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CRUDE GLYCEROL UTILISATION IN MONOLAURIN PRODUCTION USING IMMOBILISED *Rhizomucor miehei* LIPASE: OPTIMISATION AND THERMODYNAMICS STUDY

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ABSTRACT

The significant growth of biodiesel industry causes an excessive production of crude glycerol in Malaysia's market, especially being as one of the global biggest producers and exporters of palm oil. The ability of crude glycerol being reused as a substrate to produce monolaurin has been the main focus while catalysing the production efficiency using Lipozyme RM IM, a commercial immobilised Rhizomucor miehei *lipase in a solvent-free system. Optimal reaction conditions obtained were 8:1 molar ratio of crude glycerol to lauric acid, temperature 47°C, and lipase loading 1 wt% of lauric acid. Over 82% lauric acid conversion with 56 mol% monolaurin yields was achieved within 8 hr. Using Arrhenius equation and transition-state theory, thermodynamic studies on monolaurin synthesis under the optimum conditions were performed. Energy of activation (E_a) and energy of deactivation (E_d) were found to be 89.5 kJ mol^{-1} and 110.7 kJ mol^{-1}, respectively, suggesting that higher energy was required for enzyme irreversible denaturation to occur. Values of enthalpy ($86.90 \leq \Delta H \leq 86.84$ kJ mol^{-1}), entropy ($0.29 \leq \Delta S \leq 0.28$ kJ mol K^{-1}), and Gibbs free energy ($-2.53 \leq \Delta G \leq -5.22$ kJ mol^{-1}) of the esterification were also determined.*

Keywords: crude glycerol, monoglycerides, enzymatic reaction, lipase, thermodynamics.

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INTRODUCTION

Due to the rising concerns of global warming and energy deprivation, biodiesel as a renewable alternative fuel has been introduced and intensively produced in the last few decades. As a result, the main by-product from biodiesel synthesis, glycerol, has been massively produced. However, due to the costs associated with processing, capital, and transportation, thus, purification practice for the crude glycerol would not be a profitable option for biodiesel producers. If crude glycerol can be utilised as a building block for higher value-added compounds such as monoglycerides, the economic viability of the biodiesel production might be improved. Monoglycerides or monoacylglycerols (MAG) are widely used as synthetic surfactant or emulsifier in pharmaceutical, cosmetic, food, detergent, and plasticiser formulations. They represent about 70% of the synthetic emulsifiers used in food and pharmaceutical industries (Bruschweiler and Dieffenbacher, 1991; Ferreira-Dias *et al*., 2001; Chen and Terentjev, 2018).

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Besides the bulk applications as emulsifiers, monolaurin also known as glycerol monolaurate, a special type of MAG formed from lauric acid and glycerol, has been widely reported of having anti-microbial activities. For instance, this monoester was able to cause leakage of cell membrane of *Staphylococcus aureus*, a type of Gram-positive bacteria (Tangwatcharin and Khopaibool, 2012; Zhang *et al*., 2009). Besides, monolaurin also shows certain extent of virucidal effects towards measles, HIV, Herpes simplex-1, visna virus, vesicular stomatitis and cytomegalovirus (Lieberman *et al*., 2006).

At the industrial scale, MAG are manufactured by chemical glycerolysis of fats and oils at high temperature (200°C-250°C), using inorganic catalysts under a nitrogen gas atmosphere (Gupta, 1996; Satriana *et al*., 2016; Hermida *et al*., 2017). However, this method creates many drawbacks. It consumes a lot of energy, generates dark coloured by-products and gives a low yield (30%-50%). Therefore, an additional distillation step is often required to obtain high purity MAG. To overcome these problems, enzymatic productions of MAG employing lipase have been developed due to milder conditions required. Several lipase-catalysed esterification approaches have been employed to produce MAG in organic medium (Cetina *et al*., 2011; Dom *et al*., 2014; Kapoor and Gupta, 2012; Zeng *et al*., 2010), in solvent-free systems (Freitas *et al*., 2010; Ghamgui *et al*., 2006), or using microemulsion as reaction media (Itabaiana Jr *et al*., 2013). Solvent-free systems are more attractive because they avoid toxicity issues of the solvent and reduce purification steps, therefore lowering the cost of end products. Furthermore, MAG have potential use in food products, a solvent-free system is preferable.

The enzymatic glycerolysis and esterification of pure glycerol have been widely studied but there is an absence of information available on the enzymatic synthesis of MAG using biodiesel-derived crude glycerol. On top of that, thermodynamic studies concerning esterification of crude glycerol catalysed by lipase in a solvent-free system has not been disclosed in any published literature yet. In this work, biodiesel-derived crude glycerol was utilised for the synthesis of monolaurin catalysed by lipase from *Rhizomucor miehei* (Lipozyme RM IM). The esterification reaction was performed without any solvent or surfactant. In addition, crude glycerol was characterised and used without any further treatment. To study the thermostability of the immobilised enzyme in esterification reaction, optimal conditions of monolaurin yield was first being determined. Then, the thermodynamic aspect of lipase-catalysed synthesis of monolaurin was explored.

MATERIALS AND METHODS

Materials

Crude glycerol was kindly provided by a local biodiesel production plant, Lereno Sdn Bhd located in Perak, Malaysia and used for this work without prior purification. Lipozyme® RM IM (*Rhizomucor miehei* lipase immobilised on macroporous anion-exchange resin) was purchased from Sigma-Aldrich. Monolaurin, dilaurin, trilaurin, n-Tetradecane and *N,O*-Bis(trimethylsilyl)acetamide were obtained from Sigma-Aldrich. Chloroform was purchased from Fisher Scientific. All the chemicals used are of analytical grade.

Crude Glycerol Characterisation

Sample of crude glycerol was sent to SGS laboratory in Selangor, Malaysia for characterisation. Determination of glycerol content was in accordance with British Standard (BS 5711-3:1979). Gravimetric analysis was performed to determine the amount of insoluble impurities. Determination of fatty acids content was based on American Oil and Chemical Society (AOCS) Official Method.

Esterification of Lauric Acid with Crude Glycerol

The esterification reactions were performed in 50 ml screw-capped flasks. Crude glycerol and lauric acid were prepared into the flasks according to their molar ratio (2:1 to 10:1). Lipozyme RM IM of various loading (0.2 to 1.2 wt %) was added to the reaction mixture. The reaction mixture was then incubated at the desired temperature (40°C to 60°C) for 8 hr with shaking at 200 rpm, using Ecotron shaker from INFORS HT.

Sample Derivatisation for Gas Chromatography (GC) Analysis

The reaction mixture obtained from esterification reactions were centrifuged at 3500 rpm for 10 min to separate into oil, glycerol, and enzyme layers. Derivatisation was achieved according to Watts and Dills (1969) with slight modifications. Fifty µl of sample was withdrawn from the resulting oil layer and diluted with 1 ml chloroform. Then, 0.1 ml of internal standard (n-Tetradecane) was added followed by 0.2 ml of TMS reagent [*N,O*-Bis(trimethylsilyl)acetamide]. The resulting mixture was incubated at 70°C for 30 min. Samples were analysed using GC with flame ionisation detector. SGE BPX5 (5%-Phenyl Polysilphenylene Siloxane) non-polar capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) was used.

GC Analysis of Glycerides and Fatty Acid Conversion

Temperature program was set up as described previously by Bruschweiler and Dieffenbacber (1991). Fatty acid conversion was calculated based on the amount of lauric acid converted into products, as given in Equation (1).

$$\text{Fatty acid conversion } (\%) = \left(\frac{A-B}{A}\right) \times 100\% \quad \text{Equation (1)}$$

where, A and B are the peak areas for lauric acid-TMS before and after esterification, respectively.

Monolaurin and dilaurin contents were expressed as the sum of weight of their regioisomers. The mol % of each component was calculated as shown in Equation (2), Equation (3), and Equation (4).

$$Monolaurin\ (mol\ \%) = \frac{Monolaurin}{Monolaurin+Dilaurin+Trilaurin+Lauric\ Acid} \times 100\%$$

Equation (2)

$$Dilaurin\ (mol\ \%) = \frac{Dilaurin}{Monolaurin+Dilaurin+Trilaurin+Lauric\ Acid} \times 100\%$$

Equation (3)

$$Trilaurin\ (mol\ \%) = \frac{Trilaurin}{Monolaurin+Dilaurin+Trilaurin+Lauric\ Acid} \times 100\%$$

Equation (4)

Optimisation of Monolaurin Synthesis Using RSM

A 2^k central composite design (CCD) of three parameters (substrate molar ratio, temperature, enzyme loading) was employed to obtain the optimal conditions for the esterification reactions. The randomised experiment was tabulated, and the responses were analysed by statistical software Design Expert® Version 7.0.0 (Stat-Ease, Inc., Minneapolis). Experimental data obtained were fitted into a second-order polynomial equation [Equation (5)] for generating response surface, which was used for predicting the optimum esterification point (Y):

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 + \varepsilon \quad \text{Equation (5)}$$

where, Y is the predicted response variable; β_0 is the constant coefficient; β_i ,β_{ii},β_{ij} are the coefficients for the linear, quadratic, and for the interaction effects, respectively; X_i and X_j are the coded level of variables x_i and x_j respectively and ε is the error of the model (residual term).

Thermodynamics Studies of Lipozyme RM IM on Monolaurin Synthesis

The optimal conditions (substrate molar ratio and lipase loading) obtained from the optimisation part were further analysed for thermodynamic properties of monolaurin synthesis represented by the changes in enthalpy (ΔH), Gibbs free energy (ΔG) and entropy (ΔS). The thermodynamic studies consisted of investigating the effect of temperatures from 40°C to 60°C in accelerating or decelerating monolaurin production rate. Aliquots were withdrawn at periodic intervals to determine the initial reaction rate, v_o.

A plot of ln k versus 1/T was used to determine the activation energy E_a as described by Arrhenius equation:

$$\ln k = \left(-\frac{E_a}{RT}\right) + \ln A \quad \text{Equation (6)}$$

where, ln k is the reaction rate constant, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature (K) and A is the pre-exponential factor. The E_a can be determined from the slope of the graph as described earlier (Khor *et al*., 2010; Yu and Li, 2006). Gibbs free energy ΔG enthalpy, energy ΔH and entropy energy ΔS of the enzymatic reaction can be calculated according to the following equations (Jin *et al*., 2015; Pogaku *et al*., 2012):

$$\Delta H = E_a - RT \quad \text{Equation (7)}$$

$$\Delta G = \Delta H - RT \quad \text{Equation (8)}$$

$$\ln k = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \quad \text{Equation (9)}$$

Irreversible Denaturation and Thermal Stability of Lipase

Thermal stability of Lipozyme RM IM was studied by incubating the enzyme in lauric acid suspension without glycerol at 50°C, 55°C and 60°C for variable periods of one to four days. In order to determine the lipase residual activity, esterification was re-performed as described above.

$$\Psi = \frac{v}{v_o} \quad \text{Equation (10)}$$

From Equation (10), Ψ is the activity coefficient, v is the initial reaction rates of unheated lipase and v_o is the initial reaction rates for lipase that has undergone thermal treatment.

The thermodynamic parameters of the irreversible denaturation of the enzyme can be then be estimated according to Eyring equation:

$$k_d = T\frac{k_B}{h} e^{\frac{\Delta S}{R}} e^{-\Delta H/RT} \quad \text{Equation (11)}$$

where k_d, T, k_B, *h* and R are deactivation constant, absolute temperature, Boltzmann constant, Planck's constant and gas constant, respectively (Khor *et al.*, 2010). Determination of k_d at each temperature will also lead to enzyme's half-life at particular temperature (Marangoni, 2003).

$$t_{1/2} = \frac{0.693}{k_d} \qquad \text{Equation (12)}$$

RESULTS AND DISCUSSION

Components in Crude Glycerol

The characteristic of crude glycerol used is shown in *Table 1*. The purity of crude glycerol was 88.35% with 10.65% moisture content plus other insoluble impurities around 0.10%. The result clearly displayed that less than 1% of the remaining content were a mixture of different types of fatty acid. As expected, C16:0 (palmitic acid) dominated the total amount of fatty acids content followed by C18:1 (oleic acid) because the crude glycerol obtained was derived from biodiesel reaction using palm oil. Therefore, no pre-treatment was conducted on the substrate due to high glycerol content.

TABLE 1. CRUDE GYLCEROL COMPOSITION

Content	Composition
Glycerol content (%)	88.35
Moisture (%)	10.65
Insoluble impurities (%)	0.074
Fatty acids composition (%)	
-C6:0	7.8
-C8:0	9.0
-C10:0	<0.1
-C12:0	<0.1
-C14:0	<0.1
-C16:0	50.7
-C16:1	<0.1
-C18:0	<0.1
-C18:1	32.5
-C18:2	<0.1
-C20:0	<0.1
-C18:3	<0.1

Optimisation for Monolaurin Production

Analysis of variance (ANOVA) using Fisher's F-tests was conducted as presented in *Table 2*. The ANOVA revealed that the model was capable to represent the actual relationship between monolaurin yield and the significant factors. The F-value of the model (154.54) with a Prob (F) < 0.0001 indicated that the model was significant at 95% confidence level. The lack of fit F (3.46) implies that it is not significant relative to pure error. Also, the precision of the model can be checked by coefficient of determination (R^2 value). The R^2 of 0.9929 and adjusted R^2 value of 0.9864 also showed that the model has high goodness of fit. Within the range studied, all individual factors [substrate molar ratio (A), temperature (B), and lipase loading (C)] showed significant effect towards monolaurin yield with Prob (F) value of less than 0.05. On the other hand, only one interaction effect between the factors [substrate molar ratio and temperature (AB)] was found to be significant during esterification of monolaurin.

Figure 1 shows the effect of glycerol to lauric acid molar ratio, temperature and their mutual interaction (P-value = 0.0136) on the yield of monolaurin at a fixed amount of enzyme loading (1 wt % lauric acid). At the lowest reaction temperature (40°C) with the lowest substrate molar ratio (3:1),

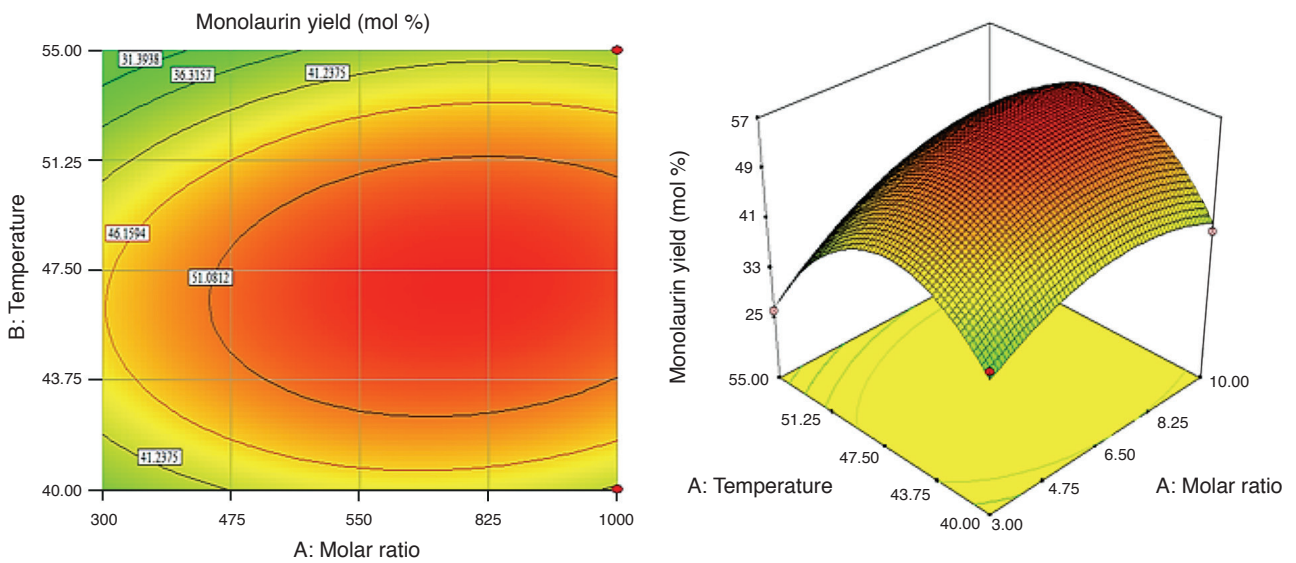

Figure 1. (a) Contour plot (b) response surface plot of monolaurin yield as a function of temperature and substrate molar ratio at fixed lipase loading of 1 wt % lauric acid.

the monolaurin yield was only 37.54 mol %. Further increase of the temperature to 47.5°C and molar ratio to 8:1 could bring a maximal yield of over 50 mol %. This indicated that temperature and molar ratio greatly affected the monolaurin yield (response), which agreed with the previous outcome of ANOVA test (*Table* 2). When the temperature increases, collisions between enzyme and substrate molecules are speeded up and causes the increase of reaction rate. However, the yield of monolaurin decreased with an increase in temperature. This negative effect might be associated with the loss of enzyme activity due to denaturation. It has been reported in numerous studies that the reaction temperature of 45°C to 55°C is optimal for Lipozyme RM IM performance on esterification reactions (Bernardes *et al*., 2007; Manurung *et al*., 2015).

Response surface plots display function of two factors at one time while keeping other factors at a constant level are useful in understanding both the main and interaction effects of these factors. The yield response for different combination of parameters levels can be easily predicted from the relevant response surface plots. Monolaurin yield (*Figure 1b*) increased with increasing molar ratio of glycerol to lauric acid (3:1 to 8:1). It has been proven that excess glycerol favoured the yield of glyceride esters (Freitas *et al*., 2007; Pereira *et al*., 2004; Zhao *et al*., 2011). This is most likely due to the excess glycerol which can push the reaction equilibrium to the right side, favouring esterification rather than hydrolysis. However, further increase in molar ratio to 10:1 led to a slight reduction in the yield. As the glycerol content increases, the solution viscosity also increases, causing mass transfer limitation. Also, it has been confirmed that alcoholic groups are terminal inhibitors of lipase (Mhetras *et al*., 2010).

The optimal conditions for lipase-catalysed monolaurin synthesis, using Lipozyme RM IM were determined by the response desirability profile. The optimised conditions with desirability of 0.977 were: 8:1 glycerol:lauric acid molar ratio, 47°C and 1 wt % lipase loading. Experiments were conducted under optimum conditions with three replications and the results were tested using single sample t-test. Under these optimum conditions, the monolaurin yield was predicted to be 56 mol %, with a p-value of 0.116, bigger than 0.05 (95% confidence level) showed that the experimental data had no significant different with the predicted data by Design Expert software. This indicates that the model was validated, and the optimisation was considered successful.

Thermodynamics Studies on Lipozyme RM IM

The initial reaction rate, v_0 of monolaurin yield under various temperature of 40°C to 60°C is presented in *Figure* 2. The figure clearly displays two different trends according to the monolaurin produced. The initial reaction rate progressively increased with temperature up to 47°C. Beyond this threshold value, monolaurin production rate decreased with increasing temperature, therefore was considered as a non-productive region. Previous studies have found that, the catalysing activity of Lipozyme RM IM was usually performed at 45°C to 50°C regardless of acyl donators and acyl acceptors (Bernardes *et al*., 2007; Ungcharoenwiwat *et al*., 2016; Yadav *et al*., 2014). The positive relationship between monolaurin production rate and temperature was in accordance with the Arrhenius model. Arrhenius plot was used to estimate the activation energy and reversible unfolding energy (*Figure 3*).

TABLE 2. ANALYSIS OF VARIANCE (ANOVA) TABLE OF QUADRATIC MODEL

Source	Sum of squares	Degrees of freedom	Mean square	F value	P-value* Prob>F
Model	4 941.76	9	549.08	154.54	<0.0001
A	194.19	1	194.19	54.65	<0.0001
B	94.86	1	94.86	26.70	0.0004
C	1 463.03	1	1 463.03	411.76	<0.0001
AB	31.76	1	31.76	8.94	0.0136
AC	1.77	1	1.77	0.50	0.4968[a]
BC	0.50	1	0.50	0.14	0.7154[a]
A^2	403.09	1	403.09	113.45	<0.0001
B^2	2 904.44	1	2 904.44	817.43	<0.0001
C^2	151.43	1	151.43	42.62	<0.0001
Residual	35.53	10	3.55	-	-
Lack of Fit	27.56	5	5.51	3.46	0.0998 [a]
Pure Error	7.97	5	1.59	-	-
Cor Total	4 977.29	19	-	-	-

Note: *A - glycerol:lauric acid molar ratio; B - temperature (°C); C - lipase loading (wt % of lauric acid).

*Statistically significant at 95% confidence level.

[a] Not significant.

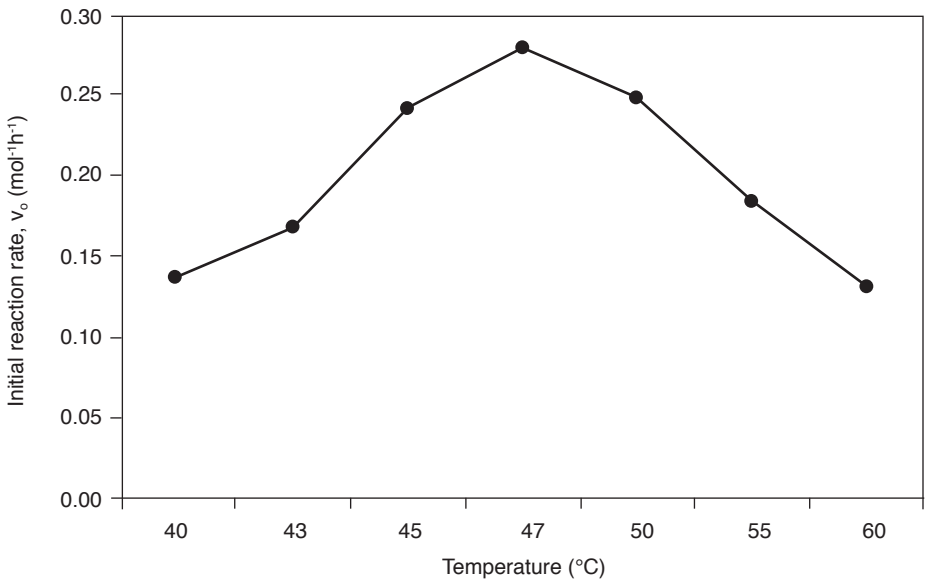

Figure 2. Initial reaction rate of monolaurin yield under different operating temperatures (reaction conditions: 8:1 glycerol/lauric acid molar ratio; 1 wt % lipase loading; 200 rpm)

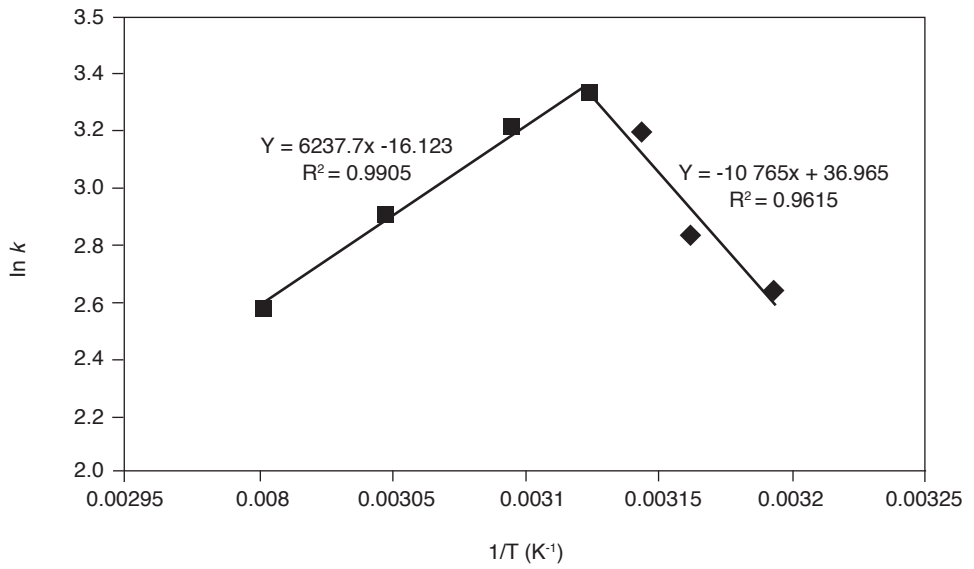

Figure 3. Arrhenius plots for the estimation of activation energy and reversible denaturation energy (40°C-60°C).

The Arrhenius plot associates the natural logarithm of the reaction rate constant (*ln k*) with the inverse of temperature (1/T). *Figure 3* presents the activation energy which was calculated from the slope below the threshold temperature [intersect value of *ln k* (47°C)] and was found to be 89.5 kJ mol^{-1} (R^2 = 0.9651), whereas the reversible denaturation energy was estimated from the slope above the threshold and the value was found to be 51.86 kJ mol^{-1} (R^2 = 0.9651) (Yu and Li, 2006). Most of the reactions have E_a values ranging from 40 kJ mol^{-1} to 400 kJ mol^{-1} (Qu *et al*., 2013). For this work, the activation energy was higher than the reversible denaturation energy, suggesting that higher energy is required for esterification reaction to go forward than unfolding of the enzyme. However, contrary to this study, previous studies reported for Lipozyme TL IM (Activation energy = 22.15 kJ mol^{-1}; Deactivation energy = 45.18 kJ mol^{-1}), tannase from *Apergillus niger* (Activation energy = 23.29 kJ mol^{-1}; Deactivation energy = 62.85 kJ mol^{-1}) and *Candida antarctica* lipase B (Activation energy = 22.15 kJ mol^{-1}; Deactivation energy = kJ mol^{-1}) have shown that these enzymes required higher energy to unfold their active sites than to activate it (Khor *et al*., 2010; Yu and Li, 2006). Hence, a probable reason is the high viscosity of the reaction mixture in a solvent-free system required higher energy to bring non-activation molecules into activation molecules. This is according to Kramers' theory, where viscosity of reaction mixture consequently would introduce friction against any proteins in the system and this

eventually restricted the protein motion, inhibiting catalysis in motile enzymes. For this work, viscosity happened by increasing of crude glycerol concentration in the absence of solvent (Kumar *et al*., 2016; Uribe and Sampedro, 2003).

Determination of Thermodynamic Properties *ΔH*, *ΔS* and *ΔG*

The enthalpy of activation (*ΔH*), entropy of activation (*ΔS*) and Gibbs free energy of activation (*ΔG*) at each activation temperature were calculated according to Equations (7) - (9). Results are presented in *Table 3*. The endothermic nature of the esterification reaction was confirmed by the positive value of enthalpy (*ΔH*). Heat needs to be input for enzyme catalysis to occur. Furthermore, these values indicate that lower enthalpy of activation was needed as the temperature increased. This is probably due to high temperature which promotes collision between the reactant molecules. Entropy (*ΔS*) is the measure of disorder in a system. Positive entropy indicates an increase of disorder in the system. The value did not show any significant changes suggesting that lipase configuration remains the same despite changing in temperature (Bhatti and Amin, 2013). The microenvironment around the enzyme could significantly affect the entropy value (Yu and Li, 2006). The Gibbs free energy (*ΔG*) shows negative value, validating that the reaction will proceed spontaneously and is said to be exergonic. This thermodynamic study demonstrates a favourable condition towards monolaurin synthesis (Aledo *et al*., 2003; Berg *et al*., 2002).

TABLE 3. THERMODYNAMIC PARAMETERS OF LAURIC ACID AND CRUDE GLYCEROL ESTERIFICATION, ESTIMATED BY THE ARRHENIUS MODEL UNDER DIFFERENT ACTIVATION TEMPERATURE

Temperature (K)	ΔH, kJ mol^{-1}	ΔS, kJ mol^{-1}K^{-1}	ΔG, kJ mol^{-1}
313.15	86.90	0.29	-5.22
316.15	86.87	0.29	-4.18
318.15	86.86	0.28	-3.78
320.15	86.84	0.28	-2.53

Irreversible Denaturation and Thermal Stability of Lipase

To evaluate the irreversible denaturation rate of Lipozyme RM IM, the enzyme was treated with various thermal conditions and its residual activity was determined. The first order rate constant of lipase deactivation, k_d at different temperature can be estimated from the slope by plotting ln Ψ versus time (days), where Ψ is the activity coefficient as shown in *Figure 4*. The experimental results indicated that k_d continuously increased from 0.073 to 0.246 day^{-1} (0.0031 to 0.0103 hr^{-1}) when the temperature increased from 50°C to 60°C. Enzyme stability decreased with increasing k_d at a certain temperature (Khor *et al*., 2010). To validate Lipozyme RM IM stability, its half-life, $t_{1/2}$ was determined. Half-life of an enzyme is the time it takes for its activity to reduce to a half of the original activity. The calculated values for half-life of the lipase were 9.5 (228 hr), 5 (120 hr) and 3 (72 hr) days for 50°C, 55°C, and 60°C, respectively. Both k_d and $t_{1/2}$ values showed that the lipase has moderate thermal stability over a period of time. Based on the denaturation constant, the energy of irreversible denaturation obtained from Arrhenius equation was 110.7 kJ mol^{-1}, higher than that of the activation energy (89.5 kJ mol^{-1}). This reveals that the enzyme activation can be attained at lower temperature, whilst higher temperature would hasten the denaturation rate of enzyme (Khor *et al*., 2010).

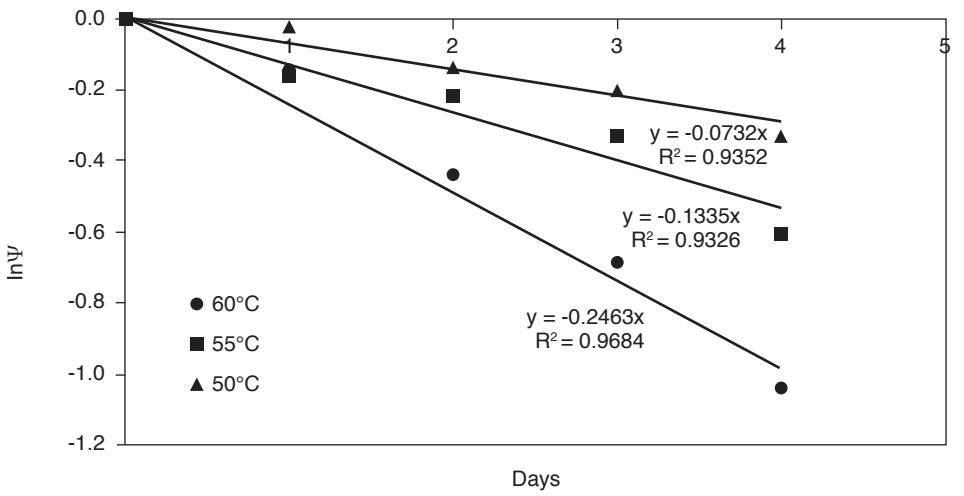

Figure 4. Initial reaction rate of monolaurin yield versus different heat treatment (50°C-60°C) for variables times (1- 4 days). Reaction conditions: 8:1 glycerol/lauric acid molar ratio; 1 wt % lipase loading; 200 rpm.

CONCLUSION

The work has demonstrated feasibility of using crude glycerol as a substrate for monolaurin production in a solvent-free system using Lipozyme RM IM as catalyst. Response surface methodology was able to predict the optimum reaction which were 8:1 crude glycerol to lauric acid ratio, 47°C and 1% wt lipase loading. At optimum conditions, monolaurin yield was attained at 56 mol %. The thermodynamic studies obtained a negative Gibbs free energy, whereas both enthalpy and entropy gave positive values, indicating endothermic, spontaneous and irreversible nature of the reaction. The optimisation and thermodynamics data obtained could be useful for process improvement and industrial utilisation of crude glycerol for monoglycerides synthesis in the future.

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