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Development and Validation of a Kinetic Model for Enzymatic Hydrolysis Using *Candida rugosa* Lipase

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Abstract

Biochemical processing involving enzymatic catalysis of hydrolysis reactions of oils and fats must overcome significant technological barriers before the full benefits of the technology can be realized. Owing to their selectivity and mild reaction conditions, lipases are becoming increasingly important as biocatalysts provided that their kinetics and optimum reaction conditions are well-understood. In this study we report on the development and validation of a kinetic model for the degradation of oils using *Candida rugosa* lipase, from which a better understanding of the influence of different reaction conditions on hydrolysis kinetics is elucidated. Variations of reaction temperature, mixing speed, enzyme loading and substrate concentrations yielded a maximum lipase activity of 25.67 lipase units (LU), and an activation energy of 4.32 Kcal/gmol. Significantly higher enzyme loading at 0.7 mg/ml was achieved, a 169% increase over most recently reported loading by other investigators. Optimum operating ranges for medium pH and substrate concentration were established to be 7.5 to 8.5, and 30 to 55%, respectively. Reported findings mark a significant improvements over previously reported much narrower ranges of 8.0 for pH and 30 to 43% for the substrate concentration under similar experimental conditions. Developed kinetics model closely predicted and matched experimental results, rendering it suitable for biochemical engineering design application.

Keywords: Enzyme; Lipase; Hydrolysis; Kinetics; Olive oil

Nomenclature

v	Rate of reaction (mol ml min^{-1})
k_p	Adsorption rate constant (m^2 min^{-1})
k_d	Desorption rate constant (min^{-1})
k_i	Reaction rate constant (min^{-1})
k_{-1}	Reaction rate constant (min^{-1})
k_{cat}	Catalytic rate constant (min^{-1})
E	Free enzyme (mole/total reactor volume) (mol ml)
K_e	Equilibrium constant of E*S (mol ml)
E_t	Total active enzyme (mol ml)
a	Specific free interfacial area (m^{-1})
E^*	Penetrated enzyme (mole/total interfacial area) (mol m^{-2})
S	Bulk substrate concentration ('mole/total reactor volume) (mol ml)
E^*S	Enzyme/substrate complex (mole/total interfacial area) (mol m^{-2})
P	Bulk product concentration (mole/total reactor volume) (mol ml)
a_t	Total specific free interfacial area (m^{-1}).
ϕ	Oil volume fraction in the emulsion.
D_0	Emulsion droplet diameter (m).
α	Constant defined by Eqn. 3.12.
ω	Mixing speed (rpm).
T	Temperature (°C).
m	Constant defined by Eqn. 3.12.
k	Constant defined by Eqn. 3.12.
n	Constant defined by Eqn. 3.12.

Introduction

Due to the importance of hydrolysis reactions, lipase enzymes are the subject of exhaustive research for many medical applications, biological treatment processes, and pharmaceutical synthesis [1-3]. Glycerols and fatty acids produced from oil degradation are significantly important and had been used in many applications. Although the use of enzymes such as lipase in fatty acids production and glycerols manufacturing is well-known, industrial applications which require high conversion (99%) are hindered by the application of high pressure, high temperature, and the very long reaction times [4,5]. These severe operating conditions are expensive and in most cases produces dark fatty acids as a result of side polymerization and the formation of undesired by-products. Additionally, treatment and purification of final products by complex distillation methods complicates the process and add further energy concerns [4]. Research is needed to develop an efficient, simple, and low cost methods to produce clean fatty acids.

Interestingly, some reaction parameters do not consume much energy and yet have great impact on the final products. Tailoring reaction parameters is a key factor to debottleneck the difficulties that limits the application of an important reaction such as oil hydrolysis [6,7]. Lipase is a water-soluble enzyme that exhibits high activity when contacted with water and some organic solvents [8]. This important characteristic allows the control of the hydrolysis reaction conversion in applications with emulsified water/oil phases. By tailoring the substrates' droplets sizes through manipulating mixing speed, specific contacting area becomes an interesting factor in controlling the

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hydrolysis reaction [8,9]. Mixing speed and substrate concentration both significantly affect the reaction conversion and the products quantity and quality [10]. A previous study conducted by Al-Zuhair et al. on palm oil hydrolysis using lipase assumed that the area of contact between the enzyme and substrate stays constant, even if the mixing speed remains constant. This assumption can be valid only if high substrate-to-enzyme concentrations or an excellent organic solvent is used [8]. Otherwise, the interfacial contacting area has a significant effect on the lipolysis reaction. According to Tsai et al. the contact area between the enzyme and the substrate is directly affected by mixing speed [10]. In addition, the use of an organic solvent in hydrolysis reactions is not an efficient choice in most cases since lipase enzyme is insoluble in most of these materials and it suspends at the surface [11]. Moreover, some applications, such as enzymatic and biological water treatment, add more complexity to the application of such reactions, not only for the fact that they include other solvents like water, which affects lipase solubility, but also because of the variety of process designs which includes different agitation speeds. There are concerns about the validity of previous models that do not consider these important factors [8,12]. Moreover, substrate concentration is a key factor that affects oil hydrolysis reactions. In an ideal conditions, the rate of reaction should increase with the increase of substrate concentration; however, with high substrate loading other issues arises. High substrate loading tends to clog enzymes' active sites causing what is popularly known as substrate inhibition [13].

Considering all addressed issues relating to oil hydrolysis using lipase enzyme, determining optimum conditions and parameters is very crucial for efficient oil hydrolysis applications. In this study, focus is placed on the effect of the interfacial contacting area, substrate concentration, temperature, pH, and enzyme loading on olive oil hydrolysis kinetics. In order to study all of these parameters, one must determine the reaction rate and the amount of fatty acids liberated form the hydrolysis of a specified amount of oil by lipase [14,15].

Studying olive oil hydrolysis kinetics enables a clear understanding of important reactions such as esterification, transesterification, peptide synthesis, and selective acylation of glycols [2,16]. Glycerin produced from esterification re-acylation is used in cosmetic applications, pharmaceuticals, and animal food industries. In addition, long-chain fatty acids produced such as tripalmitin and triolein are widely used in many industrial applications [3,17-19].

The reaction mechanism proposed here for olive oil lipolysis reaction is a continuum of previous work conducted by Alzuhair et al. and Tsai and Chang [8,10]. The underlined hypothesis is that lipase enzyme molecules are absorbed into the water interface to yield the activated enzyme, E*. Lipase absorption is assumed to be proportional to the free specific area, a, and free enzyme concentration, E. The enzyme-substrate complex, E*S, is assumed to be the product of the interaction of the substrate, S, with the free enzyme active sites. The generated product, P*, produced from the complex, E*S, at the interface and the enzyme, E*, is reproduced again hence it will not be consumed in the reaction. Subsequently, the product, P*, is desorbed from the interface to the bulk phase as final product, P. The simplified mechanism steps are illustrated in Eqns. (1) to (3),

$$E + a \xleftrightarrow{k_a, k_d} E^* \quad (1)$$

$$E^* + S \xleftrightarrow{k_1, k_{-1}} E^*S \quad (2)$$

$$E^*S \xrightarrow{k_{cat}} E^* + P^* \quad (3)$$

As proposed by Tsai and Chang, a quasi linear (steady state) was assumed for the enzyme-substrate adsorption/desorption system. Proportional relation between the interfacial product, P*, and the final desorbed product, P, was also assumed [20]. All assumptions stated above were applied to the model, hence, Eqns. (1) to (3) could be rewritten as:

$$k_a E.a - (k_d + k_1 + k_{cat})E^*S = 0 \quad (4)$$

$$k_1 E^*.S - (k_{-1} + k_{cat})E^*S = 0 \quad (5)$$

$$a_t = a + A_m(E^* + E^*S)a_t \quad (6)$$

$$E_t = E + a_t(E^* + E^*S) \quad (7)$$

whereas, $P*=CP/a_t$ as reported by Al-Zuhair et al. [8]. By applying assumptions stated previously, the rate of reaction for products formation, v would be:

$$v = \frac{dP}{dt} = \frac{a_t}{C}.\frac{dP^*}{dt} = \frac{a_t}{C}k_{cat}E^*S \quad (8)$$

Eqns. (4) to (8) must be solved simultaneously to yield the final expression of the reaction rate. Similar derivations have been investigated in the literature Al-Zuhair et al.; Mukataka et al.; Tsai and Chang [8,20,21]. Following the same method and applying assumptions stated above, Eqns. (1) to (8) could be solved and simplified to yield the following:

$$v = \frac{k^*_{cat}E_tS}{K_e\left[\frac{k_d}{k_a a_t^2} + 1\right] + S} \quad (9)$$

Where, $K_e = (k_{cat} + k_{-1})/k_1$ and $k^*_{cat} = k_{cat}/\mathrm{C}^*$

Experimental Part

Materials

Candida rugosa lipase was purchased from Sigma Aldrich Co., US. Purified high quality olive oil was supplied locally from Hail Agricultural Development Co., KSA. Gum Arabic purchased from Sigma Aldrich Co., US. Absolute ethanol, hydrochloric Acid, sodium hydroxide, potassium and sodium phosphate buffer, phenolphthalein, and other chemicals that have been used were supplied from local companies. All chemicals used were analytical grade reagents.

Equipment

A batch reactor with 250 ml Litre working volume, water bath circulator, and a stirrer were used for the hydrolysis reactions, see Figure 1. Reaction temperature was controlled using the water path jacket via an external controller, and an electrical motor was used to agitate the reaction mixture. By varying the impeller speed, substrate droplets' size was varied and controlled precisely. Subsamples were extracted using an injection sampler. During the reaction and with adjusted time periods, subsamples were collected out of the reactor for quenching. The effects of other conditions such as pH, substrate concentration, and enzyme loading on the reaction rate and lipase activity were investigated using a similar setup.

Reaction rate and enzyme activity measurements

Lipase activity was measured by lipase units (LU), defined as the quantity of lipase capable of degrading 1 μ mol of olive oil per min, at standard reaction conditions recommended by assay protocol (temperature of 37°C, olive oil/gum Arabic substrate, agitation speed of 200 rpm, and

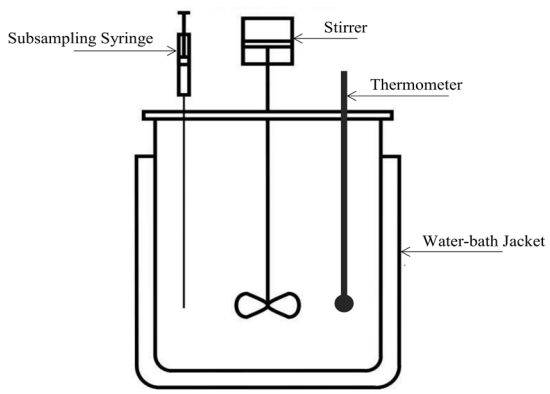

Figure 1: Experimental setup for the lipolysis of olive oil emulsion. Water jacket is used to control reaction temperature. Stirrer impeller is used to mix the emulsion substrate during the reaction. In a pre-adjusted periods, sub-sampler syringe is used to collect the samples.

sodium phosphate buffer for pH of 8.0) [15]. Lipase units and enzyme specific activity is represented by Eqns. (10) and (11) below:

$$Sp_{act} = \frac{v_0(v + v_E)}{m_E} \quad (10)$$

$$LU = Sp_{act} \times m_E \quad (11)$$

Where,

Sp_{act} is the specific activity (mol min^{-1} mg^{-1})

v_o is the initial reaction velocity (mol ml min^{-1})

v is the substrate volume (ml)

v_E *is the enzyme volume (ml)*

m_E *is the amount of enzyme (g)*

It is well-known that the hydrolysis of olive oil produces free fatty acids and glycerol as shown in Figure 2. Since lipase activity is defined based on the amount of fatty acids produced, liberated acids are the key factors for reaction rate determination in this study. Direct titration of the fatty acids resulting from oil hydrolysis with diluted NaOH (0.05 N) was a suitable and accurate procedure to calculate reaction rate and lipase activity. The amount of consumed base represents liberated free fatty acids from the lipolysis reaction.

As recommended by the assay protocol [15], enzymatic reaction was conducted using olive oil/gum arabic emulsion as a substrate. To prepare this substrate, 10 g of olive oil and gum arabic were added and mixed in a 400 ml beaker and the volume was brought to 200 ml using 50 mM sodium phosphate buffer with pH of 8.0. The mixture was then gently homogenized for five minutes in a household blender. Lipase was then added to the emulsion mixture and incubated in 37°C water bath with continuous stirring. Samples were withdrawn at regular intervals and quenched immediately in ethanol to break the reaction. Titrating the resulted mixture with NaOH (0.05 N), yielded an accurate determination of the enzyme's specific activity [15].

Hydrolysis reaction was initiated by adding lipase to the substrate emulsion. Ten milliliters of ethyl alcohol was placed in Erlenmeyer flask and three droplets of 1 wt% phenolphthalein indicator were added in each flask to prepare the quenching.

Fifty milliliters of olive oil-gum Arabic substrate was incubated at 37°C for 15 minutes with continuous stirring. After incubation, 50 mg of lipase was added to the emulsion substrate in the batch stirred reactor shown in Figure 1 to start the reaction. Temperature was maintained at 37°C, and constant amounts of 5 ml were sampled from the reactor at a predetermined reaction periods and transferred to an isolated flasks which contained the titration cocktail prepared previously. Immediately, the amount removed was swirled in ethanol to quench the reaction. As a preparatory step for the titration process, quenched samples were turbid and held for 2 hours at room temperature. The flasks' content were titrated using 0.05 M NaOH until a light purple color showed. A plot of free fatty acids concentration (mol/ml) versus time (min), representing initial velocity (v_o) was generated.

Lipase loading, temperature, pH, and mixing speed

The effect of enzyme loading on reaction rate was studied using same procedure stated previously. By fixing the reaction time at 30 min and varying the amount of enzyme from 0.1 mg/ml (lipase/substrate) to 1 mg/ml, the effect of lipase concentration on reaction rate was determined. Holding the other parameters constant, the reaction temperature was varied from 20 to 60°C and the relevant effects on the reaction and activation energy were clearly observed. To study the effect of hydrogen ions, the substrate pH was varied from 4.0 to 9.0 under the same standard conditions. The effect of hydrogen ions was observed and addressed carefully.

Surface area and oils droplet size effects

Many methods have been developed for predicting the interfacial area of oil/water emulsion's droplets [9,21-23]. The famous Calderbank model (Equation 12) for interfacial area estimation was modified by Albasi et al. and later on by Al-Zuhair et al. [8,22].

Calderbank Model:

$$a_t = \frac{6\phi}{D_0} = \frac{\alpha\omega^m\phi T^k}{1 + n\phi} \quad (12)$$

Where, a_t is the total specific free interfacial area (m^{-1}), ϕ is the oil volume fraction in the emulsion, D_0 is the emulsion droplet diameter *(m),* ω is mixing speed (rpm); and α, m, k, and n are constants defined by Eqn. 3.12.

Albasi et al. modified the model and investigated the effect of increasing the oil fraction, φ, on the accuracy of the results [22]. Their proposed model (Equation 13) predicts up to 60% of the oil fraction. Al-Zuhair et al. further modified the model by including the effect of temperature on the interfacial area and droplet size distribution (Equation 14) [8].

$$A = 2.05 \times \omega^{0.745}\phi^{-1.269} \quad (13)$$

$$a_t = \frac{0.024\omega^{0.6}\phi T^{1.7}}{1 + 3\phi} \quad (14)$$

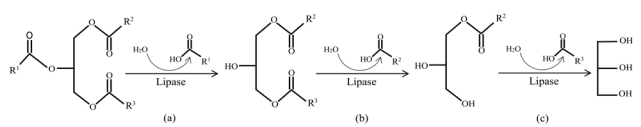

Figure 2: Hydrolysis of triglycerides (oils) into simpler fatty acids and glycerols catalyzed by lipase. (a) Primary hydrolysis of triglycerides into diglycerides, (b) decomposition of diglycerides into monoglycerols, (c) final hydrolysis step into free fatty acids and glycerols [17].

Figure 1 shows the stirred reactor that was used to study the effect of mixing speed and oil drop size on lipase activity. The last model, which was modified by Alzuhair et al. equation 14, was used to determine the droplet sizes and distribution for the oil water mixture [8].

Results and Analysis

In this section, results and analysis for olive oil catalytic lipolysis will be presented. The effect of reaction conditions (temperature, pH, reaction time, and substrate concentration) on the catalytic activity of lipase is presented first. Then the derived kinetic model for oil degradation by lipase is addressed and validated.

Lipase activity

Enzyme activity was studied according to the method described above and mentioned in Pinsirodom and Parkin. As shown in Figure 3, the amount of fatty acids liberated increased rapidly with the reaction rate. Because of the hydrophilic nature of lipase, it is being absorbed into the water phase and gradually started to be activated. As time elapsed, more substrate was introduced to the enzyme active sites and reacted at the interface. Figure 3 indicates that the specific activity, Sp_{act}, for lipase enzyme at the stated conditions reached up to 2.57 μmol/(mg × min). Initial rate of reaction (v_0) was obtained from the linear fitting of the experimental data. Enzyme unit, *LU*, was calculated accordingly and found to be 25.67. Krakowiak et al. reported similar results while studying lipase immobilization on chitosan polyphosphate. They reported an activity of 22 LU [24].

Effect of enzyme loading on reaction rate

Figure 4 depicts the enzyme concentration's effect on the lipolysis reaction, where the reaction mixture reached a saturation point at 0.7 mg/ml; beyond this point, adding more enzymes did not affect the reaction rate. This is due to the substrate saturation, thus, no more substrate was left to be degraded by the extra free lipase. Alzuhair et al. studied *Candida rugosa* lipase and found that the rate of palm oil hydrolysis remained unchanged after 0.1 mg/ml [8]. The low enzyme loading that they reported is most probably due to the high temperature that was used (45°C) which is slightly above the optimum range as seen in Figure 11, and reported by other studies [25]. Saktaweewong et al. reported a maximum lipase loading of 0.26 mg/ml, which is closer to the one observed in this study because of the similar experimental conditions [26]. The main reason behind the inhibition of the enzyme after this critical point is the formation of multilayers at the interface where the reaction takes place. Below this concentration a monolayer dominated the adsorption at the interface and the rate of reaction linearly increased with the lipase concentration (Figure 4). Increasing the enzyme loading increases the layers around the substrate and, therefore, decreases the interaction between the enzyme and the substrate bulk.

Effect of pH on the lipolysis reaction

Figure 5 shows that the enzyme activity remains high at a pH range from 7.5 to 8.5 and decreases at lower and higher values. These results confirm previous work in the literature Krakowiak et al., Sharma et al. and Soares et al. [3,26,27]. Gupta et al. reported an optimum pH of 8.0 when they characterized *Candida rugosa* lipase after immobilization on membranes at nearly similar conditions. The clear decrease of enzyme activity below and above this optimum range is because of the reversible reaction that occurred due to the ionization and deionization of basic or acidic groups in the active sites of the lipase [28].

Table 1: Fitting of experimental data for olive oil hydrolysis to modified Michael-Menten model using Hanes-Woolf and Mathematica software.

Model	Maximum Velocity	Substrate Affinity	R^2
	V_{max}, (μmol/ml.min)	Km (g/ml)	
Hanes-Woolf Model	22.727	0.1364	0.89
Mathematica Fit	20.514	0.0975	0.992

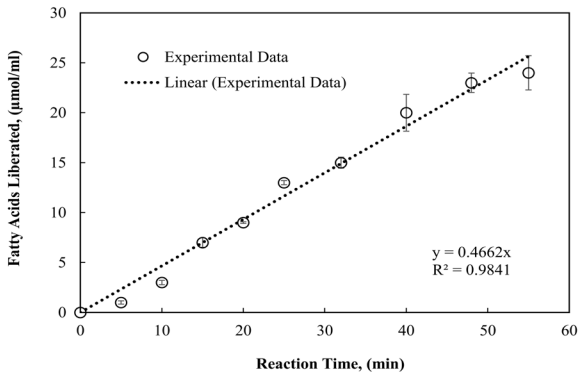

Figure 3: Initial reaction rate (velocity) calculations using linear fit, ([E]=2 g/L (lipase/substrate), ω=200 rpm, T=37°C, pH = 8.0).

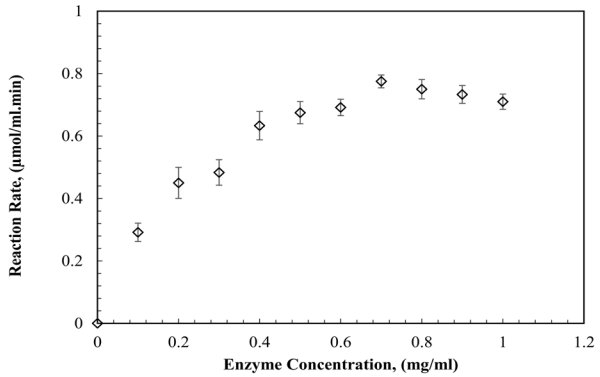

Figure 4: Enzyme concentration effect on the reaction rate and activity, (t=30 min, ω=200 rpm, T=37°C, pH=8.0)..

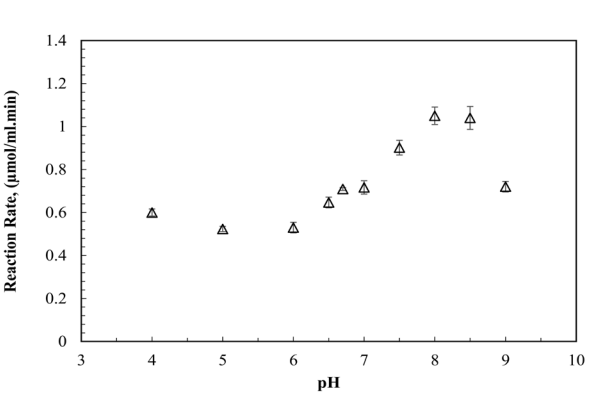

Figure 5: Effect of pH on the reaction rate and enzyme activity, ([E]=1 g/L, t=30 min, ω=200 rpm, T=37°C).

Substrate concentration effect and kinetic modeling

Substrate concentration has a significant effect on lipolysis reactions. Figure 6 depicts the increase in reaction rate with increasing olive oil concentration in the emulsion. This is due to the high enzyme capacity compared to the oil involved at the beginning of the reaction. However, from Figure 8, and at an oil fraction range of 30 to 40%, the reaction rate became constant. A saturation point was observed and the rate of reaction remained unchanged at about 17 mol/(L min). This is because after this critical concentration enzyme active sites cannot absorb more substrate and are completely saturated. Al-Zuhair et al. reported similar results when studying lipase hydrolysis of palm oil under similar conditions. They reported that, after 43% of oil fraction, the reaction rate decreases and the substrate causes inhibition [8]. In this case, increasing olive oil fraction up to 55% did not significantly affect the rate of reaction. It is clear from these results that olive oil substrate has relatively low affinity to the enzyme, since K_m for the enzyme was found to be about 0.097 g/ml. This K_m is quit high as compared to results obtained in the other previous works [29,30].

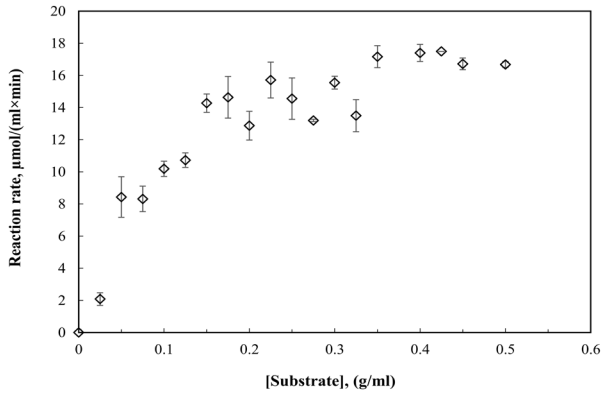

Figure 6: Substrate concentration effect on reaction rate, (t=30 min, ω=200 rpm, T=37°C, pH=8.0).

Experimental data fitting and constants evaluation

Many studies recommend Hanes-Woolf model to fit enzymatic experimental data to a modified Michaelis-Menton Model such as Eqn. 9 ("Kinetic model for the enzymatic hydrolysis of tributyrin in O/W emulsions") [31]. Figure 7 shows results after using Hanes-Woolf fitting. The plot of [S]/v versus [S] yields accurate results as recommended by Nimmo et al. [31]. Using this fit, estimated values for V_{max} and K_m as shown in Table 1 were found to be 22.727 µmol/(ml min) and 0.1364 g/ml, respectively.

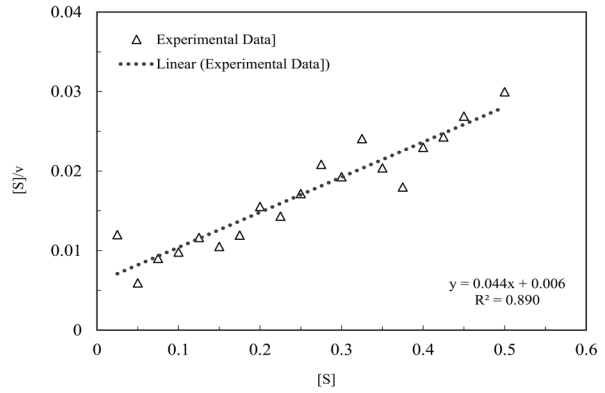

Figure 7: Hanes-Woolf linear fitting for experimental data to Michaelis-Menten model.

A nonlinear fit of the data to the proposed model was directly obtained using Mathematica software. As shown in Figure 8, high correlation coefficient (R^2=0.992) was obtained and the estimated values for V_{max} was about 20.514 µmol/(ml min). This result is very close to the Hanes-Woolf fitting as represented in Table 1. The substrate affinity K_m was found to be 0.0975 g/ml which confirms the results obtained by Al-Zuhair et al. since they report a value of 0.076 g/ml [8]. This slight difference may be caused by two major differences: 1) they studied palm oil as a substrate which has lower affinity; and 2) and they used higher mixing speed since increasing the stirring and mixing decreases the affinity to the substrate concentration (800 rpm) [8,22].

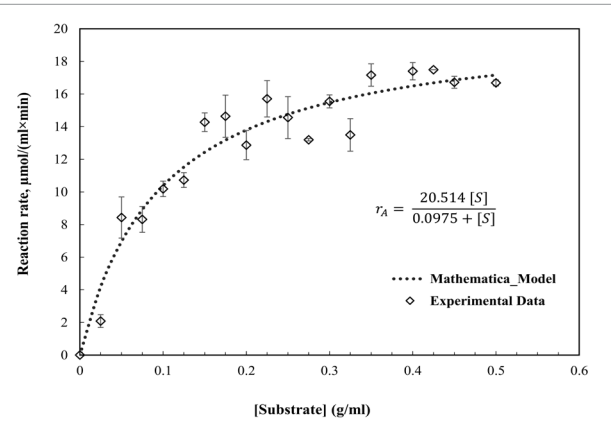

Figure 8: Nonlinear fitting of experimental data to Michaelis-Menten model (Mathematica®).

Effect of mixing speed on the rate of reaction

The effect of mixing speed on the interfacial area between the enzyme and the substrate is shown in Figure. It is well-known that increasing mixing and agitation leads to smaller oil droplets dispersion and subsequently affects the rate of reaction. Equation (14) was used to estimate the specific area for olive oil/water emulsion. As seen in Figure 9, the specific interfacial area increases proportionally with mixing speed. Subsequently, Figure 10 shows that the rate of reaction also increases as the mixing speed increases. However, after 900 rpm no more effect occurs on the reaction rate. At this point the enzymes active sites are fully saturated and no more oils could be added. Many studies reported similar behaviour for oil hydrolysis using lipase, hence, the specific interfacial area has no effect at very high mixing speeds [8,22,32]. By applying Equation 14 for specific interfacial area, new coefficients for Equation 9 could be obtained.

Activation energy and temperature effect

As shown in Figure 11, the reaction temperature had a significant effect on the rate of reaction. Increasing the temperature increased the initial reaction rate rapidly in the range below 40°C, primarily due to two phenomena: (1) lipase's nature as an enzyme which is affected severely by temperature and its activity being high inside this range, and (2) the increasing temperature increases the interfacial area due to the thermal effect and mixing enhancement. Above 40°C, a significant decrease on the reaction rate was observed. This is mainly due to the deactivation (denaturation) of the enzyme. High temperatures affect

the effect of temperature and activation energy on reaction rate was examined using the Arrhenius equation:

$$k_{cat}^{*} = k_o exp\left[\frac{E_{act}}{RT}\right] \quad (15)$$

From Figure 12, values for k_o and E_{act} were determined, and the

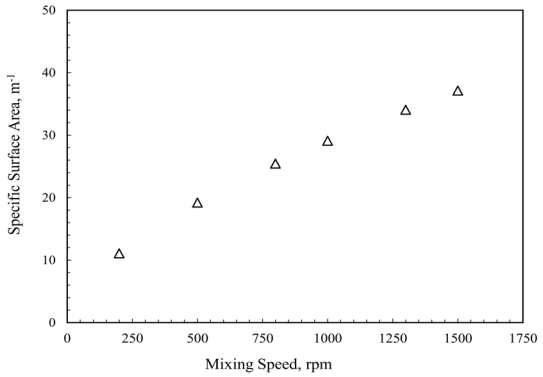

Figure 9: Mixing speed effect on specific interfacial area, (φ=0.05, T=37°C).

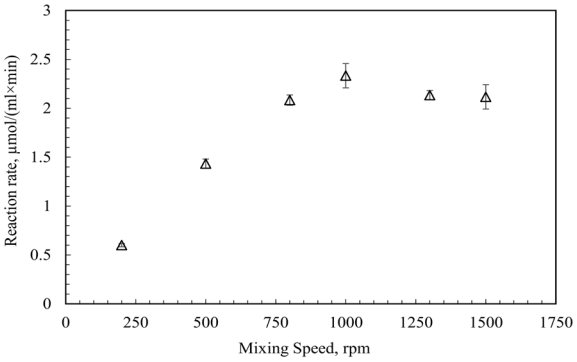

Figure 10: Mixing speed effect on the reaction rate and enzyme activity, ([E]=1 g/L, t=30 min, pH=8, T=37°C).

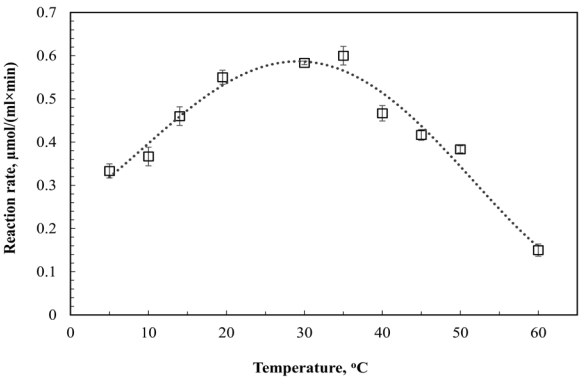

Figure 11: Reaction temperature effect on the reaction rate and enzyme activity, ([E]=1 g/L, t=30 min, ω=200 rpm, pH=8.0)

the enzyme severely, and it is completely damaged outside its ideal temperature range [33].

The reaction constant k_{cat} was estimated from Figure 11, and

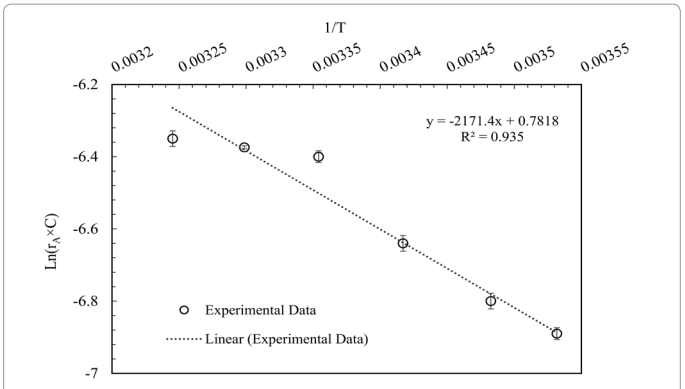

Figure 12: Activation energy for the lipolysis reaction, ([E]=1 g/L, t=30 min, ω=200 rpm, pH=8.0).

activation energy was found to be 4.32 Kcal/gmol. This is reasonably acceptable and closely comparable to literature reported values for enzymatic reactions [33]. Kim and Chung reported an activation energy of 7.0 Kcal/gmol for the hydrolysis of palm oil in reversed micelles system. Al-Zuhair et al. reported an activation energy of 1.2 Kcal/gmol for the hydrolysis of the palm oil using batch stirring reactor [8]. Nevertheless, the closest result in the literature to those reported here is 5.3 Kcal/gmol, reported by Semeriva and Desnulle when they studied the hydrolysis reaction for oil/water emulsions [34].

Discussion and Conclusions

In this study, we derived a kinetic model for enzymatic hydrolysis using *Candida rugosa* lipase, and experimentally verified its response. Model parameters were manipulated and carefully studied to determine the optimum conditions for the catalytic hydrolysis.

The derived model provided a reasonably good fit to the experimental data, with an R^2 value of 0.992. As shown in Table 1, the reported substrate affinity (K_m) was found to be 0.0975 g/ml. This is comparable to what has been reported in previous studies [29,30]. Although, previous reported values are slightly lower than the one we report here, the higher values are the result of using low mixing speed in this study [8]. It is known that increasing mixing speed results in an interactive phase between the enzyme and substrate molecules. Consequently, this increases enzymatic activity in the surrounding microenvironment.

Interestingly, it was observed that after the olive oil fraction exceeded 40%, the rate of reaction became constant at 17 µmol/(ml min). This is due to the formation of multilayers at the interface where the reaction takes place. Below this concentration a monolayer dominated the adsorption at the interface, and the rate of reaction linearly increased. To avoid this phenomenon, either the amount of added enzyme be decreased or the mixing speed must be increased to allow more agitation and refresh the surface of the enzymes. Increasing the mixing speed, however, has its limitations (Figure 10) since the reaction rate remained constant after the mixing speed exceeded 1000 rpm [10]. At this point, the mixed droplets' sizes did not have an overriding effect on the reaction rate and other parameters dominated.

Reaction parameters were varied and adjusted carefully to study their effects. It was observed that lipase activity toward olive oil hydrolysis decreases below and above a pH range of 8 to 8.5. This is due to the reversible reaction that occurs as a result of ionization and deionization of basic/acidic groups in the active sites of the lipase [28].

Temperature was found to be a critical parameter lipase activity. Above 35°C, a significant decrease of the reaction rate was noticed as shown in Figure 11. This is owing to the denaturation of the enzyme since high temperatures destroy and damage the enzyme [33]. A sharp rapid decrease in the enzyme activity immediately after the previous stage is also due to the previous effect and to the fact that the interface between active enzymes and the substrate is blocked by the inactive enzymes. This adds more resistances to the hydrolysis reaction. The activation energy for the hydrolysis reaction was found to be 4.32 Kcal/gmol, in a complete agreement with literature reported values [8,34].

The derived kinetic model and optimized parameters for olive oil hydrolysis by lipase was estimated using both mathematical and experimental data and can be readily used to predict the behaviour of similar hydrolysis reactions under similar conditions.

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