phospholipa     EKMPDIPLAVLQAGDDKIVSKYAVRDWFDSLDVTEKYYKEWKGLYHEVFN
Alteribacillus_persepolensis       DRFPNVPLLVMQAGEDYIVDKYAAHRWFNRIETADRSMKEWKGLYHELLN
Bthuringiensis_Lysophospholipa     DDFPDVPLLLMQACEDKLVDKTRVRTWFDNVKISDKAFKEWPNCYHELLN
                                   : :*::** ::** :* :..:  .  **: :. :::  *** . ***::*

CM1_lysophospholipase              EPEREAVFQYTCFFIEQQLS-------100%
Bpseudalcaliphilus_phospholipa     EPEKEVVFRHAVGIMNLWT-------- 57%
Alteribacillus_persepolensis       EPEREEVFQFMMNFINQRL-------- 54%
Bthuringiensis_Lysophospholipa     EYERDEILNYIQSFTEIRINNIIETNK 43%
                                   * *:: ::..   : :
```

Fig 5 Alignment of deduced amino acid of *Bacillus halodurans* CM1 phospolipase compared to other amino acid phospholipase from other resources. Bpseudalcaliphilus_phospholipa: lysophospholipase from *Bacillus pseudalcaliphilus*; Alteribacillus_persepolensis: lysophospholipase from *Alteribacillus_persepolensis*; Bthuringiensis_Lysophospholipa: lysophospholipase from from *Bacillus thuringiensis*.

false insert DNA, the positive clone showed clear zone around the colony. The clear zone was could be considered appeared from the gene inserted in pGEM. The extracellular expression could be come from the enzyme expressed in the cells which leaked into extra of the cells, since *E. coli* is usually cannot secreted the enzyme (Helianti *et al.* 2010).

The isolation of this gene is the first step to express the gene product in suitable host and produce and apply the gene product.

ACKNOWLEDGMENT

Part of this study was funded by Insinas Research Incentive Program of Ministry of Research, Technology, and Higher Education 2018-2019 granted to IH.

REFERENCES

Aisyah A, Mangunwardoyo W, Trismilah, Suhendar D. 2017. Optimization and concentration of lipase from *Bacillus halodurans* CM1. Al Kauniyah: J Biology. 10(2): 114-123.

Borrelli GM, Trono D. 2015. Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. Int J Mol Sci16: 20774—20840.

Cesarini SFIJ, Pastor, Nielsen PM, Diaz P. 2015. Moving towars a competitive fully enzymatic biodiesel process. Sustainability 7: 7884—7903.

Chandrayan SK, Dhaunta K, Guptasarma P. 2008. Expression, purification, refolding and characterization of a putative lysophospholipase from Pyrococcus furiosus: retention of structure and lipase/esterase activity in the presence of water-miscible organic

solvents at high temperatures. Protein Expr Purif. 59(2):327-33. Doi: 10.1016/j.pep.2008.02.019.

Coe JGS, Wilson CF, Sorrel TC, Latouche NG, Wright LC. 2003. Cloning of CnLYSO1, a novel extracellular lysophospholipase of the pathogenic fungus *Cryptococcus neoformans*. Gene 316: 67—78.

Dale JW, Von Schantz M. 2007. *From gene to genomes: Concepts and applications of DNA technology*. John Wiley & Sons, Ltd., Chichester: xii+360 hlm.

Helianti I, Nurhayati N, Ulfah M, Wahyuntari B , Setyahadi S. 2010. Constitutive High Level Expression of an Endoxylanase Gene from the Newly Isolated *Bacillus subtilis* AQ1 in *Escherichia coli*. J Biomed Biotechnol. doi: 10.1155/2010/980567.

Helianti I, Furgeva N, Mulyawati L, Ferniah RS, Kusumaningrum HP. 2018. Cloning of a Gene Encoding Protease from *Bacillus halodurans* CM1 into *Escherichia coli* DH5 and Expression Analyses of the Gene Product. Makara J Sci. 22/3 (2018), 113-120 doi: 10.7454/mss.v22i3.9900.

Jiang F,Wang J, Kaleem I, Dai D, Zhou X, Li C. 2011a. Degumming of vegetable oils by a novel phospholipase B from *Pseudomonas fluorescens* BIT-18. Bioresour Technol. 102(17):8052-8056. doi: 10.1016/j.biortech.2011.05.050.

Jiang F, Wang, J, Ju L, Kaleem I, Dai D, Li C. 2011b. Optimization of degumming process for soybean oil by phospholipase B. Chem Technol Biotechnol. 86(8): 1081-1087.

Jiang F, Huang S, Imadad K, Li C. 2012. Cloning and expression of a gene with phospholipase B activity from *Pseudomonas fluorescens* in *Escherichia coli*. Bioresour Technol.104:518-22. doi: 10.1016/j.biortech.2011.09.112.

KAPA Biosystems. 2017. KAPA Taq HotStart PCR Kit Protocol. KAPA BIO. Boston, Massachusetts, USA: 2 hlm.

Merino, S., A. Aguilar, M. M. Nogueras, M. Regue, S. Swift, & J. M. Tomás. 1999. Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic aeromonas sp. serogroup O:34. Infection And Immunity 67 (8): 4008–4013.

Promega. 2015. Protocol Promega. *Technical Manual pGEM®-T and pGEM®-T Easy Vector Systems: Instruction for Use of Products A1360, A1380, A3600, AND A3610*. USA. 29 hlm. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol.pdf

Ramchuran SO,Vargas VA, Hatti-Kaul R, Karlsson EN. 2006. Production of a lipolyticenzyme originating from *Bacillushalodurans*LBB2 in the methylotrophic yeast Pichia pastoris. Appl Microbiol Biotechnol.2006 Jul;71(4):463-72. Epub 2005 Oct 12.

Ramrakhiani L, Chand S. 2011. Recent progress on phospholipases: different sources, assay methods, industrial potential and pathogenicity. Appl Biochem Biotechnol. 164(7):991-1022. doi: 10.1007/s12010-011-9190-6.

Sambrook, J. and Russell, D. W. (2001). Molecular Cloning: A Laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sharma A,Meena KR, Kanwar SS. 2018. Molecular characterization and bioinformatics studies of a lipase from *Bacillus thermoamylovorans* BHK67. Int J Biol Macromol. 107(Pt B):2131-2140. doi: 10.1016/j.ijbiomac.2017.10.092.

TakamiH, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, Fuji F, Hirama C, Nakamura Y, Ogasawara N, Kuhara S, Horikoshi K. 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Research 28 (21): 4317—4331.

Ulfah, M, Helianti I, Wahyuntari B, Nurhayati N. 2011. Characterization of a new thermoalkalophilic xylanase-producing bacterial strain isolated from Cimanggu Hot Spring, West Java, Indonesia. Microbiol Indones 5 (3): 139—143.

Zhu S. 2007. Cloning and characterization of two lipases and a lysophospholipase from *Aspergillus niger*. *Thesis*. Concordia University (Canada), ProQuest Dissertations Publishing: x+119 hlm.