Short Communication:

Ability of Ectoine to Stabilize Lipase against Elevated Temperatures and Methanol Concentrations

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Abstract: *Ectoine is one of the compatible organic molecules that can protect the protein from heating, freezing, and chemicals contact. This study aims to investigate the ability of ectoine to stabilize lipase on heating and in methanol treatments as an effort to provide a stable biocatalyst for the production of biodiesel. Various ectoine concentrations were added to lipase solutions, then the mixture was heated, and the residual activity of the lipase was determined. Similar steps were also conducted for methanol treatment. The results showed that ectoine maintained and even improved the catalytic activity of lipase after treatment with either heat or methanol. The addition of ectoine to a final concentration of 110 to 150 mM could maintain lipase activity up to 80% when heating to approximately 95 °C. Additionally, more than 20% of lipase activity increased on heating to temperatures below 75 °C in the presence of ectoine at a final concentration of 25 to 120 mM. Meanwhile, after incubation in methanol at a level of around 84% (v/v), the activity of lipase containing 40–90 mM ectoine was maintained. These results demonstrated that ectoine was highly effective in protecting lipase from heat and methanol.*

Keywords: *ectoine; lipase; heat; methanol; protection*

■ INTRODUCTION

Lipase is an enzyme that can catalyze several reactions, including lipid hydrolysis, esterification, interesterification, transesterification, alcoholysis, acidolysis, and aminolysis [1-2]. This enzyme has unique characteristics regarding substrate specificity, stereospecificity, and regioselectivity [3]. As a consequence, lipase is widely used in various industries, such as food, oil and fat, detergent, pulp and paper, leather, textile industries, and other applications, such as organic synthesis, cosmetic, medical, and pharmaceutical, a combination of excellent chemical, and biodiesel production [4].

For industrial applications operating, the catalytic process at high temperatures is more beneficial as the reaction will run faster, increase substrate solubility, reduce substrate viscosity, and avoid contamination by microorganisms [5]. For these reasons, a thermostable lipase is required to catalyze the reaction process at high temperatures. Lipase, which has thermal stability, is generally obtained from microorganisms that live in extreme environments, such as hot springs, soil,

landfills, and industrial waste [6]. Thermophilic microorganisms are one of the producers of thermostable lipase. However, the yield of the enzyme's production from these microbes is generally low [7]. As a consequence, another strategy is needed to meet the increasing industrial demand for stable thermal lipases.

Nowadays, lipases are widely used as a biocatalyst for the alcoholysis reactions of triglycerides in biodiesel production as an alternative to the use of chemical catalysts, which have several disadvantages, including wasting energy, difficulty in by-product separation, pressure in the removal of the catalyst from products, waste treatment, and the interference of free fatty acids and water in catalysis reactions [8]. For this reason, alcohol-stable lipase is the right choice of a biocatalyst for biodiesel production.

To improve lipase stability at high temperatures and in organic solvents, some researchers have immobilized enzymes on hydrophobic materials such as Fe_3O_4 nanoparticles [9], butyl and octadecyl beads [10], nano-sized clay [11], and chitosan beads [12]. However, immobilization has several disadvantages; loss or reduction in the enzyme activity, limitation in the diffusion of the substrate or catalytic product, and additional costs entailed by the immobilization process [13]. Thus, other methods need to be developed to improve lipase stability.

Lipase stability is expected to be improved by protecting it with bioactive molecules. Several researchers have studied the protecting protein from osmotic pressure, heating, freezing, drought, or contact with chemicals by using ectoine [14-16]. Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a small bioactive molecule and a compatible solute as it does not interact directly with the protein surface. Thus, the catalytic activity remains unaffected [17-18]. The previous study indicated that ectoine is able to prevent methanol inhibition of lipase on biodiesel production [19]. However, the lipase used in this study was immobilized on a hydrophobic carrier (acrylic resin) as initial protection. In addition, the biocatalysis process by the lipase was conducted at a constant temperature of 40 °C.

In this study, the ability of ectoine to protect free lipase from *Burkholderia* sp. against high temperature and methanol treatments was investigated. Park et al. (2007) reported that lipase from *Burkholderia* sp. showed the highest activity at pH 8.0 and 60 °C. However, the thermostability test showed that this enzyme could only maintain its activity about 50% after preincubated at 60 °C for 30 min. In addition, the lipase lost more than 70% of its activity after incubated at the same temperature for 1 h, indicating that the half-life of enzyme activity was very short. The enzyme was also reported to have good stability in methanol. It maintained its activity up to 95% after incubation in 50% (v/v) methanol [20]. However, for biodiesel production, lipase should stable in a high concentration of methanol.

Therefore, this study was conducted to investigate whether ectoine could improve thermal and methanol stability of lipase from *Burkholderia* sp. The effect of ectoine concentration and heating temperature as well as methanol concentration on lipase activity was determined and optimized using response surface methodology (RSM), a statistical approach for studying and optimizing the relationship between factors and responses without involving a large number of experimental trials [21-22]. The optimization results were used to predict an efficient and effective ectoine concentration that gave optimum protection and improvement of lipase activity against heating and methanol.

■ EXPERIMENTAL SECTION

Materials

Lipase used in this study was a commercial lipase from *Burkholderia* sp. (Sigma). The activity of the enzyme was 12 U/mg. One U corresponds to the amount of enzyme which liberates 1 μmol oleic acid per min at pH 8.0 and 40 °C using triolein as substrate. Acetonitrile, ethanol, methanol, Na_2HPO_4, NaH_2PO_4 were purchased from Merck (Germany). *p*-nitrophenyl palmitate and *p*-nitrophenol were purchased from Sigma (USA). All chemicals used in this study were in pro analysis grade.

Ectoine was prepared from halophilic bacteria *Halomonas elongata* BK-AG25 which has been isolated

from the mud crater of "Bledug Kuwu" located at Kuwu Village, Kradenan District, Grobogan Regency, Central Java, Indonesia. The bacteria were cultivated on a shaker at 37 °C in MM63 medium containing (per liter): 13.61 g KH_2PO_4, 4.21 g KOH, 1.98 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.0011 g $FeSO_4 \cdot 7H_2O$, 5 g glucose·H_2O, 150 g NaCl (pH 7.0). After 24 h of incubation, the bacterial cell was separated by centrifugation at 6,000 × g for 20 min at 4 °C and lyophilized. Ectoine was then extracted from the dried cell using Bligh and Dyer methods [23] with the mixture of methanol:chloroform:water (10:5:4 v:v:v) by vigorous shaking for 90 min. Equal volumes of chloroform and water were then added and shaken for 30 min. The water phase containing ectoine was separated by centrifugation at 10,000 × g for 30 min and lyophilized. The dried ectoine was resuspended in methanol and separated from concomitant precipitated hydroxyectoine by centrifugation. Ectoine was then recovered by lyophilization and stored at 4 °C. The purity of ectoine produced was about 80% based on HPLC analysis (data not shown).

Experimental Design

Experiments to investigate the stabilization of lipase by ectoine in heat and methanol were conducted with three factors, i.e., the final concentration of ectoine added to the lipase solution, temperature, and levels of methanol. The central composite design was used to obtain a range of five-levels of each parameter to be optimized (Table 1). The responses for the three factors designed were studied by determining the relative activity of the treated lipase against control (untreated lipase). The response data were then fitted to a full quadratic model using Minitab 17 software.

Procedure

Stabilization of lipase in heat and methanol

Lipase stabilization at high temperatures was investigated as follows. Ten microliters of 3.8 μg/mL lipase solution were added to 10 μL ectoine solution with various final concentrations (Table 1) and incubated at 4 °C for 1 h. The lipase solutions were incubated in a water bath at different temperatures (Table 1) for 1 h. After heating, the residual activity of lipase was assayed using *p*-nitrophenyl palmitate as a substrate. The ectoine ability to stabilize lipase was determined as the relative activity of the enzyme to the reference one, which was measured in the absence of ectoine at 37 °C (Control 1) and various temperatures (Control 2).

Lipase stabilization in the methanol was investigated as follows. Ten microliter lipase solution 3.8 μg/mL was added to 10 μL ectoine solution at different final concentrations (Table 1) and incubated at 4 °C for 1 h. The lipase solution was then added to methanol at a range of levels (Table 1) and incubated in a water bath at 37 °C for 1 h. The residual activity of lipase was assayed using *p*-nitrophenyl palmitate as a substrate. The ectoine ability to stabilize lipase was determined as the relative activity of the enzyme to the reference one, which was measured in the absence of ectoine and methanol (Control 1), and in the lack of ectoine but at various level of methanol (Control 2).

Lipase activity assay

Lipase activity was assayed using a spectrophotometric technique as proposed by Lee [24]. Substrate emulsion was prepared by mixing 10 mM *p*-nitrophenyl palmitate, phosphate buffer, and ethanol with a ratio of 1:95:4 v:v:v. A total of 20 μL enzyme was

Table 1. The experimental design for stabilization of lipase activity by ectoine under elevated temperatures and methanol concentrations

Variable	Parameter	Level				
Equation 1	Stabilization in heat	-1.414	-1	0	+1	1.414
X_1	Ectoine concentration (mM)	25	43	88	132	150
X_2	Temperature (°C)	60	65	78	90	95
Equation 2	Stabilization in methanol	-1.414	-1	0	+1	1.414
X_1	Ectoine concentration (mM)	25	40	75	110	125
X_2	Methanol (% v/v)	40	47	65	83	90

added to 480 µL of the substrate emulsion. The mixture was incubated at 60 °C for 15 min. After incubation, the reaction was stopped by the addition of 500 µL ethanol. The activity was determined by measuring the absorbance of *p*-nitrophenol at 405 nm. Lipase activity is expressed in units/mg, which is defined as the mole of the product (*p*-nitrophenol) produced by the catalytic activity of lipase per min per mg of the enzyme.

■ RESULTS AND DISCUSSION

Stabilization of Lipase in Heat

Ectoine produced by *Halomonas elongata* BK-AG25 was tested for its ability as a lipase stabilizer when heated at the temperature range of 60–95 °C. After treatment, the relative activity of lipase to Control 1 (without ectoine and incubated at 37 °C) and to Control 2 (without ectoine and incubated at the same temperature variation as the lipase tested) were determined and shown in Supplementary Table 1 and 2. Analysis of the data produced a regression model for the relative activity of lipase to Control 1 with a determination coefficient value (R^2) of 93.67%, revealing a relatively high correlation between experimental and predicted values. The predictive results of the relative activity of lipase at various heating temperatures in the range of experiments using regression Eq. (1) are shown in Supplementary Table 1.

$$\text{Relative activity}(\%) = -365 - 1.245X_1 + 15.78X_2 - 0.0045X_1 \times X_1 - 0.1297X_2 \times X_2 - 0.0273X_1 \times X_2 \quad (1)$$

The results of the t-test (significance level of 5%) for each regression coefficient of the regression model of the relative activity of lipase against Control 1 are shown in Table 2. The test results showed a very significant linear effect of heating on the relative activity of lipase (probability < 0.05). At the same time, the final concentration of ectoine did not give a significant linear effect on the relative activity of lipase. The heating temperature is harming the relative activity of lipase (linear regression coefficient of -24.04), meaning that an increase in temperature causes a decrease in the relative activity of lipase (Fig. 1(a)). These results indicate that the higher the temperature, the lower the ability of ectoine to stabilize lipase.

Table 2. Estimated coded coefficient and p-value for the regression model of the relative activity of lipase after heating

Factor	Relative activity of lipase (%)	
	Coefficient	p-Value
Linear		
X_1	3.74	0.284
X_2	-24.04	0.000*
Square		
X_1^2	-8.78	0.039*
X_2^2	-19.87	0.001*
Interaction		
$X_1 X_2$	14.93	0.014*

*Significant (p-value < 0.05)

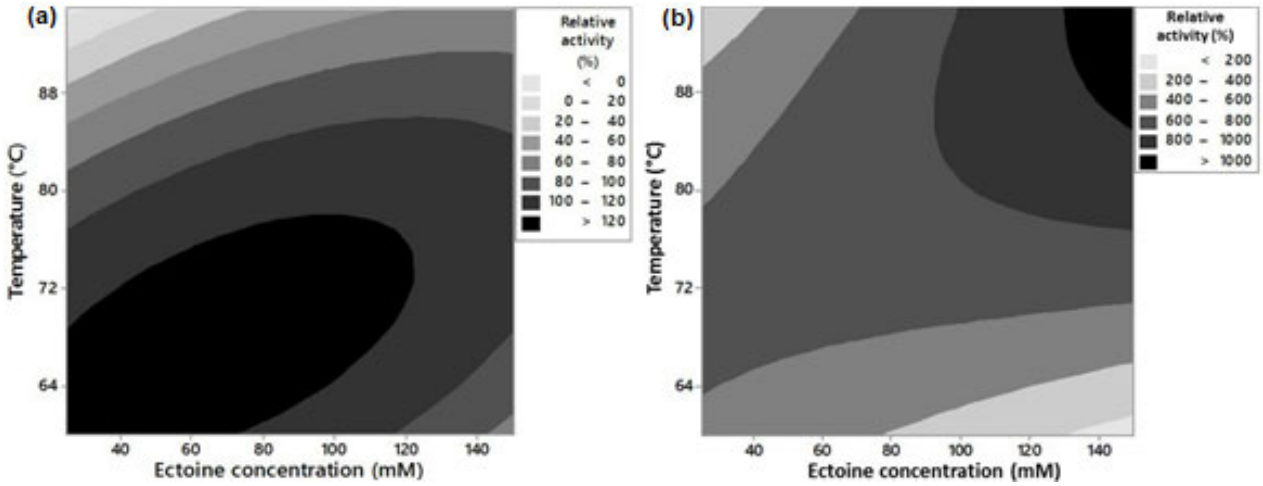

Fig 1. Effect of ectoine concentration and heating on the relative activity of lipase to Control 1 (a) and Control 2 (b)

The regression model of the relative activity of lipase also showed a significant and robust interaction between the ectoine concentration in the enzyme solution and temperature (interaction coefficient of 14.93 with a probability value of 0.014). An increase in temperature must be accompanied by an increase in the ectoine concentration added to maintain lipase activity (Fig. 1(a)). Heating lipase containing a low level of ectoine (< 80 mM) to high temperatures (> 88 °C) causes a decrease in the relative activity of the enzyme (< 20%). Likewise, at low temperatures (< 64 °C) and high concentrations of ectoine (> 140 mM), the relative activity of lipase decreases to below 80%.

Ectoine added to the lipase solution was able to maintain and even increase the enzyme's catalytic activity at high temperatures. At temperatures of around 95 °C, the addition of ectoine at the final concentration of 110–150 mM could maintain lipase activity up to 80%. The addition of ectoine at the final level of 100–150 mM was able to keep lipase activity up to 100% after being heated for 1 h at 88 °C. Heating lipase containing 60-150 mM ectoine to temperatures below 80 °C could even increase the lipase activity up to 20% (Fig. 1(a)). Also, an increase in lipase activity of more than 20% at temperatures below 75 °C was observed when 25–120 mM of ectoine was used. Other researchers have also reported ectoine's ability to stabilize other enzymes at higher temperatures, such as lactate dehydrogenase and phosphofructokinase [25-26] and phytase [27].

Lipase without ectoine showed a dramatic drop in catalytic activity when heated (Control 2) (Table 2). Consequently, the relative activity of lipase with ectoine added was significantly increased (Fig. 1(b)). These results indicate that the ability of ectoine to maintain lipase activity after heating is very impressive. Heating of lipase containing more than 90 mM ectoine at temperatures above 74 °C significantly increased the relative activity of lipase between 800–1000%. Also, at temperatures above 82 °C, lipase containing more than 130 mM ectoine was able to increase its activity by more than 1000% relative to Control 2. When compared with Control 1, the optimum activity of lipase was obtained at the relatively low final concentration of ectoine and low temperature (Fig. 1(a)). However, when compared with Control 2, optimum lipase activity was attained at high ectoine concentration and high temperature (Fig. 1(b)). These results indicate that the higher the temperature, the higher the ability of ectoine to stabilize lipase due to a drastic decrease in the activity of lipase Control 2 at high temperature.

Stabilization of Lipase in Methanol

The ability of ectoine to stabilize lipase was also tested with methanol solvents at levels of 40–90% (v/v). After being incubated in methanol for 1 h, the relative activity of lipase to Control 1 (without ectoine and methanol treatment) and to Control 2 (without ectoine and treated with methanol at the same level as the lipase tested) were assayed (shown in Supplementary Table 3 and 4). The regression model for the relative activity of lipase to Control 1 was generated from experimental data with a determination coefficient (R^2) of 88.36%. The model was then used to predict the relative activity of lipase at various methanol levels in the experimental range using the regression Eq. (2) (prediction results are shown in Supplementary Table 3).

$$\text{Relative activity}(\%) = -646 + 5.71X_1 + 19.14X_2 - 0.018X_1 \times X_1 - 0.1371X_2 \times X_2 - 0.0395X_1 \times X_2 \quad (2)$$

The significance of each coefficient of the regression model using Student's t-test with a significance level of 5% is shown in Table 3. The final concentration of ectoine in the enzyme solution did not produce a significant linear effect on the relative activity

Table 3. Estimated coded coefficient and p-value for the regression model of relative activity of lipase after methanol treatment

Factor	Relative activity of lipase (%)	
	Coefficient	p-Value
Linear		
X_1	15.67	0.085
X_2	-28.90	0.008*
Square		
X_1^2	-22.49	0.031*
X_2^2	-42.83	0.001*
Interaction		
$X_1 X_2$	-24.7	0.060*

*Significant (p-value < 0.05)

of lipase (probability > 0.05), while the methanol level significantly affected the relative activity of lipase (linear regression coefficient of -28.9 with probability < 0.05). Increasing methanol levels resulted in the reduced relative activity of lipase (Fig. 2(a)). These results indicate that at high methanol levels, the ability of ectoine to protect lipase is decreased. The regression model did not show a significant interaction between the final concentration of ectoine added and the methanol levels on the relative activity of lipase. The increase in the final concentration of ectoine gave an insignificant stabilization effect on lipase activity (Fig. 2(a)).

In general, ectoine was effective in maintaining and even improving lipase stability in methanol. The addition of ectoine with a final concentration of 40–90 mM was able to keep lipase activity up to 100% after being treated with around 84% (v/v) methanol. At methanol levels below 78% (v/v), the addition of ectoine with a final concentration of 40–125 mM could maintain and improve the relative activity of lipase at more than 100%. Interestingly, the lipase activity significantly increased by more than 50% at methanol levels below 65%, with the final concentration of ectoine above 70 mM (Fig. 2(a)). Other researchers have also reported the ectoine's capacity to stabilize lipase in methanol; the addition of ectoine increased biodiesel synthesis from cottonseed oil using immobilized lipase as a biocatalyst and a three-step methanol addition process [19].

The relative activity of lipase compared to Control 2 was higher and showed a different optimum area (Fig. 2(b)). When compared with Control 1, the optimum relative activity of lipase (> 150%) was obtained at the final concentration of ectoine of 70–125 mM and levels of methanol of 45–65% (v/v). However, when compared with Control 2, the optimum relative activity of lipase (> 400%) was obtained at high methanol levels (> 60% v/v) at relatively low ectoine concentrations (< 105 mM). It was caused by a significant decrease in the activity of lipase Control 2 at high methanol levels. These results indicate that ectoine can maintain and even increase lipase activity in methanol.

Based on the results obtained, heat treatment produced a more detrimental effect on lipase stability than methanol, indicated by a significant decrease in the activity of lipase Control 2 after heating at various temperatures (Table 2). This finding was in accordance with Park et al. reports, who stated that lipase from *Burkholderia* sp. has a short half-life when heating at a temperature of above 60 °C [20]. As a consequence, the adequate protection of lipase from the heat was obtained at high ectoine concentrations of above 130 mM (Fig. 1(b)). Conversely, sufficient stability of lipase in methanol was attained at moderate levels of ectoine of under 110 mM (Fig. 2(b)). In accordance with Park et al. work, it reported that lipase from *Burkholderia* sp. showed sufficient stability in methanol [20], hence the concentration of ectoine required for adequate protection of lipase against methanol was lower than that for heat protection. High temperatures cause proteins to denature [28], so catalytic activity will decrease drastically or even completely disappear. Meanwhile, polar organic solvents such as methanol and

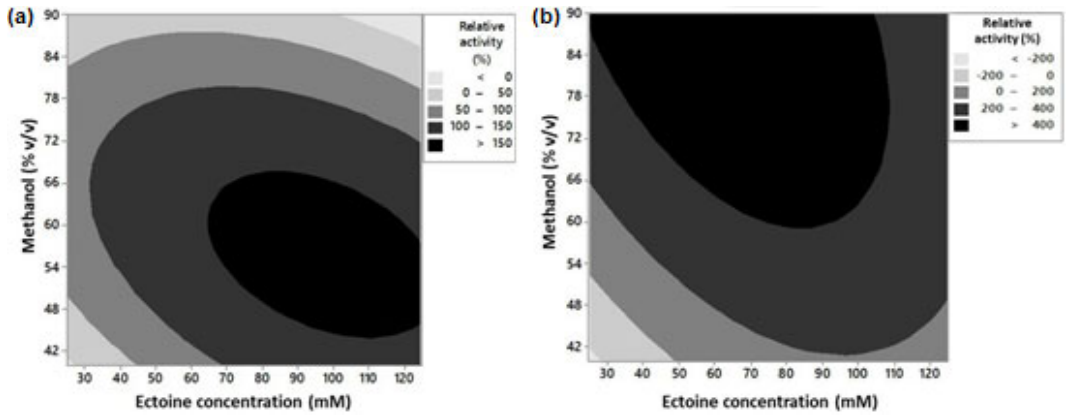

Fig 2. Effect of concentrations of ectoine and methanol on the relative activity of lipase in Control 1 (a) and Control 2 (b)

ethanol reduce enzyme activity by attracting the water molecules present on the enzymes' surface that play a role in maintaining their catalytic functions [29]. Ectoine added to the enzyme solution can secure the layer of water on the surface of the enzyme so that the native conformation of the catalyst remains stable. Therefore, the protective effect of ectoine on lipases treated with methanol (Fig. 2(a)) was better than that treated with heat (Fig. 1(a)).

For optimum protection and increased activity, we recommend an efficient and effective ectoine concentration to protect lipase of *Burkholderia* sp. against heating and methanol. For about 0.038 µg lipase, we need a final ectoine concentration of around 60 mM to protect and improve the enzyme activity up to 50% against methanol concentration up to 70% [v/v]. However, we need a higher concentration of ectoine of around 100 mM to protect and improve the enzyme activity up to 20% when heating at a temperature up to 80 °C.

■ CONCLUSION

Ectoine was highly effective in protecting lipase from heat and methanol. At moderate temperatures or methanol levels, ectoine could even improve the activity of the lipase. The heat had a more negative effect on lipase stability than methanol, so more ectoine was required to protect lipase from the effects of heating. An efficient and effective ectoine concentration to protect and improve the activity of 0.038 µg lipase from *Burkholderia* sp. against 70% [v/v] methanol was around 60 mM. However, it needed around 100 mM ectoine to protect and improve the enzyme activity against heating at temperatures up to 80 °C. Our study has proved that ectoine is a potential stabilizer for lipase and suggests that it should be used in industrial processes.

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