Malaysian Journal of Microbiology, Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

# Expression and characterization of a novel cold-adapted lipase from psychrotolerant bacteria *Psychrobacter* sp. S1B

**Andreas Adhi Satya[1], Anastasia Aliesa Hermosaningtyas[2], Esti Puspitasari[2], Antonius Suwanto[1]***

[1]Department of Biotechnology, Atma Jaya Catholic University, 12930 Jakarta, Indonesia.
[2]Department of Research and Development, PT. Wilmar Benih Indonesia, 17530 West Java, Indonesia.
Email: asuwanto@indo.net.id

Received 19 July 2018; Received in revised form 12 June 2019; Accepted 26 September 2019

## ABSTRACT

**Aim:** This study aimed to isolate, express and characterize the lipase derived *Psychrobacter* sp. S1B in *Escherichia coli* expression system.
**Methodology and results:** Exploration towards S1B lipase characteristic was conducted where shotgun cloning method was applied to obtain lipase encoded gene and *E. coli* expression system through pET28a was used to overexpress S1B lipase. Lipase activity was measured by using *p*-nitrophenol method. The S1B lipase gene is 1005 bp in length with molecular weight of 46 kDa, optimum pH was 10.0, showed hydrolytic activity preference toward *p*-nitrophenyl caprylate (C8) and *p*-nitrophenyl hexanoate (C6) substrates (C6 < C8). The best temperature for S1B lipase activity was at 30 ºC while exhibited high activity at lower temperature (10-25 ˚C) with above 90% of maximum activity, therefore it is classified as cold adaptive lipase. In addition, S1B lipase showed stability against various metal ions, including $Cu^{2+}$ and $Zn^{2+}$ which commonly act as inhibitors of lipases derived from *Psychrobacter* species. Moreover, S1B lipase exhibited great tolerance against up to 50% (v/v) hexane and some non-ionic detergents such as 1% (v/v) DMSO and 1% (v/v) Triton X-100.
**Conclusion, significance and impact of study:** The study proposes a novel cold-adapted lipase which has potential as a biocatalyst for synthesis caprylic acid ester.

*Keywords:* Cold-adapted lipase, genomic DNA library, recombinant lipase, *Psychrobacter* sp.



## INTRODUCTION

Lipases (*Triacylglycerol hydrolase*, EC 3.1.1.3) are class of enzyme that acts as a catalyst in the long chain acylglycerol hydrolysis reaction. These enzymes were easily found in nature such as animal tissues, plants, and microorganisms. Lipase is widely used as a catalyst in the synthesis process of organic chemical and biotechnology applications. Lipase becomes an important biocatalyst in many industrial applications because it has stable characteristics in organic solvents, broad substrate specificity, and has region- and stereo-selectivity (Park *et al.*, 2009; Lin *et al.*, 2010).

Cold-adaptive lipases may offer additional opportunities for novel biotechnological applications due to their high catalytic activity at low temperatures and unique specificities. These properties offer potential economic benefits in detergents, textile and food industries, also in bioremediation of polluted soils and waters. Therefore, prospecting for novel lipase genes from psychrophilic bacteria become a great interest. Several reports showed lipase gene from genus *Psychrobacter* was successfully isolated, cloned, and expressed it in *E. coli* (Zhang *et al.*, 2007; Lin *et al.*, 2010; Chen *et al.*, 2011; Novototskaya-Vlasova *et al.*, 2013a and 2013b; Maiangwa *et al.*, 2014; Koo *et al.*, 2016; Zhang *et al.*, 2018).

Polymerase Chain Reaction (PCR) methods have been developed for screening lipase encoding genes from environmental microorganisms (Park *et al.*, 2009; Fan *et al.*, 2009). However, low homology among different lipase genes making the PCR screening method inefficient and ineffective to find a novel lipase genes. Another approach is constructing genomic DNA library which is no sequence homology is needed for lipase gene screening and detection. Chen *et al.* (2011) was successfully isolated *Psychrobacter* sp. C18 lipase gene by constructing genomic DNA library using shotgun cloning.

S1B isolate which was obtained from fish cold storage at supermarket in Bogor, showed lipolytic activity in rhodamine B screening medium. This isolate had optimum growth temperature at 20-25 °C which is classified as thermotolerant bacteria, while from its 16s rRNA gene sequence was identified as *Psychrobacter* sp.

***Corresponding author**


ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

with 100% similarity. Isolation of the S1B lipase encoding gene employing PCR approach was failed. This result suggested that S1B lipase gene might be a novel gene. Therefore, this study were aimed to isolate the S1B lipase gene employing genomic DNA library generated from shotgun cloning, to express, and characterize S1B lipase in *E. coli* expression systems.

## MATERIALS AND METHODS

### Source of strains

The microorganisms in this research are *Psychrobacter* sp. S1B isolate, *E. coli* DH5α isolate (Invitrogen, USA), and *E. coli* BL21 (DE3) pLysS (Merck, Germany) obtained from PT. Wilmar Benih Indonesia (Cikarang, West Java, Indonesia).

### Genomic isolation and shotgun cloning

*Psychrobacter* sp. S1B genomic DNA was isolated by using Wizard® Genomic DNA Purification Kit (Promega, USA). The genomic DNA was then digested with endonuclease restriction enzyme (*Eco*RI) and ligated to pGEM-T easy cloning vector that has control fragment from the kit and was digested with the same enzyme. Recombinant plasmids were transformed with the heat shock and cold treatment protocol into *E. coli* DH5α competent cells prepared by the calcium chloride protocol with some modification (Li *et al.*, 2010). Recombinant *E. coli* DH5α dispersed on Luria selective medium with ampicillin supplementation (100 mg/L) and glyceryl tributyrate (TBN) as lipase substrate. Colonies that have lipolytic activity, characterized by clear zone, are taken and tested furthermore.

### Plasmid mapping and bioinformatics analysis

The plasmid mapping was done by plasmid digestion using several restriction endonucleases and visualized with agarose gel electrophoresis DNA. After that, sub cloning to reduce the size of the DNA insertion was done by plasmid digestion based on plasmid map prediction.

Next, the fragment was re-ligated into other pGEM®-T easy cloning vector. All subcloned plasmids that still have lipolytic activity are verified by Sanger Sequencer ABI 3130 (Thermo Scientific, USA). Furthermore, ORF-finder(https://www.ncbi.nlm.nih.gov/orffinder/) was used to find lipase-encoding gene from *Psychrobacter* sp. S1B; LipoP web based software (http://www.cbs.dtu.dk/services/LipoP/) was used to predict the propeptide sequence; BPROM web based software (https://omictools.com/bprom-tool/) was used to predict the promoter sequence and position; and, Geneious R10 software was used to visualize, and construct all plasmid.

### S1B lipase gene cloning and expression in heterologous *E. coli*

Primers for the amplification of the S1B lipase gene were synthesized based on sequences obtained from sequencing results. Furthermore, the lipase gene was amplified by using specific primers (Table 1), digested with *Eco*RI and *Not*I, then ligated to pET28a expression vector (Novagen, USA), and transformed into *E. coli* DH5α as a cloning host. Verified plasmid is then transferred to *E. coli* BL21 (DE3) pLysS and S1B lipase gene was expressed in Luria Broth medium supplemented with kanamycin (25 mg/L) and chloramphenicol (35 mg/L) antibiotics, induced by β-D-1-thiogalactopyranoside (IPTG) (Biobasic, Canada) with final concentration 0.5 mM, and incubated at 28 °C 200 rpm for 20 h. S1B lipase was obtained by sonicating the pellet cells in Tris-HCl 0.1 M pH10 for 10 min, centrifuged 13000 rpm 10 min, and collected the supernatant. Then, the supernatant was concentrated with Amicon® Ultracel -30K (Merck, Germany).

### Lipase activity and protein content assay

Catalytic activity of S1B lipase was tested by using *p*-nitrophenyl methods as describe by Arifin *et al*. (2013) with some modification. The mixture containing 0.94 mL of 0.1 M glycine-NaOH buffer pH 10.0; 0.04 mL of ethanol absolute; 0.01 mL of enzyme; and 0.01 mL of *p*-nitrophenyl decanoate 0.02 M were used to measure

**Table 1:** List of specific primers that used in this study.

| Name | Sequence (5'→ 3') | Restriction enzyme (Underlined) |
|---|---|---|
| M13 Forward | GTAAAACGACGGCCAG | |
| M13 Reverse | CAGGAAACAGCTATGAC | |
| T7 Promoter | TAATACGACTCACTATAGGG | |
| T7 Terminal | GCTAGTTATTGCTCAGCGG | |
| 930 Forward | TTCATGAATTCGTCACCTCAGAAGAAATAACC | *Eco*RI |
| 1005 Forward | GATCGAATTCATGTTAAACAACGCTATTTATCC | *Eco*RI |
| 1050 Forward | CGATCAGAATTCATGAACCTTACATACAAAAAGACC | *Eco*RI |
| 1300 Forward | TATGGAATTCTGTGTTATTCGCGATGCTAT | *Eco*RI |
| Lipase Reverse | ATGAGCGGCCGCCTATACACAGTTTTTATAATCGTAA | *Not*I |



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

lipase activity. The mixture was then incubated at 30 ºC for 5 min and the concentration of hydrolysis product, *p*-nitrophenol (pNP), was measured using a spectrophotometer at 405 nm. One unit of lipase activity was defined as the amount of lipases required to release 1 µmol of pNP under the assay conditions (Natalia *et al.*, 2014). Then, protein concentration was determined by BCA assay kit (Merck, Germany).

### Characterization of S1B lipase

Molecular weight of S1B lipase was determined by using SDS-PAGE and zymogram as described by Xie *et al.* (2012) with some modification. SDS-PAGE was performed using 12% (w/v) polyacrylamide gels and the protein was stained with Coomassie Brilliant Blue R-250 (Applichem, USA). Gels used for zymograms were washed with 1% (v/v) TritonX-100 solution 2 times 10 min, continued with 0.1 M Tris-HCl (pH 8.0) 2 times 10 min, and final washed with distilled water for another 10 min. Renatured protein activities were checked by attaching gels to TBN agar and incubating at 30 °C for 16 h.

The optimum lipase reaction temperature was determined by measuring the lipase reaction rate to release *p*-nitrophenyl molecule from pNP-caprylate at different temperatures (10-65 °C). The lipase thermostability was carried out by measuring the residual lipase activity after incubating the lipase at different temperatures (10-50 °C) for 1 h.

The optimum lipase reaction pH was determined between pH 7.0 and pH 12.0. The lipase pH stability was carried out by measuring the remaining lipase activity after incubating the lipase at 30 ºC for 1 h in different buffer at range pH 5.0 to pH 12.0. Buffer that used in this assay are 0.1 M citrate buffer (pH 5.0, 6.0); 0.1 M phosphate buffer (pH 6.0, 7.0, 7.5, 8.0); 0.1 M Tris-HCl buffer (pH 8.0, 8.5, 9.0); and 0.1 M glycine-NaOH buffer (pH 9.0, 9.5, 10.0, 10.5, 11.0, 12.0).

The effect of various metal ions, organic solvent, and detergent on the lipase activity were performed by measuring the remaining lipase activity after incubating the lipase for 1 h at 30 °C with a final concentration of 0.001 M ions solution ($MgCl_2 \cdot 6H_20$, $FeCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $CaCl_2$, and $MnCl_2$); 25 and 50% (v/v) of organic solvent (methanol, ethanol, isopropanol, butanol, hexane, and acetonitrile); and 1% (v/v) of dimethyl sulfoxide (DMSO), Tween 80, Triton X-100; also 1% (w/v) of SDS solution.

Furthermore, experiment of substrate specificity of the lipase was carried out by using *p*-nitrophenyl ester substrates with variable chain length (C4 to C18) at 30 °C and pH 10.0. Substrates of 0.02 M *p*-nitrophenyl with short chain length (C4 to C10) were diluted in acetonitrile. While, substrates of 0.01 M *p*-nitrophenyl with long chain length (C14 to C18) were diluted in isopropanol. *p*-nitrophenyl dodecanoate (C12) was used to standardize the diagram by tested in same condition as short chain and long chain substrates.

## RESULTS

### Shotgun cloning and S1B lipase gene isolation

S1B genome was digested by *Eco*RI overnight and fragmented into 3 main parts: 3-6 kb; 6-10kb; and above 10kb. Each fragment was purified and ligated to pGEM-T easy vector which had been previously treated with the same restriction endonuclease. Recombinant plasmids were transformed into *E. coli* DH5α competent cells. Single colony of *E. coli* DH5α which inserted with above 10 kb fragment gave a halo zone in lipase selective medium, which later called pGem S1B C6. Then, several restriction enzymes (*Nde*I, *NcoI*, and *Not*I) were used to map the pGem S1B C6. Triple digest with those enzymes generates 5 bands beside of pGEM®-T easy vector backbone (3kb). Multiple bands generated as shown as Figure 1A was used to calculate and predict the location of enzyme restriction sites (Figure 1).

Plasmid pGem S1B C6 was fully digested with *NcoI* to locate the putative lipase. Single colony of *E. coli* DH5α which contained fragment iv gave a halo zone in lipase selective medium which later called pGem S1B C6 IV. This indicates that lipase gene was located in the 6 kb of fragment iv. After that, the pGem S1B C6 IV was isolated and digested by *Sac*I, *Spe*I, and *Sph*I to locate S1B putative lipase precisely. Multiple bands produced by digestion were used to calculate and predict the location of enzyme restriction sites (Figure 2).

**Table 2:** Bioinformatic analysis and prediction of S1B lipase gene.

| Size of S1B gene | Remarks |
|---|---|
| 930 bp | Mature S1B lipase without propeptide |
| 1005 bp | First Open reading frame of S1B lipase (Methionine to STOP) |
| 1050 bp | Second Open reading frame of S1B lipase (Isoleucine to STOP) |
| 1300 bp | Including 288bp promoter region upstream of first ORF S1B Lipase gene |

### Bioinformatics analysis

S1B lipase gene has been identified through sub cloning and sequencing of pGem S1B C6 IV (Figure 2B). Bioinformatics software predicted 4 different sizes of S1B lipase genes for expression study (Table 2). Each gene size was then ligated to plasmid pET28a and expressed in *E. coli* BL21 (DE3) pLysS. ORF-finder (NCBI) software identified 2 types of open reading frame; first from methionine to STOP (1005 bp) and the other is from isoleucine to STOP (1050 bp). The first open reading frame (1005 bp) was analysed by sequence and translated sequence comparison using the BlastN and BlastP (NCBI) tools where this gene was similar to *Psychrobacter* sp. DAB_AL43B genome sequence



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

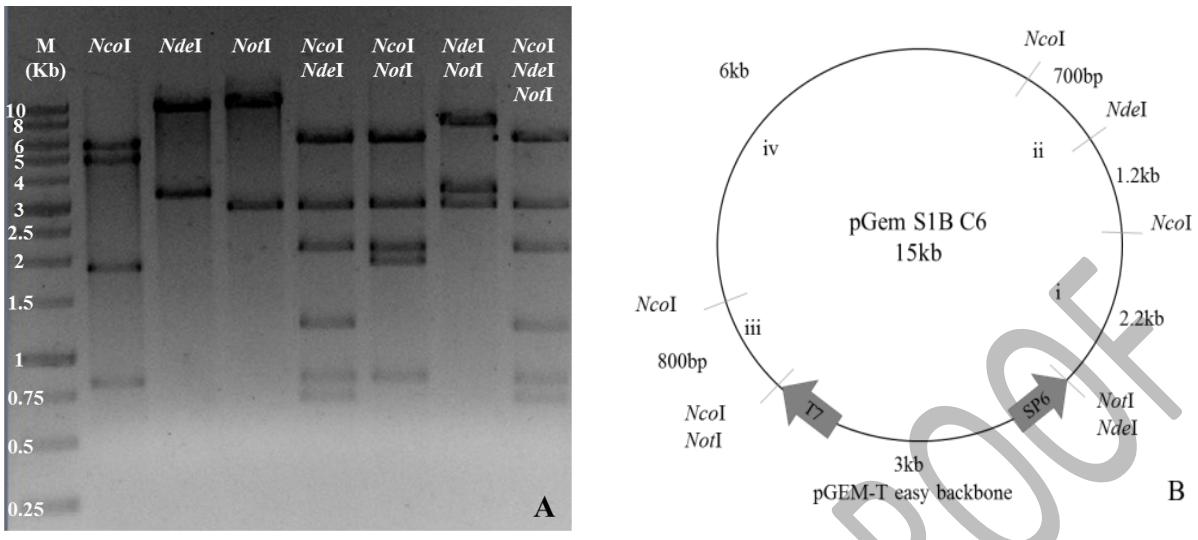


**Figure 1:** Plasmid mapping of pGem S1B C6. (A) DNA gel electrophoresis results and (B) simulation of restriction enzyme sites.

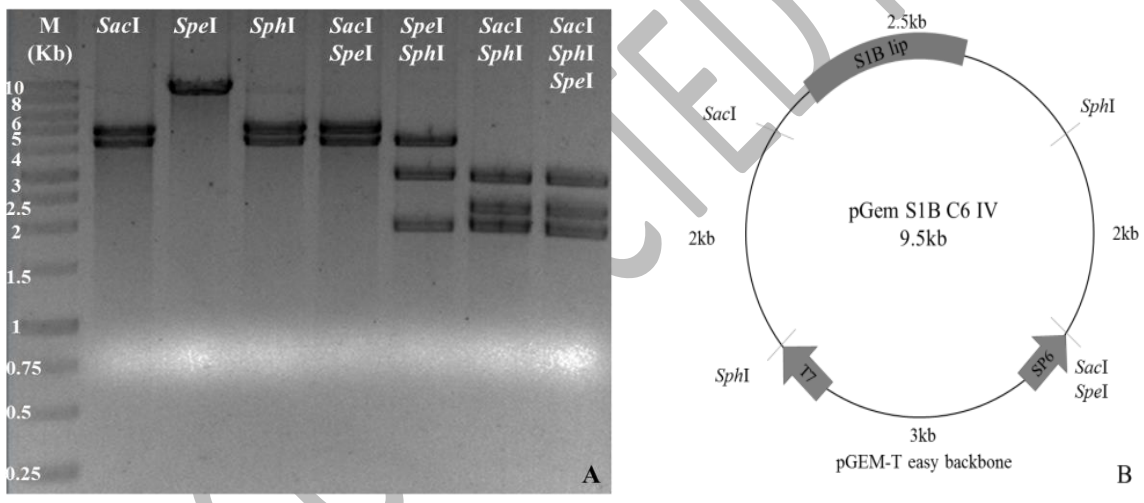


**Figure 2:** Plasmid mapping of pGem S1B C6 IV. (A) DNA gel electrophoresis results and (B) simulation of restriction enzyme sites where the S1B lipase gene located at 2.5 kb fragment between *Sac*I and *Sph*I sites.


**Figure 3:** S1B lipase gene designs in pET28a from T7 promoter until T7 terminal.



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

**Table 3:** BlastN and BlastP analysis of 1005 bp predicted S1B lipase gene sequence.

| Analysis | Description | Organism | E-Value | %Pairwise Identity | Accession |
|---|---|---|---|---|---|
| BlastN | *Psychrobacter* sp. DAB_AL43B genome assembly, chromosome I | *Psychrobacter* sp. | 0.0 | 80.6% | LT799838 |
| BlastP | Lipase [*Psychrobacter* sp. JCM 18903] | *Psychrobacter* sp. JCM 18903 | 6.91e-160 | 90.2% | GAF62011 |

(Accession number: LT799838) and lipase from *Psychrobacter* sp. JCM 18903 (Accession number: GAF62011), respectively (Table 3). Therefore, this lipase gene could be identified as a novel lipase.

Furthermore, LipoP software was used to predict propeptide sequence of S1B lipase. S1B mature lipase was preferred to be expressed in this study to prevent proteolytic degradation in heterologous expression system (Novototskaya-Vlasova *et al.*, 2012). Software (BPROM) predict S1B lipase putative promoter at 288 bp upstream the S1B lipase first open reading frame (Table 2; Figure 3).

**Production of S1B lipase**

All recombinant S1B lipase proteins were expressed in *E. coli* BL21 (DE3) pLysS which harbor pET28a containing S1B lipase genes. All samples including negative control (pET28a without insert) was expressed with same conditions. Then, S1B lipase activity was measured by using *p*-nitrophenyl method. Truncated gene with 1005 bp length have the highest activity, the second is 1300 bp, followed by 1050 bp, and 930 bp, respectively. S1B lipase with 1005 bp length was then selected for further analysis. Sample with size of 930 bp has the lowest activity and hypothesized to be inactive. The inactive protein may cause by the forming of inclusion body. This result was similar with experiment conducted by Novototskaya-Vlasova *et al.* (2013b) where deletion mutant of *Psychrobacter cryohalolentis* K5$^T$ lipase formed inclusion body which leads to decrement of its activity (Figure 4).

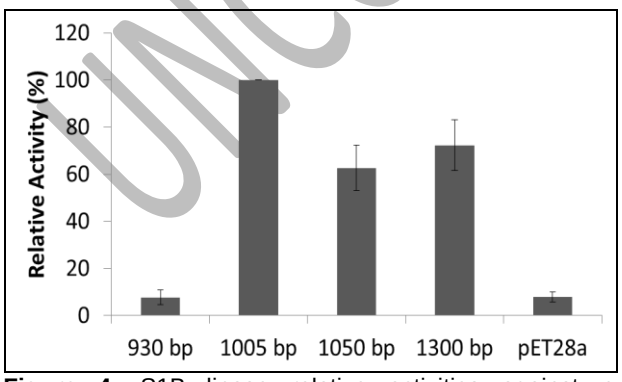


**Figure 4:** S1B lipase relative activities against *p*-nitrophenyl caprilate substrate based on the size of the gene.

**Molecular weight of S1B Lipase**

SDS-PAGE and zymogram results support the expression of S1B lipase in E. coli BL21 (DE3) pLysS harboring pET28a S1B lipase 1005 bp. Based on SDS-PAGE result, 2 different bands occurred with molecular mass of 31 and 46 kDa compared to negative control (pET28a) (Figure 5, arrow). To determine which band is S1B lipase protein, zymogram using TBN as substrate was performed. A halo zone in TBN agar supported that S1B lipase molecular weight was around 46 kDa (Figure 5, rectangle).

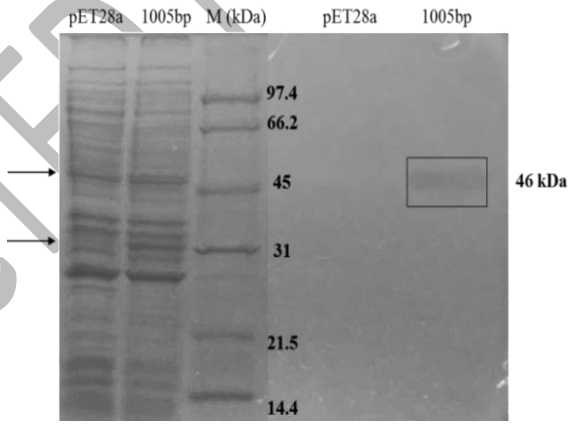


Figure 5: Characterization of S1B lipase molecular weight using SDS-PAGE and TBN zymogram.

**Effect of temperature on S1B lipase activity and stability**

The concentrated S1B lipase showed its maximum activity at temperature 30 °C. S1B lipase is more active at low temperature than high temperature condition. At temperature in the range 10-25 °C the lipase activity amounted to above 90-98% and its lipase activity decreased as long as the increment of temperature above 30 °C (Figure 6A). The thermostability of the recombinant S1B lipase was determined by preincubating the enzyme at different temperature (10, 20, 30, 40, and 50 °C) for 1 h, then the remaining lipase activity was measured at 30 °C. The result revealed that the recombinant lipase activity was stable at temperature from 10 to 30 °C and declined rapidly at temperature 40 °C. Preincubating the S1B lipase for 1 h at 50 °C reduced the enzyme activity more than 95% (Figure 6B).



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

**Effect of pH on S1B lipase activity and stability**

The S1B lipase was active in the pH range 9.0–11.0, with a maximum activity at pH10.0. Application with Tris-HCl buffer decreased the enzyme activity by 13 and 14% at pH 8.0 and 9.0 respectively (Figure 7A). S1B lipase was stable at a pH range of 7.0–8.0 for 1 h incubation on ice, retaining more than 80% of the maximum activity. The S1B lipase enzyme not stable in acidic or basic condition for 1 h incubation, the remaining activity decreased along increment of acidity and alkalinity (Figure 7B).

**Effect of metal ion on S1B lipase activity**

Metal ion assay revealed that S1B lipase is relative stable in the presence of various metal ion with final concentration 1 mM. Addition of metal ion $Fe^{3+}$ and $Mn^{2+}$ has no effect on S1B lipase activity, while the other metal ion slightly inhibited the activity of S1B lipase (Table 4).

**Effect of organic solvent and detergent on S1B lipase activity**

Organic solvent assay showed that addition of 25% (v/v) of each organic solvent except hexane decrease the S1B lipase activity and the highest remain activity was butanol. Then, preincubating the enzyme with 50% (v/v) final concentration of hexane for 1 h at 30 °C sharply enhance the catalytic activity up to 40% of its maximum value. Zhang *et al.* (2018) reported that the increasing of the concentration of organic solvents, enzyme activity was boosted in the hydrophobic organic solvents, such as in hexane. Furthermore, the enzyme remains stable at 1% (v/v) DMSO and Triton X-100. At the same time, the S1B lipase was significantly inhibited in the presence of the 1% (v/v) tween 80 and SDS (Table 5).

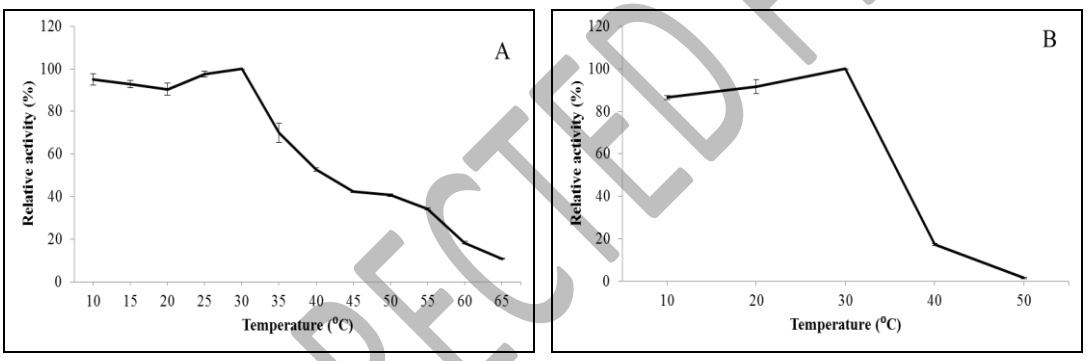


**Figure 6:** Effect of temperature on S1B lipase activity. (A) Temperature optimum of S1B lipase catalytic activity (B) Effect of temperature on the enzyme stability. Deviation standard n = 3.

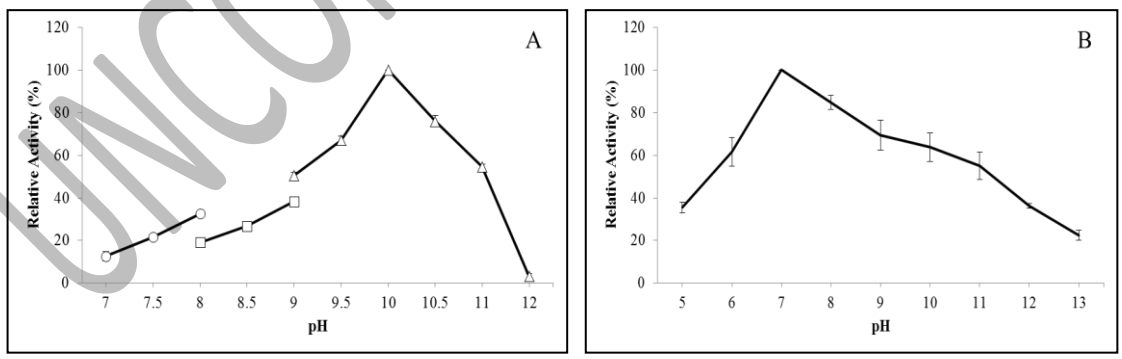


**Figure 7:** Effect of pH on S1B lipase activity. (A) pH optimum of S1B lipase catalytic acticvity (B) Effect of pH on the enzyme stability. Deviation standard n = 3.



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(2) 2020, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.180177

**S1B lipase substrate specification**

The S1B lipase substrate specificity assay indicated that medium chain fatty acids of *p*-nitrophenyl esters were better substrate for S1B lipase, and the highest lipase activity was achieved with the C8 substrate (Figure 8). This result was different with previously studies of cold adapted lipase from *Psychrobacter* species which is better at long chain fatty acid as a substrate (Zhang *et al.,* 2007; Lin *et al.*, 2010; Chen *et al.*, 2011; Novototskaya-Vlasova *et al.*, 2013a and 2013b; Koo *et al.*, 2016; Zhang *et al.*, 2018).

**Table 4:** Effect of various metal ions on S1B lipase activity.

| Metal Ions (1 mM) | Relative activity (%) |
|---|---|
| None | 100.0 |
| $MgCl_2.6H_2O$ | 88.3 ± 3.0 |
| $FeCl_3.6H_2O$ | 99.9 ± 6.2 |
| $CuCl_2.2H_2O$ | 78.8 ± 1.4 |
| $ZnCl_2$ | 82.9 ± 7.7 |
| $CaCl_2$ | 74.0 ± 2.4 |
| $MnCl_2$ | 97.7 ± 1.9 |

*Deviation standard n = 3

**Table 5:** Effect of various organic solvents and detergents on S1B lipase activity.

| Organic solvent | Relative activity (%) | | Detergent (1%) | Relative activity (%) |
|---|---|---|---|---|
| | 25% | 50% | | |
| None | 100.0 | 100.0 | None | 100.0 |
| Methanol | 35.1 ± 7.0 | 9.3 ± 4.0 | DMSO | 98.9 ± 1.5 |
| Ethanol | 22.7 ± 5.1 | 13.5 ± 1.5 | Tween 80 | 16.9 ± 0.7 |
| Isopropanol | 19.8 ± 1.7 | 21.6 ± 2.6 | Triton X-100 | 104.3 ± 1.2 |
| Butanol | 48.5 ± 2.8 | 27.0 ± 3.3 | SDS | Undetected |
| Hexane | 99.1 ± 3.2 | 139.7 ± 2.7 | | |
| Acetonitrile | 23.2 ± 5.4 | 17.6 ± 5.9 | | |

*Deviation standard n = 3

**DISCUSSION**

In this study, *Psychrobacter* sp. S1B lipase encoding gene was detected, successfully cloned and expressed in heterologous *E. coli* BL21 (DE3) pLysS system. We first conducted PCR method to isolate of S1B lipase encoding

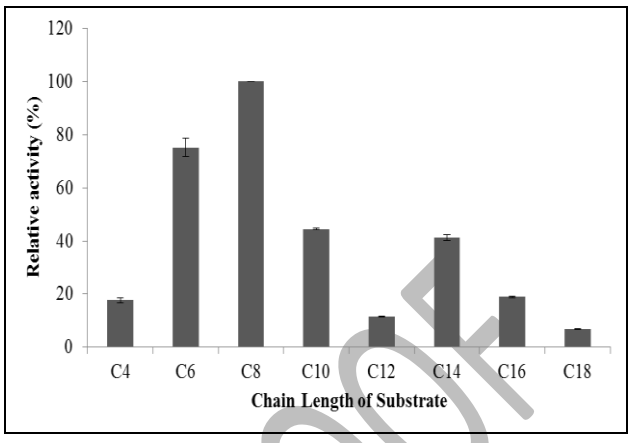


**Figure 8:** Substrate specificity of S1B lipase. Deviation standard n = 3.

gene. The degenerate primers were designed, based on lipase gene sequence from a number of lipolytic *Psychrobacter* which closely related with *Psychrobacter* sp. S1B 16s rRNA sequence identity. However, those degenerate primers were failed to amplify the S1B lipase encoding gene, which might be caused by low homology among the lipase gene sequences. Therefore, we employed shotgun cloning method to isolate the gene encoding S1B lipase. The S1B lipase gene was found to be novel according to the highest sequence identity (80.6%) with *Psychrobacter* sp. DAB_AL43B lipase gene sequences from GenBank (accession number: LT799838). At the amino acid level, the highest identical sequence of S1B lipase was the *Psychrobacter* sp. JCM 18903 lipase (accession number: GAF62011), with 90.2% similarity. Those BlastN and BlastP reference sequences only annotated lipase gene through the use of whole genome sequencing method. To the best of our knowledge, this study is the first which cloned, expressed, and characterized the cold-adapted lipase from *Psychrobacter* sp. S1B in *E. coli* expression system.

SDS-PAGE and zymogram analysis showed that S1B lipase molecular weight was 46 kDa and that is approximately 6 kDa larger than its theoretically predicted molecular weight (40 kDa). The difference condition between theoretically prediction and visualized of SDS-PAGE commonly called “gel shifting”, and the cause of gel shifting is still unclear. Guan *et al.* (2015) showed that the composition of negative amino acid residues (>11%) can inhibit protein migration in SDS-PAGE. In addition, Rath *et al*. (2009) found that protein migration in SDS-PAGE had a strong correlation with SDS-proteins binding. The form of hairpin protein (helix-loop-helix) and concentration SDS can affect the gel shift behavior. Moreover, web based software https://swissmodel.expasy.org/ developed by Guex *et al*. (2009) cannot estimate the 3-dimensional structure of 81 N-terminal amino acids of S1B lipase (data not shown). So, it was hypothesized that this section affected the gel shift behavior in S1B lipase SDS-PAGE.



 ISSN (print): 1823-8262, ISSN (online): 2231-7538

There are several previous studies that found that *Psychrobacter* strains can produced cold-adapted lipase (Zhang *et al.*, 2007; Lin *et al.*, 2010; Chen *et al.*, 2011; Novototskaya-Vlasova *et al.*, 2013a and 2013b; Koo *et al.*, 2016; Zhang *et al.*, 2018). Results from temperature effect assay indicated that recombinant S1B lipase is a cold-adapted lipase, with the optimum reaction temperature at 30 ºC and stable in low temperature (10-30 ºC) with remaining activity more than 85% of its maximum value after incubation for 1 hour. These results of temperature effect assay were similar to the other cold-adapted lipase.

Metal ions experiment showed that S1B lipase was more stable than several previously reported *Psychrobacter* cold-adapted lipases, such as LipX from *Psychrobacter* sp. C18 (Chen *et al.*, 2011); Lip1Pc and Lip2Pc from *Psychrobacter cryohalolentis* $K5^T$ (Novototskaya-Vlasova *et al.*, 2013a and 2013b); and ArcL13-lip from *Psychrobacter* sp. ArcL13 (Koo *et al.*, 2016). Commonly, $Cu^{2+}$ and $Zn^{2+}$ ions are inhibitor to cold adapted *Psychrobacter* lipase. But, S1B lipase remaining activity pattern was similar with lipase ZC12 where $Cu^{2+}$ and $Zn^{2+}$ ions slightly inhibited the lipolytic reaction (Zang *et al.*, 2018).

Furthermore, S1B lipase exhibited great tolerance to hexane. The lipase became more active with higher hexane concentration. This result was similar with lipase ZC12 from *Psychrobacter* sp. ZY124. Hexane has log*P* value 3.5 and it's the highest value between the organic solvent used in this study. Higher log*P* values of organic solvents indicate the more hydrophobic organic solvents and it is beneficial for non-aqueous enzymology such as lipase (Zhang *et al.*, 2018).

S1B lipase showed highest catalytic rate against substrate *p*-nitrophenyl caprilate (C8) and at low temperature which is may be useful for synthesis of the flavoring compound, caprylic acid esters. Joseph *et al.* (2007) reported the use of cold active lipase from *Pseudomonas fluorescens* P38 in the synthesis of the flavoring compound, butyl caprylate in n-heptane at low temperature. Syamsul kamar *et al.* (2017) also reported the use of lipase from *Candida antartica* to synthesis nonyl caprilate, citrus and rose flavor, with optimum temperature at 30 °C.

## CONCLUSION

In this study, S1B lipase gene was successfully cloned from *Psychrobacter* sp. S1B using shotgun cloning method and expressed in *E. coli* system using pET28a as expression vector. Based on BlastN and BlastP analysis, its similarity with references showed that this lipase gene classified as a novel gene. This gene consists of 1005 bp in length with molecular weight of 46 kDa and showed the best lipolytic activity (2.21 U/mg) against *p*-nitrophenyl caprylate (C8) as substrate. S1B lipase shows optimum at temperature 30 ºC, pH 10, tolerant with all metal ions test, tolerant to hexane up to 50% (v/v) final concentration, also tolerant to 1% (v/v) of DMSO and 1% (v/v) Triton X-100. S1B biochemical properties may provide potential application in biocatalysis of caprylic acid ester which is used as a food flavor.

## ACKNOWLEDGEMENTS

Author would to thank PT. Wilmar Benih Indonesia for research facilities and funded this study.

## REFERENCES

**Arifin, A. R., Kim, S. J., Yim, J. H., Suwanto, A. and Kim, H. K. (2013).** Isolation and biochemical characterization of *Bacillus pumilus* lipases from the Antartic. *Journal of Microbiology and Biotechnology* **23(5), 661-667.**

**Chen, R., Guo, L. and Dang, H. (2011).** Gene cloning, expression and characterization of a cold-adapted lipase from a psychrophilic deep-sea bacterium *Psychrobacter* sp. C18. *World Journal Microbiology Biotechnology* **27(2), 431-441.**

**Fan, Z., Yue, C., Tang, Y. and Zhang, Y. (2009).** Cloning, sequence analysis and expression of bacterial lipase-coding DNA fragments from environment in *Escherichia coli. Molecular biology reports* **36(6), 1515-1519.**

**Guan, Y., Zhu, Q., Huang, D., Zhao, S., Lo, L. J. and Peng, J. (2015).** An Equation to estimate the difference between theoretically predicted and SDS-PAGE displayed molecular weights for an acidic peptide. *Scientific Reports* **5, 13370.**

**Guex, N., Peitsc, M. C. and Schwede, T. (2009).** Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis* **30, 162-173.**

**Joseph, B., Ramteke, P. W. and Thomas, G. (2007).** Cold active microbial lipases: some hot issues and recent developments. *Biotechnology Advances* **26(5), 457-470.**

**Koo, B. H., Moon, B. H., Shin, J. S. and Yim, J. H. (2016).** A novel cold-active lipase from *Psychrobacter* sp. ArcL13: Gene identification, expression in *E. coli*, refolding, and characterization. *The Korean Journal of Microbiology* **52(2), 192-201.**

**Li, X., Sui, X., Zhang, Y., Sun, Y., Zhai, Y. and Wang, Q. (2010).** An improved calcium chloride method preparation and transformation of competent cells. *African Journal of Biotechnology* **9(50), 8549-8554.**

**Lin, X. Z., Cui, S. S., Xu, G. Y., Wang, S. A., Du, N. and Shen, J. H. (2010).** Cloning and heterologous expression of two cold-active lipases from the Antarctic bacterium *Psychrobacter* sp. *Polar Research* **29(3), 421-429.**

**Maiangwa, J., Ali, M. S. M., Salleh, A. B., Rahman, R. N. Z. R. A., Shariff, F. M. and Leow, T. C. (2014).** Adaptational properties and applications of cold-active lipases from psychrophilic bacteria. *Extremophiles* **19(2), 235-247.**

**Natalia, A., Kristiani, L. and Kim, H. K. (2014).** Characterization of *Proteus vulgaris* K80 lipase immobilized on amine-terminated magnetic

microparticles. *Journal of Microbiology and Biotechnology* **24(10), 1382-1388.**

**Novototskaya-Vlasova, K., Petrovskaya, L., Yakimov, S. and Gilichinsky, D. (2012).** Cloning, purification, and characterization of a cold-adapted esterase produced by *Psychrobacter cryohalolentis* $K5^T$ from Siberian cryopeg. *FEMS Microbiology Ecology* **82, 367-375.**

**Novototskaya-Vlasova, K., Petrovskaya, L., Kryukova, E., Rivkina, E., Dolgikh, D. and Kirpichnikov, M. (2013a).** Expression and chaperone-assisted refolding of a new cold-active lipase from *Psychrobacter cryohalolentis* K5 T. *Protein Expression and Purification* **91(1), 96-103.**

**Novototskaya-Vlasova, K. A., Petrovskaya, L. E., Rivkina, E. M., Dolgikh, D. A., Kirpichnikov, M. P. (2013b).** Characterization of a cold-active lipase from *Psychrobacter cryohalolentis* $K5^T$ and its deletion mutants. *Biochemistry* **78, 385-394.**

**Park, I. H., Kim, S. H., Lee, Y. S., Lee, S. C., Zhou, Y., Kim, C. M., Ahn, S. C. and Choi, Y. L. (2009).** Gene cloning, purification, and characterization of a cold-adapted lipase produced by *Acinetobacter baumannii* BD5. *Journal of Microbiology and Biotechnology* **19(2), 128-135.**

**Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G. and Deber, C. M. (2009).** Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *PNAS* **106(6), 1760-1765.**

**Syamsul Kamar, M. W., Salina, M. R., Siti Salhah, O., Hanina, M. N. and Mohd Basyaruddin, A. R. (2017).** Optimization of lipase catalyzed synthesis of nonyl caprylate using response surface methodology (RSM). *Journal of Biotechnology and Biomaterial* **1(3), 106-110.**

**Xie, W., Khosasih, V., Suwanto, A. and Kim, H. K. (2012).** Characterization of Lipases from *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from human facial sebaceous skin. *Journal of Microbiology and Biotechnology* **22(1), 88-95.**

**Zhang, J., Lin, S. and Zeng, R. (2007).** Cloning, expression, and characterization of a cold-adapted lipase gene from an Antarctic deep-sea psychrotrophic bacterium, *Psychrobacter* sp. 7195. *Journal of Microbiology and Biotechnology* **17(4), 604-610.**

**Zhang, Y., Ji, F., Wang, J., Pu, Z., Jiang, B. and Bao, Y. (2018).** Purification and characterization of a novel organic solvent-tolerant and cold-adapted lipase from *Psychrobacter* sp. ZY124. *Extremophiles* **22(2), 287-300.**



 ISSN (print): 1823-8262, ISSN (online): 2231-7538