EFFECT OF CALCIUM IONS TO THE ACTIVITY AND STABILITY OF EXTRACELLULAR LIPASE PRODUCED BY MODERATE HALOPHILIC BACTERIA *Halomonas meridiana* BK-AB4

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ABSTRACT

This study reported the isolation and biochemical studies of extracellular lipase produced by a moderate halophilic bacterium, *Halomonas meridiana* BK-AB4. The estimated molecular weight of the lipase BK-AB4 is about 50 kDa. The study of metal ions effect to the enzyme activity revealed the strong dependency towards the presence of Ca^{2+} ion. Further studies showed that the optimum pH of the enzyme was similar in the presence and the absence of Ca^{2+} ions. However, the presence of Ca^{2+} ions could improve the enzyme thermal stability as indicated by the shift of its optimum temperature from 45°C - 50°C. The addition of Ca^{2+} ions was also enhancing residual activity towards various inhibitors, such as SDS and PMSF up to 55% and 68%, respectively. Another interesting effect of the addition of Ca^{2+} ions were noted when various organic solvents were added to the enzyme solution in the ratio of 1:1. The activity of the enzyme increased up to 122% and 126% relative to the control when methanol and n-butanol were added, respectively. This study thus showed that the addition of Ca^{2+} ion not only improved the catalytic activity of lipase BK-AB4 but also made the enzyme more tolerable against various perturbations.

Key words: Extracellular lipase, halophilic bacteria, *Halomonas meridiana* BK-AB4, metal ions activator

INTRODUCTION

Lipase (EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis reaction of ester bonds in triacylgliseride to produce fatty acids and glycerol and is able to catalyze the reverse reaction, which is synthesis of esters of free fatty acids and glycerol or another substrate (Pera *et al*., 2006). Lipase with esterase activity has been intensively used in various industries, such as the industry for biodiesel production. Biodiesel is a product of the transesterification reaction between triacylgliseride and alcohol. Ester synthesis and transesterification reaction catalyzed by lipase occurred in an organic environment containing a small amount of water (Pera *et al*., 2006). Therefore, searching the source of lipase that has high tolerance in wide range of organic solvent polarity is still intensively carried out.

One approach to obtain organic solvent tolerant lipase is to isolate it from extremophile micro-organisms that live in habitats with low water activity that is similar to the organic solvents-rich environment. Microorganisms that are able to adapt to the habitat with high salinity is known as halophilic microorganisms. The results of several studies showed that some halophilic microorganisms have potential to produce lipase that stable in high temperature, high pH, and organic solvents (Guzman *et al*., 2008).

Indonesia is a vast archipelago country that has many types of halophilic habitats including marine and terrestrial ones. One of the unique terrestrial halophilic habitats in Indonesia is a mud crater situated in Purwodadi region in the Central Java. This crater is periodically spitting brine to the surface despite its location is far from the sea. In the previous studies, we have isolated and identified six bacterial isolates based on their potential to produce extracellular lipase (Parwata *et al*., 2014; Asy'ari *et al*., 2014). The six bacterial isolates are *Pseudomonas stutzeri* BK-AB12, *Pseudomonas alcalipila* BK-AG13, *Chromohalobacter japonicus* BK-AB18, *Halomonas eurihalina* BK-AB15,

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Halomonas elongata BK-AB8, and *Halomonas meridiana* BK-AB4. Lipases from hree of these six halophilic bacteria have been isolated and characterized and among them are lipases from *Pseudomonas stutzeri* BK-AB12 (Parwata *et al.*, 2014), *Chromohalobacter japonicus* BK-AB18 (Hertadi & Widhyastuti, 2015), and *Halomonas eurihalina* BK-AB15 (Pangesti, 2015) showing dependency towards metal ions and better stability in various organic solvents.

Parwata *et al.* (2014) has studied that lipase isolated from *Pseudomonas stutzeri* BK-AB12 is one of the potential organic solvent stable lipase. *Pseudomonas stutzeri* BK-AB12 is a lower halophilic bacterium that can live in the environment with the salt concentration in the range of 0.1%–7.5%. Lipase from *Pseudomonas stutzeri* BK-AB12 has activity within temperatures range of 25-70°C, pH range of 6.0-10.0, and showing its dependency towards Ca^{2+} ion which can increase the relative lipase activity of up to 27%. Ca^{2+} ion binding was also able to increase the enzyme activity and stability in various organic solvents such as n-hexane, n-propanol, and isoamyl alcohol.

We also have studied lipase isolated from the moderate halophilic bacteria, *Chromohalobacter japonicus* BK-AB18. This lipase has a working temperature in the range of 25-85°C, pH range of 4.0-11.0, and also showed the dependency on the presence of Ca^{2+} ion as its activity increased up to 74%. In the presence of this activator the relative activity of lipase in organic solvents, such as n-hexane, isoamyl alcohol, and chloroform, increased (Hertadi & Widhyastuti, 2015). Lipase from the other moderate halophilic bacteria, *Halomonas eurihalina* BK-AB15, was also studied. This lipase has activity at temperature range of 25-80°C, pH range of 5.0-10.5 and the enzyme activity is also enhanced by the presence of Ca^{2+} ion. This activator also improved the relative activity in various organic solvents, such as n-hexane, n-butanol, and chloroform and tolerant of SDS (Pangesti, 2015).

Description biochemical properties of the three lipases above shows that each lipase have different characteristics even though they were endogenous from the same habitat and are also affected by the same ion as the activator. Therefore, it is still necessary to perform study on extracellular lipase produced by different halophilic bacteria in order to determine variations in biochemical properties of their lipase. Collection of many lipase variants will become a useful database to accommodate the industrial needs. In the present study, we used another halophilic bacteria *Halomonas meridiana* BK-AB4 as a source of lipase. This bacterium belongs to the class of moderate halophilic bacteria or medium category with salt tolerance range from 0.5 to 22.5%. In this study we showed that this

bacterium produces lipase with different biochemical and biophysical properties such as those described above.

MATERIALS AND METHODS

Materials
All inorganic salts, such as NaCl, $CaCl_2$, $MgCl_2$, KCl, and buffer components, such as phosphate and Tris-HCl were purchased from Merck. Organic solvents, such as methanol, ethanol, propanol, n-hexane were also purchased from Merck. Medium components, such as yeast extract, peptone and lipase substrate pNPD (p-nitrophenyl decanoate) were purchased from Sigma.

Methods
Bacterial cultivation
Halomonas meridiana BK-AB4 was cultivated in Luria Bertani (LB) medium containing 0.1% tryptone, 0.05% yeast extract, and 10% NaCl. The bacterial culture was incubated at 37°C with 150rpm agitation rate for overnight.

Growth and lipase activity profile of *Halomonas meridiana* BK-AB4
As much as 1%(v/v) of the overnight culture *Halomonas meridiana* BK-AB4 was transferred into a fresh medium containing 0.5% peptone, 0.5% yeast extract, 0.05% $CaCl_2$, and 5% NaCl, which is later called as the production medium. The culture medium was incubated at 37°C with the agitation rate of 150 rpm for 24 hours. Bacterial growth was monitored every hour by measuring the optical density at 600 nm with UV/Vis spectrophotometer. The crude enzyme was also monitored every hour by measuring its activity using pNPD as the substrate. Procedure to measure the enzyme activity is described below.

Production and partial purification of lipase
A single colony of the selected bacteria was grown in a 10 mL starter medium containing 0.5% peptone, 0.5% yeast extract, 0.05% $CaCl_2$, and 5% NaCl. The medium was then incubated at 37°C with the agitation rate at 150 rpm for 24 hours. A total of 1%(v/v) of the starter culture was subsequently transferred into the production medium and incubated at 37°C in a rotary incubator with the agitation rate at 150 rpm for 14 hours. The crude extract of lipase was then separated from bacterial cell using cold centrifugation at 10^4 rpm for 30 minutes.

Purification of lipase was carried out as described by Scopes (1994). The crude extract of lipase was partially purified with ammonium sulfate precipitation method. Three sequential ammonium

sulfate concentration ranges were used; 0-30%, 30-60%, and 60-70%. Adding the ammonium sulfate solution gently to the crude extract, while stirring until the saturation reached about 30%, 60% and 70%, carried out the precipitation. The mixture was allowed to stand for 30-60 minutes and then it was centrifuged at 10^4 rpm for 30 minutes at 4°C. The supernatant was separated from the precipitated proteins for the subsequent fractionation. The precipitated proteins in each fraction were dissolved by 50 mM buffer phosphate pH 8.0 and then they were dialyzed using 20 mM with the same buffer. The lipase activity in each fraction was measured as described below.

Measurement of specific activity for extracellular lipase BK-AB4

Lipase activity was measured following the procedure described by Lee *et al.* (1999). The substrate emulsion was prepared by mixing 10 mM pNPD (p-nitrophenyl decanoate) and the mixture of buffer and ethanol in the ratio of 1:95:4 (v/v/v). A total 50 μL of the enzyme solution was added to 550 ìL of the substrate emulsion, and then immediately incubated at a given temperature for a certain time (minutes). Activity was determined by measuring the absorbance of pNP (p-nitrophenol) at 405 nm. Lipase activity was expressed in units/mg, which is defined as the ìmole product (p-nitrophenol) produced by the catalytic activity of lipase per minute per mg of the enzyme.

Protein concentration was determined using Bradford method (1976). Enzyme solution was reacted with Bradford reagent in ratio of 1:1 (v/v). After that, the mixture was incubated for 5 minutes at room temperature and the absorbance was measured at 595 nm (Bradford, 1976).

SDS PAGE, zymography, and determination molecular weight of lipase

SDS PAGE, staining and distaining methods were carried out following the standard protocol described by Sambrook and Russel (2001). The gel was analyzed with the zymography method by incubating it in 50 mM of a buffer solution containing 3 mM α-naphthyl acetate and 1 mM fast blue. The positive result was indicated by the appearance of brown band on the gel. For the determination of molecular weight of lipase, proteins left the SDS PAGE gel renatured using 100 mM phosphate buffer pH 8.0 containing 0,5% triton X-100 for 4 hours at 4°C. The gel was incubated in 50 mM buffer solution containing 3 mM fast blue dye and 1 mM α-naphthyl acetate at the optimum temperature for 8 hours (Soliman *et al.*, 2007).

Effect of metal ions and inhibitor on lipase activity

Effect of metal ions and inhibitors on the lipase activity was studied by incubating the enzyme in 50 mM phosphate buffer pH 8.0 containing 5 mM metal ions and inhibitor: Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, K^+, Co^{2+}, Fe^{2+}, Fe^{3+}, EDTA, SDS and PMSF at 45°C for one hour (Uttatree *et al.*, 2010). Lipase activity was assayed following the same procedure as described above. As a control, the metal ion solution was replaced with phosphate buffer.

Effect of pH and temperature on lipase activity

Effect of pH on lipase activity was studied at 45°C using 50 mM buffer with a pH range from 5.0 to 10.0. The buffer solution used in this study was acetate buffer (pH 5.0), phosphate buffer (pH 6.0-8.0), tris-HCl buffer (pH 8.5-9.0), glycine-NaOH buffer (pH 9.5-10.0). Effect of temperature on lipase activity was determined by measuring lipase activity at various temperatures in the range from 25-70°C. Lipase activity was assayed following the same procedure as described above.

Effect of organic solvents on lipase activity

Effect of organic solvents on lipase activity was evaluated by incubating enzyme and organic solvents in the ratio 1:1 at 45°C for 30 minutes. Lipase activity was assayed following the same procedure as described above. Organic solvents used were methanol, ethanol, 2-propanol, n-butanol, acetonitrile, acetone, chloroform and n-hexane. As a control, reagents were replaced with phosphate buffer.

Effect of Ca^{2+} on lipase activity

Effect of Ca^{2+} on lipase activity was studied by incubating the enzyme in 50 mM phosphate buffer pH 8.0 containing 5 mM Ca^{2+} ion at 45°C for one hour in various conditions, such as pH, temperature optimum, organic solvents, inhibitors, and thermal stability. Lipase activity was assayed following the same procedure as described above. As a control, lipase activity was assayed without the addition of Ca^{2+} ion.

RESULTS AND DISCUSSION

The growth profile and lipase activity of *Halomonas meridiana* BK-AB4

The growth curve of lipase was prepared by measuring optical density of the bacterial cell every hour at 600 nm (OD_{600}) and lipase activity was measured every hour of the cultivation period in the production medium up to 21 hours at 37°C with the

agitation rate at 150 rpm. The growth profile showed that the initial lag phase was observed for about 2 hours, before it enters seven-hours of log phase period, where the OD600 sharply increased from 0.1 to 1.7. After the log phase, the bacterial cell enters the stationary phase for about 20 hours (Figure 1). Some studies showed that the bacteria maximally produced the extracellular lipase in the stationary phase (Li & Yu, 2012). In our study, lipase was also maximally produced in the stationary phase, specifically at 14th hours of the cultivation period.

Estimating molecular weight and purification of lipase from isolated bacteria

The crude extract of the enzyme was purified with ammonium sulfate fractionations. The activity of lipase was quantified as a specific activity, which is a unit activity per mg of a total protein, and the best specific activity was found in the fraction of 30-60% (Table 1). We then used lipase in fraction 30-60% ammonium sulfate for further characterization.

In order to identify molecular weight of the partially purified lipase, we used SDS-PAGE and zymography methods (Figure 2). The results showed that the estimated molecular weight of lipase was about 50 kDa. The electrophoregram (Figure 2B) showed one band in all fractions indicating that there was only one type of active lipase in each fraction.

Effect of metal ions on lipase activity

Some enzyme requires metal ion as cofactor to improve its catalytic capacity. Adding various metal ions to the solution of lipase BK-AB4 gave different effects to the activity of the enzyme. The addition of Ni^{2+}, Zn^{2+}, Co^{2+}, dan Fe^{3+} ions reduced the enzyme activity significantly, while adding K^+, Mg^{2+} dan Fe^{2+} ions gave less effect to the activity (Figure 3). When Ba^{2+} and Ca^{2+} ions were added, however, the enzyme activity increased by about 116% and 124% relative to the control. As described above, metal ions exhibited different levels of effect on the lipase BK-AB4 activity.
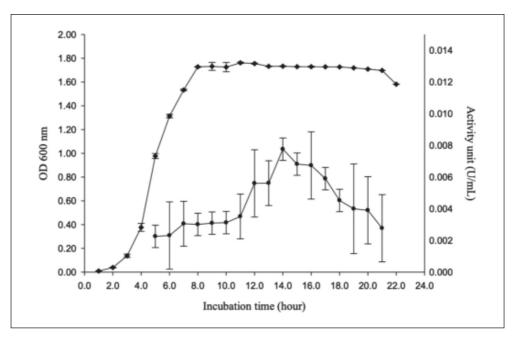

Fig 1. The growth and activity profiles of *Halomonas meridiana* BK-AB4 and its extracellular lipase.

Table 1. Ammonium sulfate fractionations of lipase produced by *Halomonas meridiana* BK-AB4

Ammonium Sulfate Concentration Range	Total Protein (mg/mL)	Activity Unit (U/mL)	Specific Activity (U/mg)
Crude Extract	0.040 ± 0.003	0.0015 ± 0.006	0.038 ± 0,006
0 – 30%	0.142 ± 0.022	0.0106 ± 0.024	0,075 ± 0.024
30 – 60%	0.123 ± 0.008	0.0389 ± 0.003	0.315 ± 0.003
60 – 70%	0.131 ± 0.005	0.0400 ± 0.003	0.304 ± 0.003

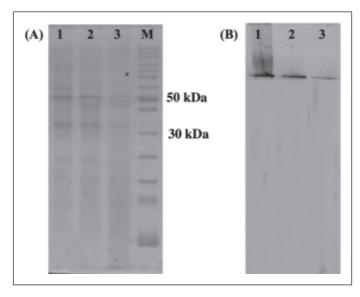1 Unit defined as 1 µmol p-nitrophenyl hidrolized per minutes.

Fig 2. Electrophoregram of SDS-PAGE (A) and zymography (B) lipase. Lane 1, 2, 3, and M for 0-30% fraction, 30-60% fraction, 60-70% fraction, and Marker.
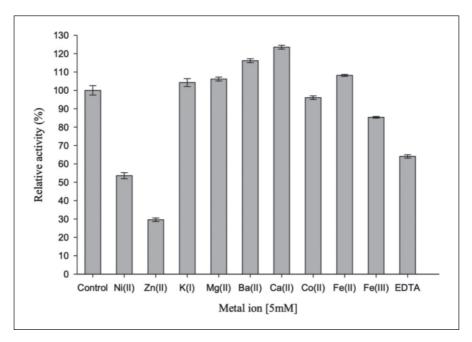

Fig 3. The activity of lipase in the presence various metal ion.

In order to evaluate whether the lipase is true metaloenzyme, we added EDTA (ethyldiamine-tetraacetic acid) as chelating agent to the enzyme solution. The result showed that the activity of lipase decreases by about 36%, thus it can be predicted that the activation of lipase by some ions apparently was not occurred via a complex formation with residues inside the active site of the enzyme (Parwata *et al*., 2014). Variation of lipase activity upon the addition of various metal ions revealed that different ion induced different level of conformational change to the enzyme (Hertadi & Widhyastuti, 2015). Because Ca^{2+} ion gave the highest improvement to the enzyme activity, we further studied the effect of the presence and the absence of Ca^{2+} ion to biochemical and biophysical property of the enzyme.

Effect of Ca^{2+} ion to the activity of lipase BK-AB4 in various pH and temperature

Effect of pH on lipase activity was studied in pH range of 5.0-10.0 (Figure 4). By visual
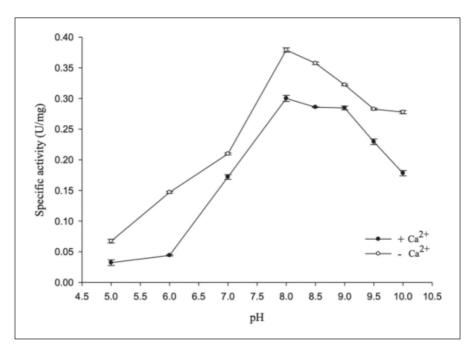
Fig 4. Profil of pH dependence to the activity of lipase BK-AB4 at the condition presence and absence addition Ca^{2+} ion.
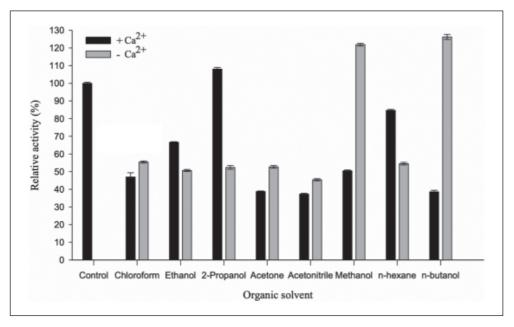

Fig 5. Profile of temperature dependence to the activity of lipase BK-AB4 at the condition presence and absence addition Ca^{2+} ion.

inspection, the activity of lipase BK-AB4 in the presence of Ca^{2+} was higher within pH 5.0 to 10.0 but the optimum pH was relatively similar, in both conditions it was observed around pH 8. Most of lipase has the optimum activity around pH 7.0 to 10.0 (Parwata et al., 2014; Uttatree et al., 2010; Kanjanavas et al., 2010; Ozcan et al., 2009). The effect of pH on the catalytic activity of lipase mainly affects the electrostatic potential of the surface and the catalytic site of enzymes. In the

optimum pH, the electrostatic potential of protein surface and its catalytic site is in optimum state to support binding of its substrate, stabilizing the transition state, as well as increasing the releasing rate of the product (Parwata et al., 2014).

Effect of temperature on lipase activity was studied in the temperature range of 25.0–75.0°C (Figure 5). Different profiles on the temperature dependency were observed at the condition with and without the addition of Ca^{2+} ion. Without the

addition of Ca^{2+} ion, the highest activity occurred at 45°C but after the addition of Ca^{2+} ion the highest activity was at 50°C. This analysis thus suggested that the effect of Ca^{2+} ion has improved the thermal stability of the enzyme.

Effect of Ca^{2+} ion to the activity of lipase BK-AB4 in various organic solvents

Stability of lipase in organic solvents was studied by measuring the enzyme activity in a 1:1 mixture of aqueous and organic solvent. The component of organic solvent varied from polar to non-polar ones. The result showed the activity of lipase has different dependency against the solvent polarity in the condition with and without the addition of Ca^{2+} ion (Figure 6).

Without the addition of Ca^{2+} ion, lipase BK-AB4 activity reduced significantly when chloroform, ethanol, acetone, acetonitrile, methanol, and n-butanol, except n-propanol were used as the organic solvent component. Polar organic solvents generally impose negative effect on lipase activity because they can attract water layer surrounding an enzyme, thereby lowering its catalytic function (Parwata *et al*., 2014; Wehtje & Adlercreitz, 1997), while nonpolar ones typically disrupt the hydrophobic interactions in the protein's core. The addition of Ca^{2+} ion showed significant enhancement in the relative enzyme's activity when methanol and n-butanol were used as organic solvent component up to 122% and 126% but in the rest of the solvents, its activity decreased.

The enhanced relative activity of lipase BK-AB4 in the presence of methanol, shows that this enzyme has potential to be applied as biocatalyst for transesterification reaction in biodiesel production.

Effect of Ca^{2+} ion to the activity of lipase BK-AB4 in various inhibitors

Effect of organic inhibitors such as SDS and PMSF on lipase activity was also studied in our study. PMSF (phenylmethylsulphonyl fluoride) was employed to identify the type of hydrolase group of lipase BK-AB4. The result showed that the addition of PMSF reduced the relative activity of lipase (Figure 7) in either with or without the addition of Ca^{2+} ion. PMSF inhibits the activity by interacting with a serine residue in the active site of the enzyme suggesting that lipase BK-AB4 is included in the serine hydrolase group. Similar to PMSF, the addition of SDS (sodium dodecyl sulfate), an anionic surfactant, also reduced the activity of the enzyme either with or without the addition of Ca^{2+} ions. SDS molecules inhibited the activity by perturbing lipid-water interface, which is very vital for the enzyme functions (Parwata *et al*., 2014; Gupta *et al*., 2004). In addition, this surfactant also weakens the hydrophobic core of the protein that might partially denature the protein. However, it was noted that the addition of Ca^{2+} ion was able to maintain its relative activity up to 55% suggesting that Ca^{2+} ion binding contributed to the extra stability of the protein against the surfactant perturbation.
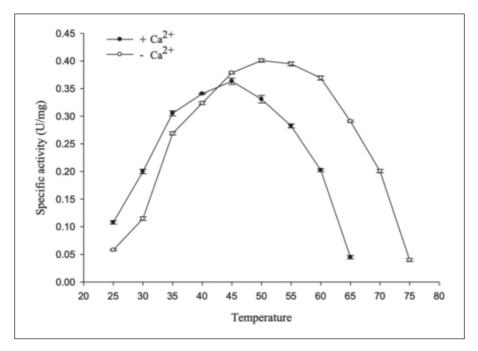

Fig 6. Profile of organic solvent dependence to the activity of lipase BK-AB4 at the condition presence and absence addition Ca^{2+} ion.
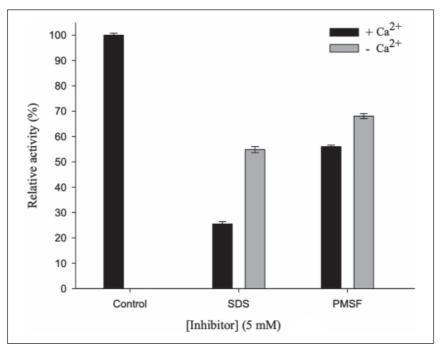
Fig 7. Profile of inhibitor dependence to the activity of lipase BK-AB4 at the condition presence and absence addition Ca^{2+} ion.

REFERENCES

Asy'ari, M., Parwata, I.P., Aditiawati, P., Akhmaloka & Hertadi, R. 2014. Isolation and Identification of Halostable Lipase Producing Bacteria from the Bledug Kuwu Mud Crater Located at purwodadi-Grobogan, Central Java, Indonesia, *Journal of Pure and Applied Microbiology*, **8(5)**: 3387-3396.

Bradford, M.M. 1976. A Rapid and Sensitif Method for The Quantitation of Microgram Quantities of Protein Utilizing The Principles of Protein-Dye Binding, *Journal Analytical Biochemistry*, **72**: 248-254.

Gupta, R., Gupta, N. & Rathi, P. 2004. Bacterial Lipases: an Overview of Production, Purification, and Biochemical Properties, *Applied Microbiology Biotechnology*, **64(6)**: 763-781.

Guzman, M.N., Vargas, V.A., Antezana, H. & Svoboda, M. 2008. Lipolytic Enzyme Production by Halophilic/Halotolerant Microorganisms Isolated From Laguna Verde, Bolivia, *Revista Boliviana De Quimica*, **25**: 14-23.

Hertadi, R. & Widhyastuti, H. 2015. Effect of Ca^{2+} Ion to The Activity and Stability of Lipase Isolated from *Chromohalobacter japonicus* BK-AB18, *Procedia Chemistry*, **16**: 306-313.

Kanjanavas, P., Khuchareontaworn, S., Khawsak, P., Pakpitch Pothivejkul, K., Santiwatanakul, S., Matsui, K., Kajiwara, T. & Chansiri, K. 2010. Purification and Characterization of Organics Detergent Tolerant Lipase from Thermotolerant *Bacillus* sp. RN2, *International Journal of Molecular Sciences*, **11**: 3783-3792.

Lavanya, B. 2014. Metal ion activated lipase from halotolerant *Bacillus* sp. VITL8 displays broader operational range, *International Journal of Biological Macromolecules*, **67**: 380-386.

Lee, D., Koh, Y., Kim, B., Choi, H., Kim, D., Suhartono, M.T. & Pyun, Y. 1999. Isolation and Characterization of Thermophilic Lipase from *Bacillus thermoleovorans* ID-1, *FEMS Microbiology Letters*, **179**: 393-400.

Li, X. & Yu, H. 2012. Characterization of a Novel Extracellular Lipase from a Halophilic Isolate, *Chromohalobacter* sp. LY7-8, *African Journal of Microbiology Research*, **6**(1): 3516-3522.

Ozcan, B., Ozyilmas, G., Cokmus, C. & Caliskan, M. 2009. Characterization of Extracellular Esterase and Lipase Activities From Five Halophilic Archaeal Strains, *Journal of Industrial Microbiology Biotechnology*, **36**: 105-110.

Pangesti, E.R. 2015. Isolasi dan Karakterisasi Lipase Stabil Detergen dari Bakteri Halofilik Kategori Sedang *Halomonas eurihalina* BK-AB15, Thesis Program Magister, Institut Teknologi Bandung, 3-20.

Parwata, I.P., Asy'ari, M. & Hertadi, R. 2014. Organic Solvent-Stable Lipase from Moderate Halophilic Bacteria *Pseudomonas stutzeri* Isolated from the Mud Crater of Bledug Kuwu, Central Java, Indonesia, *Journal of Pure and Applied Microbiology*, **8**: 31-40.

Pera, L.M., Romero, C.M., Baigori, M.D. & Castro, G.R. 2006. Catalytic Properties of Lipase Extracts from Aspergillus niger, *Journal Food Technology Biotechnology*, **44**: 247-252.

Sambrook, J. & Russel, D.W. 2001. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Press, New York.

Scopes, R.K. 1994. *Protein Purification: Principles and Practice* (3rd Edition), Springer Publisher, New York.

Soliman, N., Knoll, M., Abdelfattah, Y., Schid, R. & Lange, S. 2007. Molecular Cloning and Characterization of Thermostable Esterase and Lipase rom Geobacillus thermoleovorans YN Isolated from Dessert Soil in Egypt, *Process Biochemistry*, **42**: 1090-1100.

Uttatree, S., Winayanuwattikun, P. & Charoenpanich, J. 2010. Isolation and Characterization of a Novel Thermophilic-Organic Solvent Stable Lipase From Acinetobacter baylyi, *Applied Biochemistry Biotechnology*, **44**: 1113-1119.

Wehtje, E. & Adlercreitz, P. 1997. Water Activity and Substrat Concentration Effect on Lipase Activity, *Biotechnology Bioengineering*, **55**(5): 798-806.