Organic Solvent-Stable Lipase from Moderate Halophilic Bacteria *Pseudomonas stutzeri* Isolated from the Mud Crater of Bleduk Kuwu, Central Java, Indonesia

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Organic solvent stable lipase has been isolated from moderately halophilic bacteria of *Pseudomonas stutzeri* that originally cultivated from the mud crater of "Bleduk Kuwu" at Purwodadi, Central Java, Indonesia. Preliminary purification has been carried out by ammonium sulfate and acetone fractionations. We noted that acetone fractions gave better purification result, and have successfully extracted 29 kDa lipase. This lipase showed the highest activity at pH 8.5 and 50°C. Further characterization of the enzyme showed that the addition of Zn^{2+} ions improved its activity, but declined when Cu^{2+} and Fe^{2+} ions were added. However, this enzyme was apparently not metalloenzyme because its activity still can be detected up to 56% after it was incubated in 10 mM EDTA for one hour. We also noted that PMSF did not significantly inhibit the enzyme activity implying that it probably does not belong to a group of serine hydrolases. The obtained lipase was stable against some polar organic solvents, such as methanol, ethanol, and acetone, as well as non-polar organic solvent, such as n-hexane. Therefore, lipase from *Pseudomonas stutzeri* has high potential to be developed as biocatalyst for organic synthesis reactions, such as esterification reaction in biodiesel production.

Keywords: Lipase, Organic solvent-stable, Halophilic bacteria.

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of water-insoluble long-chain triglycerides. Under certain conditions, lipase can also catalyze the reverse reaction i.e. the synthesis of triglyceride from glycerol and fatty acids[1]. Nowadays lipase has been applied to catalyze some reactions to synthesis of organic compounds, such as transformation of chemo-, regio-, and stereo-selective, synthesis of esters, and trans-esterification. The latest application has attracted many biochemists to search novel organic solvent-stable lipases by exploring microorganisms living at organic rich environment.

An organic solvent-stable lipase is generally isolated from microorganisms that inhabit in particular extreme environments (extremophiles). One of them is halophilic microorganisms that live in environments with high salinity, such as lakes, lagoons, sea, and salters. In order to survive in environments with high salinity (high osmotic stress), halophilic microorganisms use two strategies of adaptation, namely: (1) salt-in strategy, which is the accumulation of salt ions from the environment in the cytoplasm such that the levels of ions inside and outside of the cell are balanced, (2) organic-osmolyte strategy that accumulate compatible organic compounds with

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low molecular weight to balance the osmotic pressure outside the cell[2]. Based on the adaptation mechanism, it can be predicted that halophilic microorganisms can produce lipase which is stable against organic solvents. The good example is lipase from *Aeromonas sp.* Ebb-1 isolated from sea mud in Angsila, Thailand. This enzyme exhibited relatively high stability against some organic solvents, such as isoamyl alcohol, heptane, and decane[3]. Another example is lipase from *Acinetobacter baylyi* isolated from the same sea mud in Angsila, Thailand, also showed high stability in some organic solvents, such as isoamyl alcohol, benzene, heptane, and hexadecane[4].

Enzymes produced by halophilic microorganism are commonly having amino acid composition on the protein surface differ from those non halophilic ones. The exterior of halophilic proteins are frequently containing more acidic amino acids content, such as aspartic and glutamic acid, than non halophilic and thermophilic ones by the ratio about 28.6:21.0:23.0. The interior of the protein, however, commonly have similar amino acid composition. Acidic amino acids on the surface of halophilic proteins play significant role to stabilize them against high salt concentration by binding the hydrated salt ions[5]. This biochemical nature of proteins in halophilic microorganisms will allow them to have some degree of stability in the medium with low water activity, such as an organic environment.

In the central Java, Indonesia, particularly at Kuwu Vilage of Grobongan Regency, Purwodadi, there is a unique mud crater that continuously producing salty water. We have isolated some halophilic bacteria from various water samples taken from that area. Some of them have been characterized and identified their species based on 16S RNA sequences. In the present study, we reported our result in isolation and characterization of organic solvent-stable lipase from one of the halophilic bacteria isolated from Bleduk Kuwu mud crater in particular from *Pseudomonas stutzeri*.

MATERIAL AND METHODS

Chemicals

All inorganic and organic reagents were purchased from Merck Germany, with pro Analysis grade, except for acetonitrile, p-nitrofenil palmitate, ethanol, $(NH_4)_2SO_4$ were obtained from Sigma-Aldrich (USA). Biochemicals reagents such as Bradford, Taq Polimerase, PCR buffer, dNTP were purchased from Fermentas, α-naphtyl acetate from Wako (USA), while sodium dodecyl sulfate and fast blue dye were provided by Bio Basic (Canada).

Cultivation of Bacteria from Salt Water

Salty water was sampled from the mud crater of "Bleduk Kuwu" located at Kuwu Vilage of Grobongan Regency, Purwodadi, Central Java. Bacteria were grown in liquid Luria Bertani (LB) medium composed of 0.1% tryptone, 0.05% yeast extract, NaCl 5%, and water samples. The medium was incubated at 37 °C with shaking at 150 rpm for 48 hours. Bacteria growing in liquid media were then grown on solid LB medium with the composition of 0.1% tryptone, 0.05% yeast extract, 5% NaCl, and 2% bacto-agar[6].

Test of Lipolytic Activity of Bacteria

A total of 20 well-grown colonies were randomly selected and tested for their lipolytic activity on solid medium composed of 0.5% peptone, 0.5% yeast extract, 0.05% $CaCl_2$, 5% NaCl, 2% bacto-agar, 2.5% olive oil, 1.0% tween 80, and 2.0% Rhodamin B. Medium were incubated at 37 °C for four days. The lipolytic activity of each colony was determined from red to yellow fluorescence around colonies under ultraviolet light[7].

Isolation of Bacteria from Lipolytic Potential Colony

A single colony of bacteria with the best lipolytic potency was grown in 30 mL liquid LB medium composed of 0.1% tryptone, 0.05% yeast extract, 5% NaCl. The medium was incubated at 37 °C with shaking at 150 rpm for 24 hours. Inoculum was than diluted in three stages, i.e. 10^2 times, 10^4 times and 10^6 times, then spread on solid LB medium with the composition of 0.1% tryptone, 0.05% yeast extract, 5% NaCl, and 2% bacto-agar. The grown colonies were then stroke onto solid LB medium with the same composition using quadrant technique[8]. The latest step was repeated four times to obtain single isolated bacteria.

Test of Bacteria Tolerance against NaCl

The obtained single isolated bacteria were then tested its tolerance against various concentrations of NaCl. The testing medium consists of 0.1% tryptone, 0.05% yeast extract, 2% bacto-agar, and various concentration of NaCl. The

medium incubated at 37°C for two to three days, and the growth of bacteria was observed relatively to the control.

Isolation of DNA, Amplification of 16s rRNA Gene and Phylogenetic Tree Construction

Isolation of chromosomal DNA of single isolated bacteria was carried out following the procedure proposed by Zhou *et.al*.[9]. Gene of 16s rRNA was isolated and amplified using polymerase chain reaction (PCR) technique. PCR mixture consisted of 15 mL ddH_2O, 3 mL PCR buffer, 3 mL dNTP (5 mM), 2 mL $MgCl_2$ (2.5 mM), 2 mL of each primer Bact27F (order: AGAGTTTGAT CATGGCTCAG) and Uni1492R (order: GGTTACC TTGTTACGACTT), 1 mL Taq polymerase, and 2 mL DNA template. Polymerization was carried out using the following steps: predenaturation (94°C for four minutes), denaturation (94°C for 30 seconds), annealing (50°C for one minute), and extension/propagation (72°C for two minutes). The cycle from denaturation to extension was repeated as many as 34 times[10]. PCR results were sequenced and analyzed using software DNA Baser version 3.2.5 (Heracle BioSoft). The acquired sequence was then aligned using BLAST (basic local alignment sequence tool). The sequence and related sequences obtained were further analyzed to construct the phylogenetic tree using MEGA (molecular evolutionary genetics analysis) version 5.0.

Production and Partial Purification of Lipase

A single colony of the selected bacteria was grown in 10 mL of starter media with a composition of 0.5% peptone, 0.5% yeast extract, 0.05% $CaCl_2$, and 0.1% NaCl. The medium incubated at 37°C with shaking at 150 rpm for 24 hours[11]. A total of 0.1% starter transferred into medium with the same composition as starter, than incubated at 37°C with shaking at 150 rpm for 24 hours. Crude extract of lipase was then separated from bacteria cell using cold centrifugation at 10000 × *g* for 20 min.

Purification of lipase was carried out by following the procedure proposed by Scopes[12]. Crude extract of lipase was partially purified using ammonium sulfate and acetone in three fractions, i.e. 0-40%, 40-60% and 60-80%. Ammonium sulfate fractionation was carried out by adding the ammonium sulfate solution gently to the crude extract, and then stirred until the saturation reach about 40%. The mixture was allowed to stand for 30-60 minutes, and then centrifuged at 10000 x *g* for 20 min at 4°C. Supernatant was separated from the precipitated proteins for the subsequent fractionation. Precipitated proteins in each fraction were dissolved in 50 mM sodium phosphate buffer (pH 8.0), then dialyzed using 20 mM of the same buffer. Each of enzyme fractions was then analyzed for its lipase activity and protein concentration. Fractionation using acetone was carried out with the same procedure as described above. Prior to be used, acetone was cooled at a temperature of -20 °C for one hour. Residual acetone in the precipitated protein was removed by evaporation in a freeze dryer for about 10 minutes. The precipitated proteins were than dissolved in 50 mM sodium phosphate buffer pH 8.0.

Determination of Molecular Weight of Lipase

The molecular weight of lipase was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and zymogram. Protein solution was mixed with one part of loading dye consisted of 3.1 mL of 1 M tris-Cl buffer (pH 6.8), 5 mL glycerol, 0.5 mL of 1% bromfenol blue, 0.1% SDS, and 1.4 mL H_2O. The mixture was heated in boiling water for 5 minutes to denature the protein, and then loaded to wells of the polyacrylamide gel. Electrophoresis was ran in a chamber soaked with buffer solution composed of 1.4% glycine, 0.3% tris base, and 1% SDS for 80 minute under a constant voltage of 120 volt. The gel was then stained for 12 hours with solution containing 45 mL methanol, 45 mL ddH_2O, 10 mL glacial acetic acid and 0.25 grams of commasie brilliant blue. Distaining was carried out using solution containing of 45 mL methanol, 45 mL ddH_2O, and 10 mL of glacial acetic acid for 2-4 hours[13].

For zymogram analysis, proteins in the gel were renatured using 100 mM phosphate buffer (pH 8.0) containing 0.5% triton X-100 for four hours at 4°C. The gel was then incubated in 50 mM buffer solution containing 3 mM fast blue dye and 1 mM α-naphtyl acetate at the optimum temperature for 8 hours[14].

Determination of Lipase Activity and Protein Concentration

Lipase activity was assayed using spectrophotometric technique as proposed by Lee et al.[15]. Substrate emulsion was prepared by mixing

10 mM pNPP (p-nitrofenil palmitate) and the mixture of buffer and ethanol with a ratio of 1:95:4 (v/v/v). A total of 15 µL enzyme was added to 430 µL the substrate emulsion, and then was 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 is expressed in units/mg which is defined as the mole of product (p-nitrophenol) produced by the catalytic activity of lipase per minute per mg of the enzyme.

Protein's concentration was determined using Bradford method. Enzyme solution was reacted with the Bradford reagent with a ratio of 1:1 (v / v). The mixture was incubated for 5 minutes at room temperature, and the absorbance was measured at 595 nm[16].

Effect of pH and Temperature on Lipase Activity

Effect of pH on lipase activity was examined at 37 °C using 50 mM buffer with a pH range of 7 to 10.5. Lipase activity was assayed following the procedure in section 2.7. Buffer systems include tris-HCl buffer (pH 7.0 to 8.0), glycine-NaOH buffer (pH 8.5 to 10.5). Effect of temperature on lipase activity was determined by measuring the enzyme activity at various temperatures in the range of 12 to 80 °C. The reaction mixture was incubated at a certain temperature in a water bath, and lipase activity was measured following the procedure in section 2.8.

Effect of Metal Ions, Detergent and Inhibitors on Lipase Activity

Effect of metal ions, detergent and inhibitors on lipase activity was examined by incubating the enzyme in 50 mM glycine-NaOH buffer (pH 8.5) containing 10 mM of testing chemicals at 37 °C for 1 hour[4]. Lipase activity was then assayed following the same procedure described above. The testing chemicals used including: Ba^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, SDS, EDTA, and PMSF. As a control, the testing chemicals were replaced with H_2O.

Effect of Organic Solvents on Lipase Activity

Lipase stability against organic solvents was examined by incubating the enzyme in 50% organic solvent. Incubation performed in shaker with a speed of 150 rpm at 37 °C for 30 minutes[17]. Lipase activity was then measured following the same procedure described above. As a control, distilled water was used instead of organic solvents. Lipase stability against organic solvents was expressed in terms of activity relative to the controls. Organic solvents used were methanol, ethanol, n-propanol, n-butanol, acetonitrile, acetone, isoamyl alcohol, chloroform, isopropanol, and n-hexane.

RESULTS AND DISCUSSION

Lipolytic Activity and NaCl Tolerance of Bacteria

Among 20 assayed colonies, one colony showed lipolytic activity, as indicated by the appearance of red fluorescence emitted by the colony under ultraviolet light (Figure 1). The fluorescence was occurred due to the complex formation between cationic Rhodamin B and free fatty acids resulted from the hydrolysis of lipid substrates by lipase[18]. The lipolytic potential colonies were then further sub-cultured to obtain single isolated bacteria.

Level of NaCl tolerance was examined to

Table 1. The results of ammonium sulfate and acetone fractionation of lipase

Purification Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude extract	0.411	1.572	3.8	
Ammonium sulfate fraction				
Fraction 0-40%	0.013	0.046	3.6	3.0
Fraction 40-60%	0.030	0.382	12.8	25.1
Fraction 60-80%	0.026	0.052	2.0	3.4
Acetone fraction				
Fraction 0-40%	0.039	0.023	0.6	1.5
Fraction 40-60%	0.047	0.488	10.5	32.1
Fraction 60-80%	0.035	0.003	0.1	0.2

the potential bacteria exhibited high lypolitic activity. The result showed that the potential bacteria were able to grow in the medium with NaCl concentration from 0 to 7.5% (*w/w*). According to DasSarma and Arora, the bacteria are belonging to moderately halophilic[19].

Phylogenetic Tree of Single Isolated Bacteria

Phylogenetic tree of the single isolated bacteria was constructed based on its 16S rRNA sequences (Figure 2). The result showed that the bacteria are most likely lies on the same taxa as *Pseudomonas stutzeri*. The bootstrapping test of as many as 1000 times showed that the bacteria were 100% still remained at one taxa as *Pseudomonas stutzeri*. The bacteria included in genus *Pseudomonas Sensu stricto, Protobacteria phyla,* class *Gammaproteobacteria*[20].

Table 2. Relative Activity of Lipase in Various Organic Solvents

Organic Solvents (50%)	log $P_{o/w}$	Relative Activity (%)
Control	-	100
Methanol	-0.76	112
Acetonitrile	-0.33	80
Ethanol	-0.24	105
Acetone	-0.23	110
Isopropanol	0.05	83
n-Propanol	0.29	97
n-Butanol	0.8	61
Isoamyl alcohol	1.3	76
Chloroform	2.0	91
n-Hexane	3.5	111

$P_{o/w}$ = solvent partition in nonpolar phase (octanol) and polar phase (water)

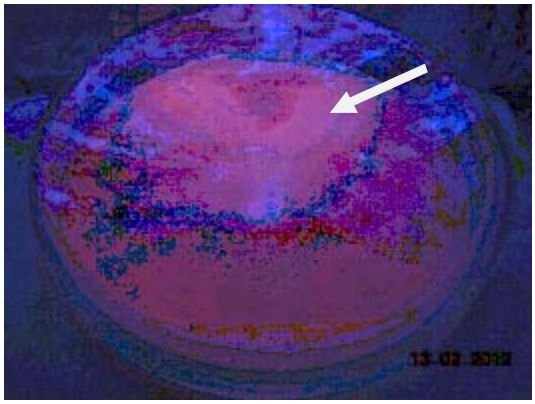

Fig. 1. The Results of lipolytic activity test of bacteria isolated from the mud crater "Bleduk Kuwu ". Red fluorescence (pointed by an arrow) shows positive result of lipolytic activity

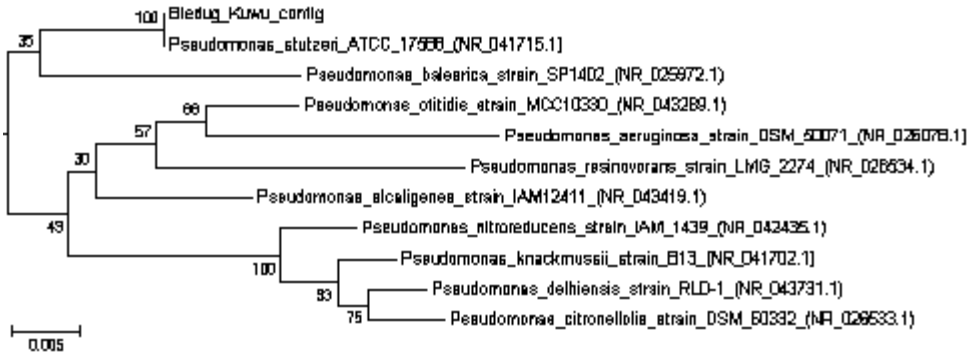

Fig. 2. Phylogenetic tree of the potential bacteria constructed from its 16S RNA sequence. The position of bacteria is shown in the box

Purification of Lipase from Isolated Bacteria

The potential haophilic bacterium strain, which has been identified above as *Pseudomonas stutzeri*, was further cultivated to produce lipase. The crude extract of the enzyme was purified with acetone and ammonium sulfate fractionations methods, and both gave the best results in fraction of 40-60% (Table 1). At this fraction, ammonium sulfate fraction gave a higher lipase specific activity than that of acetone, i.e. 12.8 and 10.5 U/mg, respectively. The yield of lipase from the ammonium sulfate fraction, however, was lower than the acetone fraction, namely 25.1 and 32.1, respectively. Referring to both comparisons, ammonium sulfate fraction provides slightly better level of purity than the acetone fraction.

Molecular Weight of Lipase

In order to estimate the molecular weight (MW) of lipase purified above, we performed SDS-PAGE and zymogram analysis (Figure 3). SDS-PAGE of crude extract showed a prominent band among other bands with approximate MW about 124 kDa (Lane 1, Figure 3A). We noted that the ammonium sulfate fraction showed fewer bands with a 71 kDa band as a prominent one (Lane 2, Figure 3A), whereas the prominent band for the acetone fraction has approximate MW about 64 kDa (Lane 3, Figure 3A). The two prominent bands of acetone and ammonium sulfate fractionations, however, showed no lipase activity as revealed by the absence of bands as characterized by zymogram method (Figure 3B). Zymogram of crude extract showed two bands that indicate lipase activity; those are with MW of 29 and 124 kDa (Lane 1, Fig. 3B). Meanwhile, each acetone and ammonium sulfate fractions exhibited only one active band with a size of 29 kDa (Lane 2&3, Figure 3B). The loss of 124 kDa active band after fractionation was likely caused by the following reason: first, 124 kDa protein is an aggregate form of lipase that apparently hard to dissolve after the fractionation process, second, this large protein is a multi-subunit lipase that may be dissociated along fractionation process, thereby lost its activity.

Characteristic of 29 kDa Lipase (40-60% Acetone Fraction)

The 29 kDa lipase obtained from acetone fraction was further characterized to study the effect of pH, temperature, metal ions, detergent, and inhibitors to its activity. In addition, we also examined the stability of the enzyme in various organic solvents.

Effect of pH on Lipase Activity

Effect of pH on lipase activity was studied from pH 7 to 10.5 (Figure 4). Lipase showed high activity in the range of pH 8 to 9.5 with the maximum activity found at pH 8.5. Most of lipases typically have optimum pH in the range of 7-10[4,21,22]

Effect of pH on the catalytic activity of lipase mainly affects the electrostatic potential on the surface and the catalytic site of enzymes. In the optimum pH, the electrostatic potential of

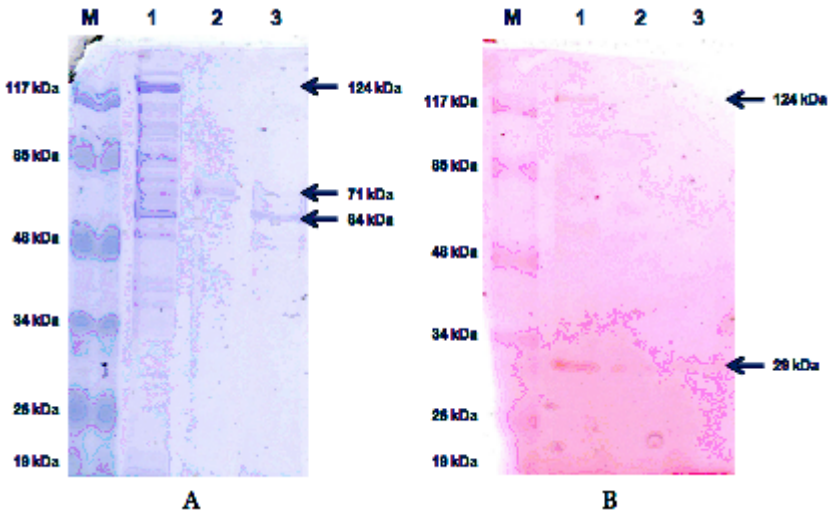

Fig. 3. SDS-PAGE (A) and Zymogram (B) of Lipase. Lane M is for proteins marker, Lane 1, 2, and 3 for crude extract, 40-60% ammonium sulfate fraction, and 40-60% acetone fraction, respectively

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. At the optimum pH, the catalytic surface of lipase tends to have a very negative electrostatic potential to support its catalytic activity[23]. However, if the potential is too negative as a consequence of full deprotonation of protein residues, the catalytic activity will decrease due to spontaneous destabilization of protein.

Effect of Temperature on Lipase Activity

Effect of temperature on lipase activity was studied in temperature range of 12 to 80°C (Figure 5). We observed that the enzyme exhibited the activity in wide temperature range (25-60°C) with the maximum activity was occurred at 50°C. Generally, lipases isolated from microorganisms show optimal activity at temperature ranges of 30-60°C[4,21,22,24,25]. At temperatures lower than 25°C, lipase activity decreased drastically due to the rigid structure of the enzyme making it difficult to bind and catalyze the substrate. In case at temperatures higher than 60°C, lipase activity decreased due to thermal denaturation of enzymes.

Effect of Metal Ions, Detergent and Inhibitors on Lipase Activity

In order to evaluate the dependency on metal ions towards the lipase activity, we examined 8 metal ions at pH and temperature optimum of the enzyme (Figure 6). We noted that lipase activity was significantly enhanced in the presence of Zn^{2+} ions, which was about 30% higher compare to the control. Addition of Ba^{2+} ion also increased the activity but only about 10%. In contrast, the presence of Fe^{2+} and Cu^{2+} were suppressing the activity about 10 to 20% compared to the control. The rest of metal ions, such as Ca^{2+}, Mn^{2+}, Mg^{2+}, and Hg^{2+} apparently showed no effect on lipase activity.

As described above, metal ions exhibited different level of influence on lipase activity. It can be predicted that the activation of lipase by some ions apparently was not occurred via complex formation with residues inside the active site of the enzyme. It was clearly indicated by the ability of lipase to maintain its catalytic activity up to 58% after the enzyme was incubated in a buffer containing 10 mM EDTA for one hour (Figure 6). The way of some metal ions in affecting the enzyme activity has been studied elsewhere. Referring to the work carried out by Sugiura et al.[26], the effect of Zn^{2+}, Fe^{2+}, and Cu^{2+} ions to lipase activity can be explained. The improvement of lipase activity by Zn^{2+} ion is most likely due to the complex formation between the ions and ionized free fatty acids derived from the hydrolysis of pNPP (The substrate). The complex can reduce the solubility of free fatty acids (products) inside the lipid-water interface such that they will readily remove from the active site, thereby opening the way of new substrates entry into the enzyme's active site. In contrast, the decreasing of lipase activity by Fe^{2+} and Cu^{2+} ions were likely caused by competitive binding between the ions and the substrate with residues in the catalytic site. The effect of other ions still unknown but generally it may affect the electrostatic potential on protein surface.

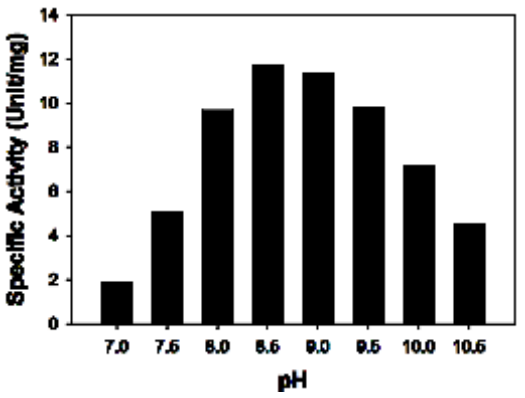

Fig. 4. Specific Activity of Lipase at Various pH (7-10,5)

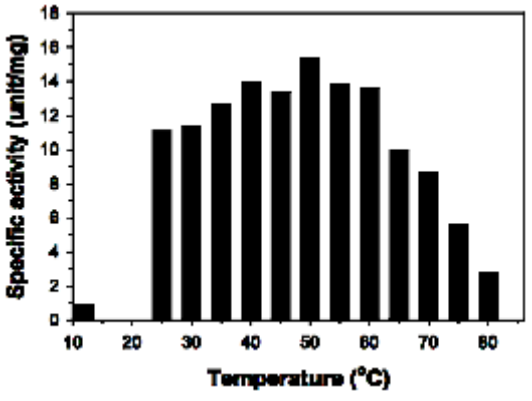

Fig. 5. Specific Activity of Lipase on Various Temperature

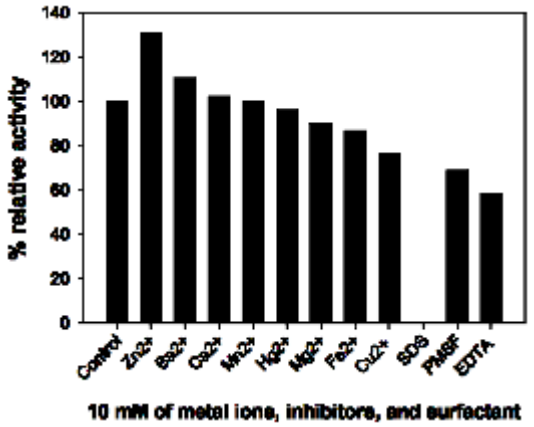

Fig. 6. Effects of metal ions, detergent, and inhibitors on lipase activity. The activity was determined after pre-incubation of the enzyme in 50 mM Glycine-NaOH buffer (pH 8,5) containing 10 mM of studied chemicals for one hour at 37 °C

Interactions between metal ions and charged residues may induce the conformational change that may optimize or deoptimize its catalytic activity[27].

Besides studying the effect of metal ions addition, we also evaluate the effect of organic inhibitors, such as SDS, and PMSF on lipase activity. The effect of these two inhibitors was different (Fig. 6). The most drastic effect was upon the addition of SDS that made the enzyme completely lost its activity, whereas the addition of PMSF reduced the activity partially. Sodium dodecyl sulfate (SDS) is a non-specific reversible inhibitor for most lipases. This surfactant inhibits lipase conformational change or affect lipid-water interface which is very vital for the enzyme functions[28]. While phenylmethilsulfonyl fluoride (PMSF) is a specific inhibitor of the serine hydrolase class of enzymes. Lipase was able to maintain its activity up to 69%. This is probably due to serine residues in the active site of lipase are sterically more protected, thereby lowering the probability to be accessed by PMSF. Nevertheless, not all of lipase inhibited by PMSF, for example the catalytic activity of lipase from *Acinetobacter byalyi* increased by PMSF[4].

Lipase Stability in Various Organic Solvents

Stability of lipase against organic solvents were studied by measuring the enzyme activity in different organic solvents and compare its activity with the one measured in the aqueous buffer (Table 2). Hydrophobicity level of organic solvents used in this study was represented with the value of log $P_{o/w}$. The higher of its value, the higher hydrophobicity level of the solvent. We noted that lipase exhibited relatively high stability either in polar organic solvents (lower value of log $P_{o/w}$), such as methanol, ethanol, acetone and n-propanol, or in non-polar solvents (higher value of log $P_{o/w}$), such as n-hexane and chloroform. However, lipase was less stable in solvents owing moderate level of logs $P_{o/w}$, such as isoamyl alcohol and n-butanol.

Polar organic solvents such as methanol or ethanol generally impose negative effect on lipase activity because they can attract water layer surrounding an enzyme, thereby lowering its catalytic function[29]. Lipase obtained in this study, however, was fairly stable in polar organic solvents such as methanol, ethanol and acetone. Lipase activity even increased slightly after incubation for 30 minutes in those polar solvent (Table 2). This suggests that the enzyme is able to maintain its surface water layer so that the conformation of the protein remains intact for catalytic process. The enzyme ability to maintain its surface water layer is probably due to the protein surface of this enzyme contains many polar and charged residues that strongly interact with water molecules. Lipase obtained in this study, therefore, can be utilized in organic synthesis reactions, such as synthesis of ester and trans-esterification in biodiesel production.

In contrast to the polar solvent, non-polar organic solvents typically do not impose negative effects on the catalytic function of lipase because the solvent is generally not able to attract water molecules on the surface of the protein[30]. The result of our study on non-polar solvent effect on lipase activity exhibited different level of influence. We noted that lipase showed a slightly increase in catalytic activity after incubation for 30 minutes in non-polar solvent such as n-hexane (Table 2). The increase of catalytic activity can be caused by the following reasons: first, the substrates are more soluble in non polar solvents, so that supporting its uptake to the active site as well as products withdrawal from the catalytic site of the enzyme and second, a hydrophobic solvent can stimulate the opening of the lid which covering the lipase active site[31]. The other two solvents i.e. n-butanol

and isoamyl alcohol showed the opposite effect on the enzyme activity (Table 2). The decrease in lipase activity by these two solvents were likely occurred by the following reasons; the organic solvents compete with the substrate to access the active site of the enzyme, breakdown the units which built the enzyme, changing the active conformation of the protein, changing the secondary structure of α-helix, and react with the enzyme[32].

CONCLUSION

Medium halophilic bacteria from Bleduk Kuwu mud crater, *Pseudomonas stutzeri*, produced 29 kDa lipase, which has been characterized as neither metaloenzyme nor serine hydrolase type of enzyme. The optimum activity of this enzyme occurred at pH 8.5 and 50°C, and can be enhanced by the addition of Zn^{2+} ion. Furthermore, the enzyme activity is also enhanced slightly in polar organic solvents, as methanol, ethanol, and acetone, as well as non-polar one like n-hexane. This organic solvent stable lipase, therefore, is highly potential to be developed as biocatalyst for crucial organic reactions, such as esterification in biodiesel production.

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