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Thermodynamic Studies on Activity and Stability of Immobilized *Thermomyces lanuginosus* in Producing Fatty Acid Methyl Ester (FAME)

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Abstract- The effect of temperature on enzymatic transesterification was studied by many researchers for process optimization purposes. The optimum operating temperature was defined on the basis of high FAME productivity or lipase activation without considering the lipase denaturation factor. High thermal energy was favored to accelerate transesterification reaction but lipases for enzymatic transesterification reaction were susceptible to denaturation even at moderate temperature operation. In this work, studies of thermal effects on lipase kinetics were carried out to propose the most desirable operating temperature that achieves high FAME productivity while preserving lipase catalyzing activity. Catalyzing activity of Lipozyme TL IM increased with temperature up to a threshold at 40°C and successive fell beyond this value have explained the occurrence of reversible biocatalyst inactivation. The reaction rates obtained for experiment under different heat treatments have confirmed the deactivation process of Lipozyme TL IM follows first-order kinetics pattern. The most desirable operating temperature for transesterification reaction is 40°C that leads to the highest productivities, 100 % FAME yield at 4 hrs while preserving acceptable stability levels of 1.09% activity lost after 1 hrs.

Index Terms **--** Biodiesel, Heat treatment, Lipase,Temperature effect, Thermodynamics

I. INTRODUCTION

Crude palm oil transesterification mediated by immobilized *Thermomyces lanuginosus* or Lipozyme TL IM in tert-butanol solvent system, have been attempted in previous study with encouraging FAME yield and initial reaction rate being achieved [1]. According to the principles of Arrhenius and Vant Hoff, transesterification reaction rate can be accelerated by either increasing operating temperature or increasing concentration of the reactants that helps to increase the rate of reactants collision between each other. The reaction is commenced in condition the colliding reactants possess sufficient activation energy to overcome the energy potential barrier. The free energy of activation, ΔG thus acts as a potential-barrier for the reaction to take place. Activation energy can be obtained from experimental studies with different reaction rate being determined at the studied ranges of operating temperature [2].

Nevertheless, transesterification reaction catalyzed by immobilized lipases under high temperature tend to expose Lipozyme TL IM to the conformational changes risk. Since lipases were easily denatured at high temperature in nature, the most operational working temperature thus must represent significantly high enzyme activity as a whole but to the extent that the lipases still retain good enzyme stability. Good thermal stability of lipase is of paramount important in transesterification to preserve high catalyzing activity for continuous process and for enzyme reuse in batch system. Thus, expensive cost of lipase production for transesterification can be circumvented by its reusability and long shelf life. Consequently, many researchers were concerned for enzyme stability under different thermal conditions [3, 4, 5].

Effects of temperature on stimulating enzymatic transesterification were initiated by many researchers for process optimization purpose. In fact the optimum operating temperature was defined on the basis of high FAME productivity or lipase activation without considering the lipase denaturation rate. In this work, thermodynamic studies were carried out as to propose the most desirable operating temperature that achieves high FAME productivity as a whole besides preserving lipase catalyzing activity. Hence, activation energy and reversible deactivation energy without thermal treatment was determined to detect the most productive temperature condition. Whereas irreversible deactivation energy together with half life time for Lipozyme TL IM were clearly defined after Lipozyme TL IM was preincubated in 45, 50, 55 and 60°C with different time periods (1 - 4 days). The desirable working temperature for the system was then justified from the FAME production rate and the extent of thermal resistance of the lipase towards temperature variations.

II. MATERIALS AND METHOD

A. Biocatalyst and Chemicals in Transesterification

Lipase *Thermomyces lanuginosus* immobilized on silica gel (Lipozyme TLIM) with catalytic activity of 170 IUN/gl converting 0.01% tristearin per minute under standard assay conditions was purchased from Novozymes (Bagsvaerd, Denmark). The unrefined crude palm oil (CPO) for transesterification reaction is procured from M.P. Mathew Palm Oil Mill Sdn. Bhd. located in Sungai Bakap, Malaysia. Analytical grade of tert-butanol and methanol in 99.0% purity are obtained from J.T. Baker and Merck respectively. Chromatographic grade of lauric acid methyl ester, palmitic acid

methyl ester, stearic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester and heptadecanoic acid methyl ester were purchased from Sigma.

B. Crude Palm Oil Transesterification

Unless otherwise stated, the detection of transesterification activities of Lipozyme TL IM when under different temperatures and heat treatments were carried out by monitoring FAME produced from the reaction between crude palm oil and methanol. The typical enzymatic transesterification reactions were carried out by adding 6.65 % enzyme loading in optimum reaction mixtures. From preliminary study, the optimum amount for crude palm oil, methanol and tert-butanol were 11.84, 3.2 and 16.96 ml respectively. 32 ml of reaction mixture in 100 ml of conical flasks were maintained in water bath shaker at 150 rpm mixing intensity and at 30°C. For thermodynamic studies, 50 µl aliquots of sample were withdrawn at 5, 10, 15 and 30 min for initial reaction rate determination. Product FAME achieved at various process conditions was analysed by gas chromatography. Each experimental run was performed in duplicates and the results were expressed as mean values $\pm$ standard deviation. The standard deviations for FAME were approximately $\pm$ 5% of the mean values.

C. Thermodynamic Studies on Lipozyme TL IM

Experimental works on thermodynamic studies for transesterification reaction were divided into two parts, mainly on the effects of temperature on lipase activity and stability. First, the effects of temperature in accelerating FAME production rate were implemented at various reaction temperatures of 30, 35, 40, 45, 50, 55 and 60 °C without imposing heat treatment on lipase. Second, the irreversible denaturation rate for Lipozyme TL IM when under different thermal treatment was studied to determine half-life time as well as the thermal resistance strength. The kinetics of irreversible denaturation was studied by incubating Lipozyme TL IM in the crude palm oil suspension without methanol at temperatures of 45, 50, 55 and 60 °C for variable times of 1, 2, 3 and 4 days. The Lipozyme TL IM residual activity was then examined by implementing typical transesterification reaction as described in section *B. Crude Palm Oil Transesterification*. The catalyzing activity for the lipase under thermal treatment was compared with the catalyzing activity for the lipase not subjected to thermal treatment. The comparison is necessary to determine the extent of heat destruction on lipase catalyzing activity.

D. FAME Determination with Gas Chromatography

Heptadecanoic acid methyl ester served as the internal standard. To quantify FAME produced, 0.4 µl sample was injected into Perkin Elmer Clarus 500 gas chromatography equipped with programmed split/ splitless injector (PSS) and flame ionization detector (FID). The split ratio was defined as 20:1. Nukol^{TM} fused silica capillary column with dimension of 0.53 mm i.d. x 15 m length x 0.50 µm film thickness (Supelco, USA) was used. The injector and detector temperature were 220°C and 250°C respectively. The column temperature was maintained at 110°C for 0.5 min, increased to 200°C at 10°C/min and maintained at this temperature for 10 min. Helium was employed as carrier gas with flowrate 1.2 ml/min.

III. RESULTS AND DISCUSSION

A. Effect of Temperature on Lipozyme TL IM- catalyzed Transesterification

The initial reaction rate of FAME yield under variation of temperature from 30 to 60 °C was graphed in Fig. 1. The graph clearly displayed two different temperature boundaries according to FAME produced. At temperature ranges of 30 – 40 °C, the FAME production rate increased linearly with temperature. Whereas temperature beyond 40 °C and thereafter was considered a non-productive region due to FAME production rate decreased with increasing temperature. Therefore, the catalyzing efficiency was largely enhanced with increasing temperature up to a threshold of 40 °C optimum point. The successive decreased in FAME production rate beyond 40 °C have prescribed the existing of irreversible denaturation phenomenon. The thermal inactivation of lipozyme TL IM might be due to the antagonistic interacting effect between solvent molecules with the "membrane-lipase" system that reversibly produces a conformational change on active structure of the lipase [6]. The positive relationship defined between FAME reaction rate and temperature in the study was in agreement with Arrhenius model. Thus, the activation energy and reversible unfolding energy were estimated from the semilog plot versus the reciprocal temperature. In the enzymatic transesterification with different types of acyl alcohols like methanol, 1-propanol, 2-propanol, catalyzing activity of Lipozyme TL IM were largely propagated at 40 °C as reported in literature [7, 8]. Thus, Lipozyme TL IM was most favour to produce FAME at 40 °C regardless of oil source and acyl acceptors.

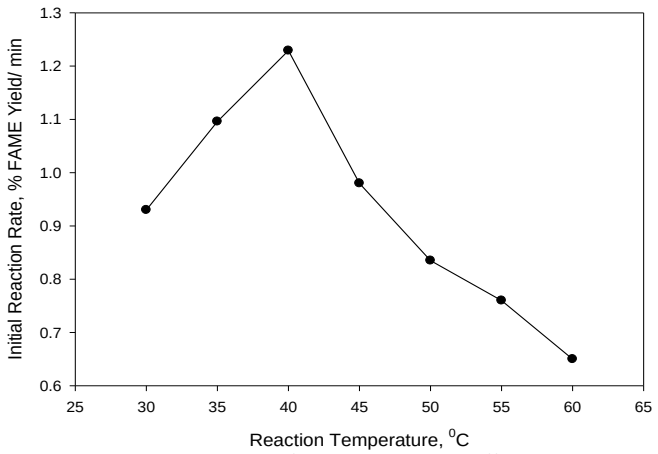

Figure 1. Initial reaction rate of FAME yield under different operating temperature.

B. Effect of Heat Treatment on Lipozyme TL IM- catalyzed Transesterification

The irreversible denaturation rate of Lipozyme TL IM was reflected by the residual activity of lipase after subjected to different thermal treatment conditions. In Fig. 2, FAME yield reaction rate were decreasing with the extended period of heat treatment. Lipase either incubated at 45 or 60 °C were experienced almost similar denaturation rate for 1 day heat treatment or 4 days heat treatment. The result enlightened the

proportional decreased of lipase activity with temperature. As clearly observed from the Fig. 2, the exponential decreased in lipase catalyzing activity was observed in 1 days of heat treatment and the rate was subsequently arrived into plateau after 4 days of heat treatment. The exponential decay of lipase activity with thermal treatment period agreed that lipase denaturation is a first-order process. Since the denaturation constant, K_d is a kinetic parameter and temperature dependent, hence the variable can be also represented by an Arrhenius-type equation.

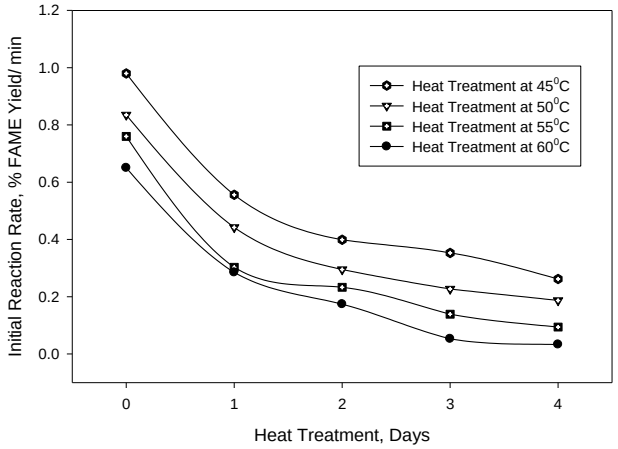

Figure 2. Initial reaction rate of FAME yield when subjected to different heat treatment conditions: 45 – 60 °C under 1 – 4 days.

C. Thermodynamic Studies on Activity and Stability of Immobilized Thermomyces lanuginosus

The dependent of initial reaction rate or enzyme activity on temperature variation is a first order process. During linear regression analysis, the relationships have been verified with high correlation coefficients of 0.9832 and 0.9913 being obtained for activation slope and denaturation slope respectively as shown in Arrhenius plot (Fig. 3). The activation energy that calculated from the negative slope of Arrhenius plot was 22 kJ/ mol whereas the reversible denaturation energy estimated from the positive slope was 26.5 kJ/ mol. The activation energy of 22 kJ/ mol for transesterification reaction suggested a low energy barrier was required for catalysis. Slightly higher reversible denaturation energy than activation energy was in agreement that lipase required much more energy to unfold lipase active conformation. This figure has been reported for immobilized *Candida antarctica* [9] lipase B and mycelium-bound carboxylesterase from *Aspergillus oryzae* [10].

The strength of lipase resistance towards heat was carried out by incubating the reaction mixture under variation of heat treatment conditions as described in materials and methods. The extent of deactivation was measured by the apparent first-order rate constant of enzyme denaturation. It was shown that denaturation constant that estimated from the slopes of plot ψ versus time, t (Fig. 4) progressively increased with increasing temperature from 45 to 60 °C. Consequently, the thermodynamic energy of irreversible denaturation that estimated based on the denaturation constants obtained from 45 to 60 °C was 45.18 kJ/ mol. It can be concluded that energy barrier as high as 45.18 kJ/ mol was required to impose thermal inactivation effect on Lipozyme TL IM because the binding of lipase to the carrier materials have greatly enhance lipase stability.

Activity of Lipozyme TL IM reached climax at 40 °C reaction temperature and beyond 40 °C, the lipase activity inactivated at faster rate with time. Therefore, the desirable working temperature for enzymatic transesterification reaction mediated by Lipozyme TL IM must be at 40 °C or below. The half life time for 30 and 40 °C operating temperatures were 112.87 and 63.23 hrs respectively. Even though Lipozyme TL IM gain higher life time when under 30 °C compared to 40 °C but the 40 °C gave 100 % FAME yield at 4 hrs reaction time. The specific lipase activity for 40 °C, 35.08 % FAME yield/ g lipase. hr was nearly 3 fold higher than the 30 °C of only 11.93 %FAME yield/ g lipase. hr. Therefore, 40 °C was selected as the optimum reaction temperature for the enzymatic transesterification reaction mediated by Lipozyme TL IM.

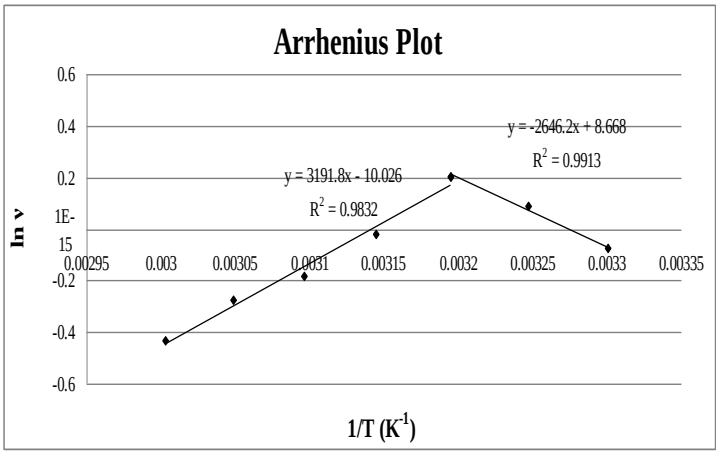

Figure 3. Arrhenius plots for the estimation of the activation energy and reversible denaturation energy.

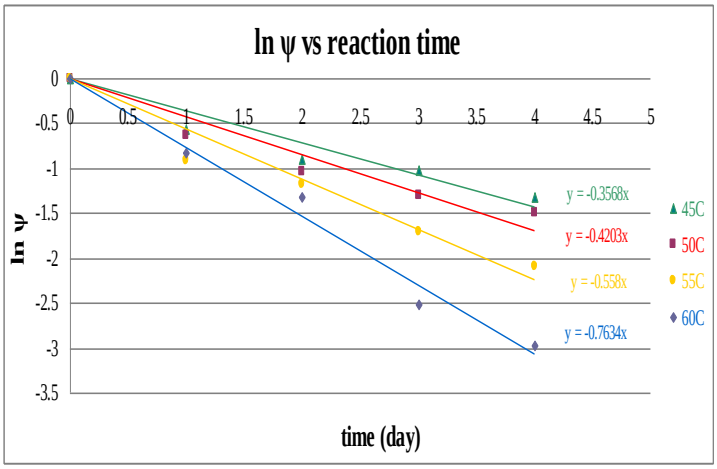

Figure 4. Semilog plots of irreversible denaturation of Lipozyme TL IM.

IV. CONCLUSION

The thermodynamic studies which involve simultaneous determination of catalyzing activity and stability of Lipozyme TL IM were particular useful to enhance production cost of enzymatic transesterification reaction. Expensive production cost of lipase for biodiesel production have resulted the lost of attention in commercializing enzymatic transesterification reaction. Thus, the appropriate operating temperature was particularly important to ensure FAME production rate generated by enzymatic reaction can compete with chemical methods besides preserving lipase activity for extensive period of lipase reuse. In this study, 40 °C was the most desirable operating temperature for enzymatic transesterification reaction where the process attained 100 % FAME yield at 4 hrs and retained 98.91 % residual activity after 1 hrs reaction time.

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