

# Kinetics of Enzymatic Synthesis of Liquid Wax Ester from Oleic Acid and Oleyl Alcohol

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**Abstract:** The kinetics of wax ester synthesis from oleic acid and oleyl alcohol using immobilized lipase from *Candida antarctica* as catalyst was studied with different types of impeller (Rushton turbine and AL-hydrofoil) to create different mixing conditions in 2l stirred tank reactor. The effects of catalyst concentration, reaction temperature, and impeller tip speed on the synthesis were also evaluated. Rushton turbine impeller exhibited highest conversion rate at lower impeller tip speed as compared to AL-hydrofoil impeller. A second-order reversible kinetic model from single progress curve for the prediction of fractional conversion at given reaction time was proposed and the corresponding kinetic parameter values were calculated by non-linear regression method. The results from the simulation using the proposed model showed satisfactory agreement with the experimental data. Activation energy shows a value of 21.77 Kcal/mol. The thermodynamic parameters of the process, enthalpy and entropy, were 21.15 Kcal/mol and 52.07 cal/mol.K, respectively.

**Key words:** wax ester, immobilized lipase, esterification, kinetics

## 1 INTRODUCTION

Wax esters are widely applied in premium lubricants, parting agents, antifoaming agents and cosmetics due to their special characteristics. Immobilized lipases for wax esters synthesis are particularly interesting for industrial purposes, since they can be easily handled. Unlike soluble lipases, a lipase in immobilized form is strongly bound to a solid phase, making it easy to separate the catalyst from the reaction mixture for repeated use<sup>1</sup>. Kinetic studies of processes using immobilized enzymes as catalysts are relatively scarce and have only become more important and studied in depth, in the last few years. Several studies have analyzed the kinetics on ester synthesis in terms of ping pong and bi-bi models<sup>2-4</sup>. However, those models are not sufficient to describe the kinetics due to the exclusion of

product levels and the reactions depend very much on the initial rate conditions. The complexity of the development of the models should not be ignored<sup>5</sup>. The kinetic equation is a basic element in the scale-up of esterification processes. The validity of such general model equations must then be tested for each particular reaction from the experimental data.

An alternative method to such models is to obtain the kinetic parameters from a single progress curve. The

**Nomenclature:** N: Agitation speed (rev/min), D: Impeller diameter (m), Ac: Oleic acid, Ao: Oleyl alcohol, Es: Ester, W: Water,  $\alpha$ : Equilibrium conversion,  $\beta$ : Parameter related to rate constant,  $t$ : Reaction time, X: Conversion  $(A_0 - Ac)/(A_0)$ ,  $k_1$ ,  $k_{-1}$ : Rate constants (forward and reverse)

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kinetic model was originally elaborated by Kosugi and Suzuki<sup>6</sup> and extended by Han *et al.*<sup>7</sup>. Recently, the model has been applied for the determination of the kinetic rate constant of pancreatic lipase in esterification and transesterification equilibrium reactions in organic media<sup>8</sup>. This simple method when compared to ping pong and bi-bi models is suitable to quantify the rate constant, in a given reaction conditions, needing only one single measurement for each substrate, even though some simplified models may be unable to capture the full behavior of the reaction. However, this simple model could be used in describing the reaction at preliminary stage and improve on the understanding of the reaction behavior.

High performance enzymatic synthesis of a liquid wax ester using immobilized lipase from *C. antartica* has been previously reported by Radzi *et al.*<sup>9</sup> and the optimized reaction conditions was achieved through response surface methodology<sup>10</sup>. The high conversion and productivity of wax ester was further studied in large scale production using batch mode stirred tank reactor with one-multi bladed impeller<sup>11</sup>. Despite all these studies, not much report has been published which deals with the kinetics and mechanism of the reaction particularly at large scale. The kinetic study is useful in obtaining the constants and kinetic parameter values that describes the kinetic behavior. In large scale production, the success of the reaction does not only depend on the optimum condition of reaction process, but also on the physical process of the reaction. The objective of the present study was to analyze the kinetics of the esterification of oleic acid with oleyl alcohol to wax ester using simplified model in stirred tank reactor using commercial lipase. Two different types of impeller were used to create different mixing conditions. The reactions were carried out at different variables such as amount of catalyst, temperature and reaction time.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Novozyme 435 as 10,000 PLU/g (from *C. antartica* lipase immobilized onto macroporous acrylin resin) was obtained from NOVO (Malaysia). Oleyl alcohol (purity >60.0%), oleic acid (purity >85.0%) and an authentic standard of oleyl oleate (purity >99.0%) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other reagents were of analytical grade and used as received.

### 2.2 Wax ester synthesis

The reaction system in a screw-capped vial consisted of 2 mmol of oleic acid, 4 mmol of oleyl alcohol, 2.0 mL of hexane and 0.3 g of Novozym 435. The mixture was incubated at 37°C using a horizontal water bath shaker. The agitation speed was set at 150 rev/min and the reaction mixture was

continuously reacted for 60 min. The reaction was then terminated by dilution with 7.0 mL of ethanol/acetone (1:1 v/v).

### 2.3 Conversion of wax ester

The conversion of wax ester was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with 0.1 M NaOH in an automatic titrator (Radiator meter, ABU 90).

Conversion of wax ester = [Volume of NaOH used (without enzyme) – Volume of NaOH used (with enzyme)] / Volume of NaOH used (without enzyme)

### 2.4 2 L stirred tank reactor

Wax ester synthesis catalyzed by Novozym 435 was also carried out in a 2l stirred tank reactor (Biostat MD, B. Braun, Melsungen, Germany) equipped with temperature and agitation control systems. The reactor consisted of a double jacketed borosilicate glass vessel, four baffles and stainless steel top plate with several opening ports for sampling and electrodes placing. The diameter of the vessel is 13.0 cm with the operating liquid height-to-vessel diameter ratio = 1.04. The reactor was equipped with a single impeller located at 3.0 cm from the oval bottom of the vessel.

Initially, the reactor was set up to the required temperature condition. The reaction was started by putting 600 mmol of oleic acid, 1200 mmol of oleyl alcohol, 90 g (900,000 PLU) of Novozym 435 and 600 ml of hexane into the vessel. At regular time interval, samples were withdrawn through the sampling port. After each sample collection, the reaction mixture was diluted with ethanol:acetone (1:1, v:v).

### 2.5 Effect of reaction time

The effect of reaction time in the wax ester synthesis was investigated by varying the reaction periods (5, 10, 15, 30, 45 and 60 min) in a 3.5 ml screw-capped vial and 2 l stirred-tank reactor. The temperature within a reactor was controlled at 37°C and the reaction rate was maintained for 1 h at 0.393 m/s of impeller tip speed. The conversion of wax ester was determined as described above.

### 2.6 Effect of enzyme amount

The reactions in a reactor were carried out using various amount of enzyme (50, 60 and 90 g) while fixing the other conditions. Samples were collected at varying reactions periods of 60 min. The conversion of wax ester was determined as described above.

### 2.7 Effect of reaction temperature

The reaction mixtures in a reactor were also incubated at various reaction temperatures (30, 37, 40, 45 and 50°C). Samples were collected at varying reactions periods of 60 min. The conversion of wax ester was determined as described above.

### 2.8 Effect of impeller design and tip speed

In order to study the effect of impeller tip speed on the reaction rate in a reactor, the enzymatic synthesis of wax ester was carried out using tip speeds of 0.262 m/s, 0.523 m/s, 0.785 m/s and 1.047 m/s, which corresponded to agitation speeds of 100, 200, 300 and 400 rev/min. Two types of impeller were used in the reactor operation study; Rushton turbine (six-bladed disc) and AL-hydrofoil. The configuration of the reactor equipped with different design of impellers as described in Table 1.

The Rushton turbine impeller is a type of radial flow impeller, which contains 6 impeller blades which are set at a vertical pitch. The liquid flow from the blades is directed towards the walls of the reactor, along the radius of the reactor. The impeller creates high shear conditions by the formation of vortices in the wake of the impeller and the high shear is effective in improving the mass transfer rate. The AL-hydrofoil impeller is an axial flow impeller blades pitched at an angle and thus directs the liquid flow towards the base of the reactor. The impeller is effective at lifting solids from the base of the reactor and exhibited low shear properties. Low shear conditions are achieved by pitching the impeller blades at an angle and by making the edge of the impeller blades thin and smooth.

**Table 1** Configurations of the 2 l Stirred-tank Reactor and Impeller Design.

Configuration	Rushton Turbine	AL-hydrofoil
$D_i$ (cm)	5.00	5.00
W (cm)	1.00	1.90
$W/D_i$	0.20	0.38
$D_t$ (cm)	13.02	13.02
L (cm)	12.50	12.50
B (mm)	10.14	10.14
$D_i/D_t$	0.38	0.38

$D_i$ : impeller diameter, W: impeller width,  $D_t$ : tank diameter, L: Liquid height, B: Baffle width.

Impeller tip speed was calculated using equation (1):  
 Impeller tip speed (m/s) =  $\pi ND$  (1)

### 2.9 Kinetic model

A simplified model based on a reversible model with second-order equations, with forward and the reverse reactions, were considered<sup>(7,8)</sup>. The kinetic model obeys a two-substrate reversible reaction mechanism and the mechanism is as follows;



The reaction rate equation was expressed as:

$$r = -dAc/dt = k_1AcAo - k_{-1}EsW \quad (3)$$

The integrated rate equation corresponding to this mechanism results in the parametric Eq. (4), containing two parameters ( $\alpha$  and  $\beta$ ) that can be determined by optimization.

$$X = [\alpha(1 - e^{\beta t})] / [e^{\beta t} [1 - \{1 + (Ac_0/Ao_0)\alpha\} + 1]] \quad (4)$$

The rate constant of the direct reaction can be calculated using Eq. (5):

$$k_1 = \beta / [(Ac_0 + Ao_0) - (2Ao_0 / \alpha)] \quad (5)$$

The kinetic model was fitted to the experimental data by non-linear regression with a Marquadt algorithm model using Sigmaplot (ver11) computer software for the optimization. The computer program was used as a search method to minimize the sum of squares of the differences between the predicted and measured values. The predicted values were then used to simulate the wax ester conversion. The deviations between the experimental and calculated data were determined to be significant or non-significant through statistical analysis. Analyses of variances for data obtained from different operating conditions experiments were performed. Duncan multiple range tests were used to determine significance among treatment means. Significance was declared at  $P < 0.05$ .

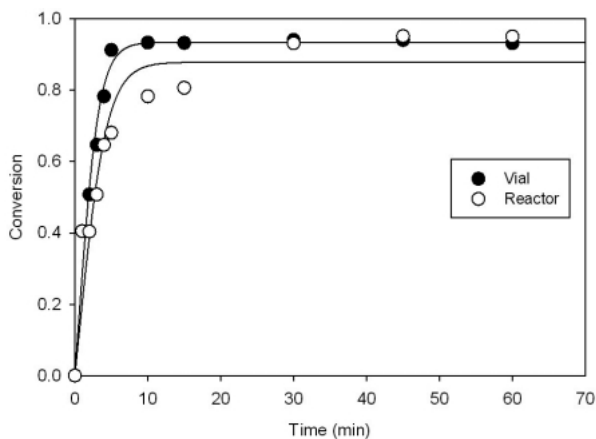
## 3 RESULTS AND DISCUSSION

### 3.1 Effect of reaction time

A good agreement between the experimental data and the values of the simulation of the conversion calculated according to Eq. (4) was observed, and the kinetic constants were summarized in Table 2. The fitness of the proposed model on the synthesis of wax ester in screw-capped vial and stirred tank reactor to experimental data is shown in Fig. 1. The calculated data fitted well to the experimental data and statistical analysis indicates that the deviations, in all cases, are not significant at probability of 5%. The progress curve is a good initial indicator for enzyme performance and reaction progress. The conversion of product was increased during the first 5 min and relatively constant up to 60 min of reaction time in the screw-capped vial. In the reactor, the conversion of wax ester was increased slowly within the first 30 min and remains constant after the initial time. The constant range after the initial time in both scale of reaction was due to the production of by-product, namely, water molecule, which was increased in an enough maximum value (after 30 min) to

**Table 2** Estimated Kinetic Rate Constant of Wax Ester Synthesis at Various Scale and Reaction Conditions.

Reaction Conditions		$\alpha$	$\beta$	$k_1$ ( $M^{-1}min^{-1}$ )	Error of Estimates	$R^2$
Screw-capped Vial		0.934	0.709	0.553	0.0493	0.9768
2L Stirred Tank Reactor		0.879	0.522	0.336	0.0901	0.9158
Catalyst (g)	50	0.247	0.096	0.007	0.0152	0.9985
	60	0.833	0.076	0.042	0.0323	0.9910
	90	0.943	0.197	0.158	0.0226	0.9503
Temperature ( $^{\circ}C$ )	30	0.835	0.076	0.038	0.0324	0.9910
	37	0.943	0.197	0.099	0.0150	0.9985
	40	0.943	0.237	0.119	0.0199	0.9974
	45	0.931	0.619	0.310	0.0205	0.9971
	50	0.940	0.612	0.306	0.0205	0.9971
Rushton Turbine Impeller (m/s)	0.262	0.938	0.425	0.336	0.0196	0.9974
	0.523	0.946	0.591	0.481	0.0081	0.9995
	0.785	0.946	0.647	0.527	0.0092	0.9994
	1.047	0.951	0.927	0.769	0.0072	0.9996
AL-hydrofoil Impeller (m/s)	0.262	0.903	0.447	0.313	0.0403	0.9878
	0.525	0.943	0.947	0.763	0.0081	0.9995
	0.785	0.947	0.739	0.604	0.0063	0.9997
	1.047	0.949	0.808	0.665	0.0051	0.9998

**Fig. 1** Comparison of Calculated and Experimental Data on the Effect of Reaction Time on Enzymatic Synthesis of Wax Ester in Screw-capped Vial and Stirred Tank Reactor.

Reaction condition: temperature;  $37^{\circ}C$ , agitation speed; 150 rev/min or 0.393 m/s, molar ratio of oleyl alcohol to oleic acid; 2:1 and enzyme amount; 90 g. Solid lines represent data calculated according to Eq. 4.

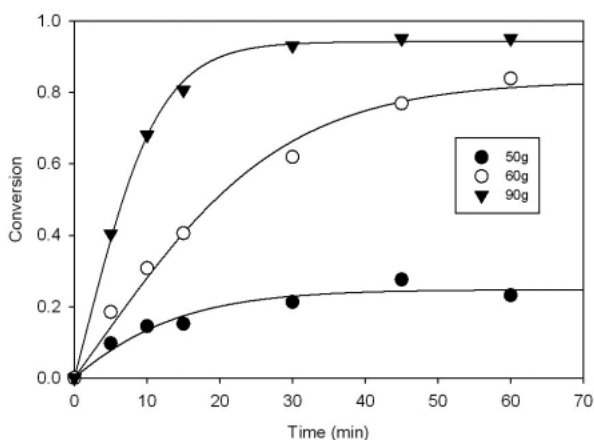
accommodate the reverse hydrolysis process<sup>12</sup>.

Generally, the conversion of wax ester was increased with increasing reaction time. The optimum time of the reaction was slightly different as compared to the screw-capped vial. The calculated data of large scale production also showed lower maximum conversion of wax ester value after 30 min of reaction compared to screw-capped vial. Furthermore, lower rate constant ( $0.336$   $1/M.min$ ) was obtained in the stirred tank as compared to screw-capped vial. This may be due to that the reaction system involved a large amount of substrates and at unfavorable conditions for the conversion. In large scale production, more time was needed for the reaction to achieve an equilibrium state as well as completing the mixing. Furthermore, the conversion of wax ester was decreased due to the large amount of water produced after 30 min of reaction. It is important to note that the reaction in a reactor was in sealed condition and with no removal of water. Similar finding was also reported by Mat Hadzir *et al.*<sup>13</sup> in the synthesis of wax ester by enzymatic alcoholysis reaction between triolein and oleyl alcohol. On the other hand, the experimental data of the reaction in large scale is superior to the calculated data after 30 min reaction time. This phe-

nomenon could be explained by the use of simplified kinetic model which may unable to capture full behavior of the reaction after 30 min of reaction using large amount of substrate. However, this model could be used to describe the reaction system at preliminary stage using different operating conditions<sup>7</sup>.

### 3.2 Effect of enzyme amount

The effect of enzyme amount on the synthesis of wax ester in stirred tank reactor is shown in Fig. 2. Generally, the rate of kinetic constant increased with the increment of enzyme amount (Table 2). Enzyme quantities corresponding to 50 g to 90 g were added and assayed at various time intervals. The conversion increased rapidly from 50 g (0.332) to 90 g (0.952) of Novozym 435 after 60 min reaction time. However, at 90 g of enzyme amount, after 30 min reaction time the conversion remained constant due to the reaction that has achieved a saturation point. In this case, substrate limitation could be the reason. Enzyme loading plays a crucial factor especially in term of production cost in the enzymatic synthesis<sup>14</sup>. Thus, the influence of enzyme loading on Novozym-catalysed synthesis of wax ester shall be assessed to determine the minimal amount necessary for achieving high conversions. In esterification reaction, the amount of enzyme influenced the total reaction time, which was required to achieve a desired conversion. However higher enzyme loading will restrict the substrates and the enzyme to move freely. Furthermore, it may cause diffusion and mass transfer limitation<sup>15</sup>.



**Fig. 2** Comparison of Calculated and Experimental Data on the Effect of Amount of Enzyme on the Enzymatic Synthesis of Wax Ester in a Stirred Tank Reactor.

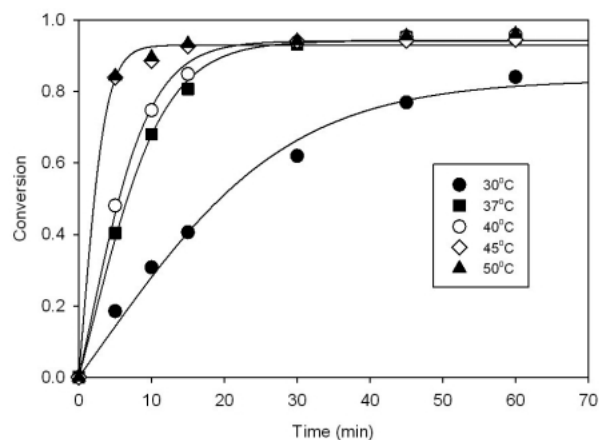
Reaction condition: agitation speed; 0.393 m/s, molar ratio of oleyl alcohol to oleic acid; (2:1) and temperature; 37°C. Solid lines represent data calculated according to Eq. 4.

### 3.3 Effect of temperature

Figure 3 is a plot of conversion versus time for five different temperatures ranging from 30-50°C at various time intervals. A good correlation between the kinetic model and experimental values was observed. Conversion was found to increase with increasing reaction temperature. Changes in the reaction temperature can affect the activity and stability of the enzymes and thus the rate of reaction<sup>16</sup>. The conversion was increased with increasing temperature from 30 to 50°C suggesting that the energy received from the heat of higher temperature was used to increase the frequency of collision of lipase to substrates<sup>16</sup>.

Higher temperature also promotes collisions between enzyme and substrate molecules to result in accelerated rates of reaction<sup>17</sup>. The natures of some enzymes also render them more effective at higher reaction temperature. The maximum conversion of wax ester was obtained at around 45-50°C (0.945-0.960) after 60 min of reaction time. High conversion was also attributed to the enzyme immobilization, which had an advantage of conferring stability to the lipase.

However, after 60 min reaction at 50°C, the conversion (0.960) was slightly higher compared to that obtained at 45°C (0.945). At this temperature (50°C), a higher water evaporation rate may shift the position of the equilibrium to the product side, in a shorter time as compared to reaction at 45°C. This enzyme profile was also simultaneously influenced by other factors such as solvent hydrophobicity, types and relative amount of reactants, enzyme amount and moisture content of reaction medium<sup>18</sup>.



**Fig. 3** Comparison of Calculated and Experimental Data on the Effect of Reaction Temperature on the Enzymatic Synthesis of Wax Ester in a Stirred Tank Reactor.

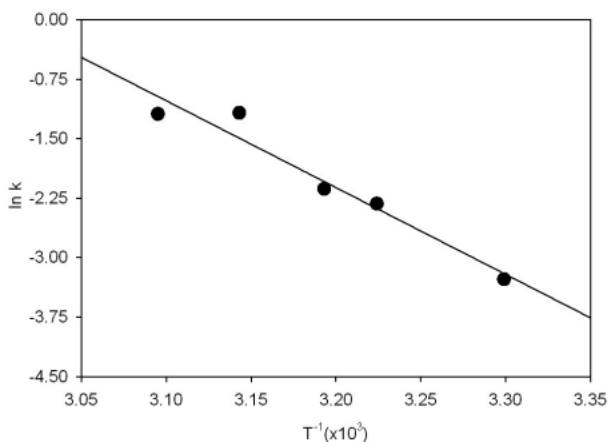
Reaction condition: agitation speed; 0.393 m/s, molar ratio of oleyl alcohol to oleic acid; 2:1 and enzyme amount; 90 g. Solid lines represent data calculated according to Eq. 4.

Generally, an increase in temperature improves solubility of a compound and reduces viscosity resulting in enhancement of the reaction rate. However, high temperatures may also cause enzyme denaturation and so, compromise the final conversion. At low temperature on the other hand, solubility was reduced with the subsequent high viscosity causing mass transfer limitations, retarding reaction rate and lowering final conversions<sup>19</sup>. Lipases are proteins and thus, usually begin to denature at temperature exceeding 40°C in aqueous media. However, immobilized lipases such as Novozym 435 showed good thermal stability at high temperature<sup>20</sup>.

Applying the Arrhenius equation and plotting  $\ln k$  versus  $1/T$  as shown in Fig. 4 and Table 3 for the forward reaction, the activation energy shows a value of 21.77 Kcal/mol and the pre-exponential factor is  $2.04 \times 10^{14}$ . The effect produced by variation of temperature on the constant is given by Van' t Hoff' s equation, which permits the estimation of enthalpy changes:

$$d \ln k/dt = \Delta H^{\circ}/RT^2 \quad (6)$$

The activation energy can be estimated from Arrhenius



**Fig. 4** Arrhenius Plot for the Synthesis of Wax Ester by Oleic Acid and Oleyl Alcohol for the Forward Reaction.

**Table 3** Thermodynamic Parameters, Pre-exponential Factor and Activation Energy.

Parameters	Values
$\Delta H^{\circ}$ (Kcal/mol)	21.15
$\Delta S^{\circ}$ (cal/mol.K)	52.07
$A$ (l/mol <sub>cat</sub> min)	$2.04 \times 10^{14}$
Activation energy, $E_a$ (Kcal/mol)	21.77

equation:

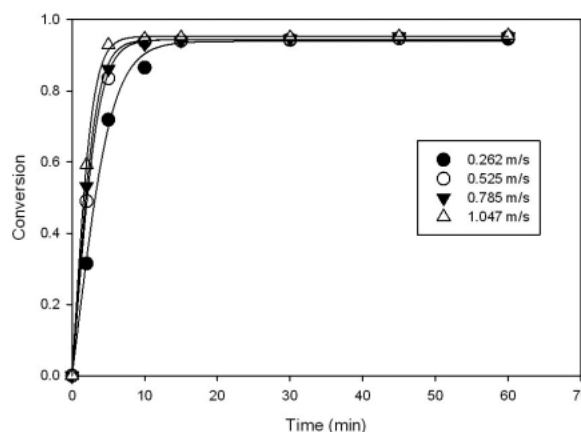
$$\ln k = \ln k_0 - (E_a/R) (1/T) \quad (7)$$

The thermodynamic parameters in the process, using the Van' t Hoff equation, the enthalpy and entropy are 21.15 Kcal/mol and 52.07 cal/mol.K, respectively.

### 3.4 Effect of impeller design and tip speed

The main factor that contributes to the success of large scale processes is efficient mixing of substrate to produce the desired product. Agitator system is the important part in a reactor system to be optimized in order to obtain complete mixing of the substrate and enzyme. The use of multi-bladed Rushton turbine impeller primarily produces radial flow and a high energy dissipation density in the proximity of the agitator and axial flow impeller (AL-hydrofoil) is particularly useful when strong vertical currents are required<sup>21</sup>.

Figure 5 depicts the effect of impeller tip speed in the range of 0.262 to 1.047 m/s on the esterification reaction using Rushton turbine impeller. Conversion of wax ester was slightly increased with increasing speed. The fitness of the proposed kinetic models was clearly shown. Interestingly to note that, from Table 2, the kinetic constants were also increased with increasing the impeller tip speed. The constants were increased gradually with increasing impeller tip speed from 0.262 m/s (0.803 1/M.min) to 0.525 m/s (0.951 1/M.min) and remain constant until 0.785 m/s (0.966 1/M.min). It was observed that substantial enzyme

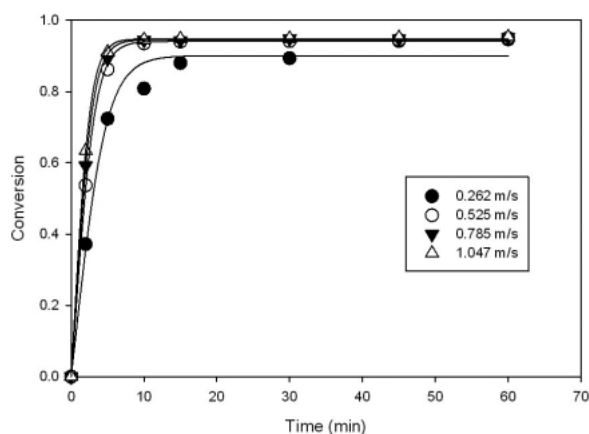


**Fig. 5** Comparison of Calculated and Experimental Data on the Effect of Impeller Tip Speed on the Enzymatic Synthesis of Wax Ester Using Rushton Turbine Impeller in a Stirred Tank Reactor. Reaction condition: enzyme amount; 90 g, molar ratio of oleyl alcohol to oleic acid; (2:1) and temperature 37°C. Solid lines represent data calculated according to Eq. 4.

particles were left at the bottom of reactor vessel at low speed resulting inefficient mixing between enzyme molecules and substrates. Agitator plays a major role in a molecule movement. Higher agitation speed can increase the movement of molecules, thus results in high interactions of enzymes to substrates. Moreover, it will also increase external mass transfer rates between the bulk phase of the reaction mixture and the surface of enzyme<sup>22</sup>.

Figure 5 and 6 show the influence of varying impeller tip speeds on the production of wax ester using Rushton turbine and AL-hydrofoil impeller, respectively. The samples were assayed at varying reaction period from 5 to 60 min of reaction time. Increasing an agitation speed led to an increase in conversion of wax ester from 0.262 to 1.047 m/s for each impeller. Noor *et al.*<sup>23</sup> reported that increase in agitation speed cause substantial increase in specific interfacial area between the substrate and the enzyme present in the non-aqueous phase by reducing the droplet size.

At 0.262 m/s, Rushton turbine impeller achieved an equilibrium constant of reaction at 15 min reaction time, whereas AL-hydrofoil needs longer time to reach an equilibrium state (45 min). Although, the maximum conversion was achieved by AL-hydrofoil impeller at 60 min reaction time, the differences in conversion for these two impellers was only 0.4% and not statistically different. However, the Rushton turbine impeller shows a significant conversion results by increasing the impeller tip speed from 0.262 to 1.047 m/s due to shorter time needed to achieve an equilibrium state. This was different with Al-hydrofoil impeller whereby, at initial impeller tip speed from 0.262 to 0.785



**Fig. 6** Comparison of Calculated and Experimental Data on the Effect of Impeller Tip Speed on the Enzymatic Synthesis of Wax Ester Using AL-hydrofoil Impeller in a Stirred Tank Reactor. Reaction condition: enzyme amount; 90 g, molar ratio of oleyl alcohol to oleic acid; (2:1) and temperature 37°C. Solid lines represent data calculated according to Eq. 4.

m/s, the conversion was slowly increased from 5 to 30 min before was maintained thereafter. This may due to the fact that the reaction system in the reactor vessel was agitated at non-optimal condition.

It could be seen that the enzyme particles remained at the bottom of the reactor vessel at low speed of 0.262 m/s. At low agitation speeds, diffusional (mass transfer) and hydrodynamic (interfacial area) effects can be a reason for the lower initial esterification rates values achieved<sup>23</sup>. However, at 1.047 m/s both impellers show a high conversion of wax ester even at 10 min reaction time due to the complete mixing. Based on the results, an impeller tip speed of 1.047 m/s could be selected for the subsequent studies in the larger scale of stirred tank reactor. Similar finding was also reported by Olivera *et al.*<sup>24</sup> in the production of large scale enzymatic synthesis of ethyl ester using a perforated rotating disk reactor.

#### 4 CONCLUSION

Kinetic analysis of esterification of oleic acid with oleyl alcohol to wax ester can be performed using a simplified model based on a reversible model with second-order equation with both forward and reverse reactions. The kinetic parameter values generated may describe the process at different operating variables in more knowledgeable manner. The highest conversion was obtained at temperature 45°C, enzyme loading of 90 g and impeller tip speed of 1.047 m/s using Rushton turbine impeller in a stirred tank reactor. AL-hydrofoil impeller could also be recommended for use in solid suspension system with no significant difference on the esterification rate as compared to Ruston turbine impeller at optimum impeller tip speed.

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