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HYDROLYSIS OF PALM OLEIN TO OLEIC ACID USING IMMOBILIZED LIPASE IN A RECIRCULATED PACKED BED BATCH REACTOR

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ABSTRACT

Hydrolysis of palm olein to oleic acid using immobilized lipase from *Asperigillus niger* with the trade name Lipozyme TL IM was studied in a recirculated packed bed batch reactor (RPBBR). This study was conducted to investigate the kinetics of the enzymatic hydrolysis of palm olein and the potential of substrate inhibition using initial velocity analysis. The temperature and pH were set at 37°C and pH 7 while the stirrer speed was set at 250 rpm and the flow rate used was 0.5 mL. min^{-1}. Hexane was selected as the solvent and gas chromatography was used to analyze the product samples. The range of substrate concentration being investigated ranged from 0.3155 mol.L^{-1} to 0.8412 mol. L^{-1}. The kinetic model of Michaelis-Menten was used to analyze the kinetic data such as the maximum rate of reaction, V_{max} and the Michaelis-Menten constant, K_m. Lineweaver-Burk plot, Eadie-Hofstee plot and Hanes-Woolf plot were used to determine the V_{max} and K_m values and the average values of V_{max} and K_m obtained from these three plots were 0.00122 mol.L^{-1}.min^{-1} and 0.167 mol.L^{-1} respectively. No substrate inhibition was observed for up to the palm olein concentration of 0.8412 mol.L^{-1}.

Key Words : Hydrolysis, Immobilized lipase, Palm olein, Kinetics, Recirculated packed bed batch reactor

1.0 INTRODUCTION

The first Industrial Master Plan of 1985 identified oleochemicals as future growth sector of the palm oil industry. Malaysia's oleochemical production was projected to reach 20% of total world production in 2004-2007. Currently, 90% of Malaysia's oleochemical production is exported. With the rapid development of enzyme technology, considerable attention has been focused on the biotechnological of lipase in the fat and oil industry [1, 2].

Lipases are a special type of enzymes that catalyse the hydrolysis of oils and fats [3]. Recently, enzymic splitting of fats has gained increasing attention, as lipase (triacylglycerol acylhydrolase) is now available at reasonable cost. Further reduction in the cost of the enzyme by genetic manipulation of the microbe producing the enzyme is expected. This would make the enzymic hydrolysis of oils and fats highly attractive. The industrial use of lipase for splitting lipids as an energy-saving process has been addressed in the literature, especially for producing high value-added products. The products, fatty acids and glycerol are basic raw materials for a wide range of applications. Fatty acids are used as a feedstock for the production of oleochemicals such as fatty alcohols, fatty amines and fatty esters. These oleochemicals are used as lubricant greases, anti-block

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agents, plastisizers, and emulsifiers and as ingredients in the manufacture of soaps, detergents, and animal feed [4].

The present method of hydrolysis of crude palm oil to fatty acids and glycerol involves high temperature and pressure operation for about 2 h to achieve the desired 96% - 99% conversion. When these extreme conditions are employed, polymerisation of fat and by-product formation takes place resulting in dark fatty acids and discoloured aqueous glycerol solution [5].To remove the colour and the by-products, further purification by distillation is required. Both hydrolysis and subsequent distillation of fatty acids are energy intensive processes. Hence, it would be advantageous to develop a lower energy process that produces a colourless product [6].

On the other hand, lipases obtained from plants and microbes, which catalyze hydrolysis of oils and fats at the oil-water interface, yielding free fatty acids and glycerol can be effectively and economically used to conduct the same reaction, under mild conditions [7]. However, a reliable kinetic model to predict the hydrolysis rate is still lacking [5]. Though several reports have appeared on the use of lipase for hydrolysis of fats and oils, very few are available on the hydrolysis of palm olein [8]. Also, most hydrolysis studies have been carried out in Erlenmeyer flasks under poorly defined agitation conditions. Hence, only limited information is available on the hydrolysis of palm olein by lipase in a packed bed reactor and there is still a need to further optimize the yield of fatty acid from palm olein. Information from these studies will help to establish the hydrolysis kinetics of palm olein by lipase and for designing a large-scale hydrolysis reactor.

2.0 MATERIALS & METHODOLOGIES

2.1 Materials

The immobilized enzyme used, known as Lipozyme TL IM, was obtained commercially from Novozymes Co. Lipozyme TL IM is an immobilized triacylglycerol lipase from Thermonyces lanuginosus produced by submerged fermentation of a genetically modified Aspergillus oryzae microorganism. Palm olein was obtained from Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia. Potassium dihydrogen phosphate, sodium hydroxide, tributyrin and hexane were obtained from Fluka Chemie AG, Switzerland. Oleic acid, stearic acid, and methyl oleate were obtained from Merck KGaA, Germany. Borontrifluoride -methanol was obtained from BDH Laboratory Supplies. All chemicals used were of analytical grade.

2.2 Determination of Enzyme Activity

The method is based on the hydrolysis of tributyrin by the enzyme and titrating the butyric acids produced with 0.1 M NaOH. 48.5 mL of 10mM potassium dihydrogen phosphate buffer (KH_2PO_4) at pH 7.0 was incubated in a thermostated vessel equipped with a magnetic stirrer at 25 °C. After addition of tributyrin (1.47 mL), the pH-meter was started to keep the pH at 7.0. When the pH stabilized, the enzymes (3 mg) were added. The consumption of sodium hydroxide (100 mM) was monitored for 30 minutes. The specific activity of the enzymes was calculated from the base consumption at the linear part of the graph using Equation 1. The enzyme activity is expressed as one unit of enzyme activity corresponds to one micromole of butyric acid liberated from tributyrin per minute per milligram of enzyme at 25 °C.

$$\text{Specific activity} = \frac{S(ml/s)}{W_E(g)} x \frac{1L}{1000mL} x \frac{60s}{1\min} x \frac{10^6 \mu mol}{1 mol} x[NaOH]M \quad (1)$$

2.3 Experimental Method

The main components of RPBBR are peristaltic pump (Masterflex,Cole-Parmer), water jacketed vessel with the maximum capacity of 50 mL and thermostat jacketed column (Pharmacia Biotech, Sweden) with solvent resistant fittings. Immobilised Lipase was packed in a XK16/20 (16 mm ID x 20 cm length) column. Substrate was fed and recirculated through the column using the peristaltic pump. The reaction mixture was stirred with magnetic stirrer in order to thoroughly mix the substrate and the solvent. A water bath (Grant Intruments, Cambridge, England) was used to maintain the temperature of reaction solution. The reactions were carried out by varying the palm olein concentrations. The experimental setup of RPBBR and its schematic diagram are illustrated in Figure 1.

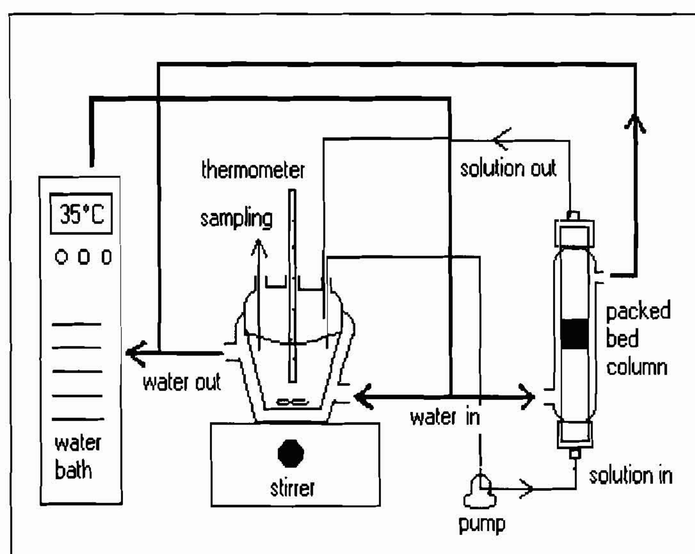

Figure 1 Schematic diagram of the experimental setup

The concentrations of palm olein were based on the ratio of volume of palm olein to volume of hexane. In order to determine the kinetics parameters, the substrate concentration was increased until a constant rate of product formation was achieved. The selected range of oil to hexane ratio started from 0.3 to 0.8 with a total volume of 38 mL consisted of palm olein and hexane only. A constant volume of 2 mL water was also added into each reaction mixture to make the total volume of the reaction mixture 40mL. The temperature and pH were being set at 37°C and pH 7. The stirrer speed was set at 250 rpm and the flow rate used was $0.5 mL.min^{-1}$. Samples were withdrawn from the reactor at regular intervals which were 15, 30, 60 and 90 minutes for analyses and the results were used to determine the initial rates of reaction. The initial rates of reaction were calculated using the slope of the hydrolysis profiles' linear portion. All experiments were conducted in triplicate and the values were averaged.

2.4 Determination of Oleic Acid Concentration

Gas chromatography was selected to provide data on the production progressive of each fatty acid. In order to determine the oleic acids using a gas chromatography, esterfication of the fatty acids to their respective methyl ester was carried out first. Samples of 0.5 mL were withdrawn from the reaction mixture each time and placed into reagent bottles. One mL of BF_3 -methanol was added and nitrogen gas was used to purge the air from the bottles. The bottles were tightly covered and heated at 100°C for 30 min for the reaction to take place. After 30 minutes, the mixtures were cooled and 1 mL of hexane was added to each bottle to extract the methyl esters. This was followed by adding 1 mL of distilled water to stop the esterification reaction and to allow phase separation. The mixture was then stirred well to separate the hexane and aqueous phases. Sample of 0.5 mL from the top portion was collected and used for GC analysis. The sampel was then injected into the gas chromatograph (Shimadzu GC-17A), equipped with a flame ionization detector, and area of each peak was determined. The temperature of injector and detector were maintained at 260 °C and the column temperature was kept at 50°C for 2 minutes and then programmed to rise 4°C per minute to reach 220°C within 15 minutes. The retention time for methyl oleate was in the range of 13.7-13.8 minutes. The peaks areas obtained with samples were compared with the calibration curve which was first developed.

2.5 Determination of Kinetics Parameter

Concentrations of oleic acid produced against sample time were plotted for each substrate concentration and the initial rate of reaction were obtained from the slope of the graph's linear portion. The initial reaction rates obtained for each substrate concentrations were rearranged and several plots including the lineweaver-Burk equation, Eadie-Hofstee equation and Hanes-Woolf equation were plotted in order to determine the kinetic parameters of V_{max} and K_m. The equation of lineweaver-Burk, the Eadie-Hofstee equation and the Hanes-Woolf equation are shown in Equation (2), (3) and (4), respectively.

$$\frac{1}{v_o} = \frac{K_m}{V_{max}} x \frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

$$v = V_{max} - K_m \frac{v}{[S]} \quad (3)$$

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}}[S] \quad (4)$$

3.0 RESULTS AND DISCUSSIONS

3.1 Enzyme Assay

A short chain of triglyceride, tributyrin, was used in the hydrolysis assay of lipases. Tributyrin was selected because it is able to form stable emulsions in aqueous media without the addition of stabilizer. The emulsions of tributyrin were hydrolyzed by lipases to form dibutyrin, monobutyrin, glycerol and free butyric acid. All of these water-soluble products of tributyrin hydrolysis did not inhibit the reaction by accumulating at the oil-water interface (Wu and Tsai, 2004).

The activity assay measured the liberation of butyric acid from tributyrin using titration method. It was conducted to check for the apparent degradation of enzyme activity. The hydrolytic activity of immobilised Lipase was found to be 8 $kU.g^{-1}$. This activity was assumed constant throughout the whole study. The consumption rate of 100 mM NaOH for the first 30 minutes is shown by the slope of Figure 2, the slope was plotted using Microsoft Excel through linear regression.

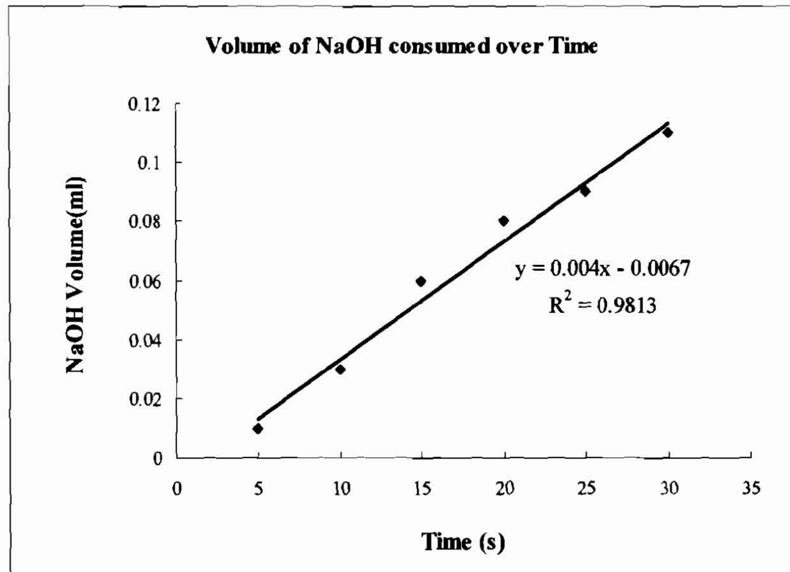

Figure 2 Consumption rate of NaOH in the activity assay

3.2 Hydrolysis Profile

In a typical enzymatic reaction, the reaction can be monitored by tracing either the loss of substrate or the formation of product. In this study, the formation of oleic acid as product was followed. Figure 3 shows the hydrolysis profile of palm olein at an initial palm olein concentration of 0.4206 mol.L^{-1}. The reaction conditions for all the runs were set at 37°C and an agitation speed of 250 rpm, without the addition of surfactant.

Based on Figure 3, the progress curve goes through 3 stages, in the first 15 minutes, there was a lag phase where the presence of product in the reaction medium were not significant (0.00004 mol.L^{-1} to 0.0005 mol.L^{-1}), the substrates was yet to bind with the desired conformation of the enzymes active site and the enzymes also required certain period of initial time to contact with the droplets of oil. From 15 to 30 minutes, the concentration of oleic acid increased exponentially and the rate of reaction was maximum. This robust rate of reaction was achieved as the active sites on the enzyme were saturated with the substrate molecules. After 30 minutes, the concentration of fatty acid did not show further increment, indicating that the reaction had reached its equilibrium state. Thus, three parts of the kinetic curve can be distinguished, the lag phase, the initial increasing part and the plateau region.

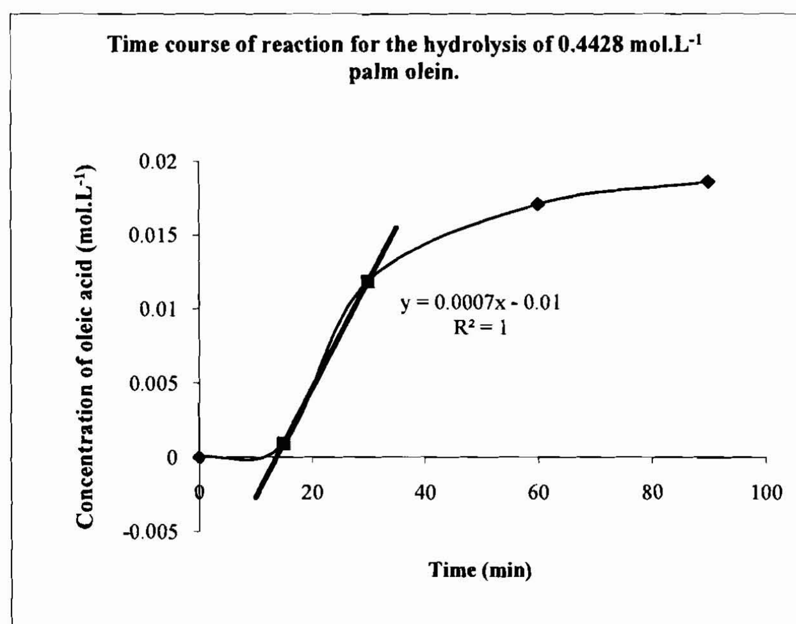

Figure 3 Concentration of oleic acid produced versus time at an intial substrate concentration of 0.4206 mol.L^{-1}.

Similar hydrolysis profiles were obtained for other experiments with different initial substrate concentrations ranging from 0.3155 mol.L^{-1} to 0.8412 mol.L^{-1} as shown in Figure 4.

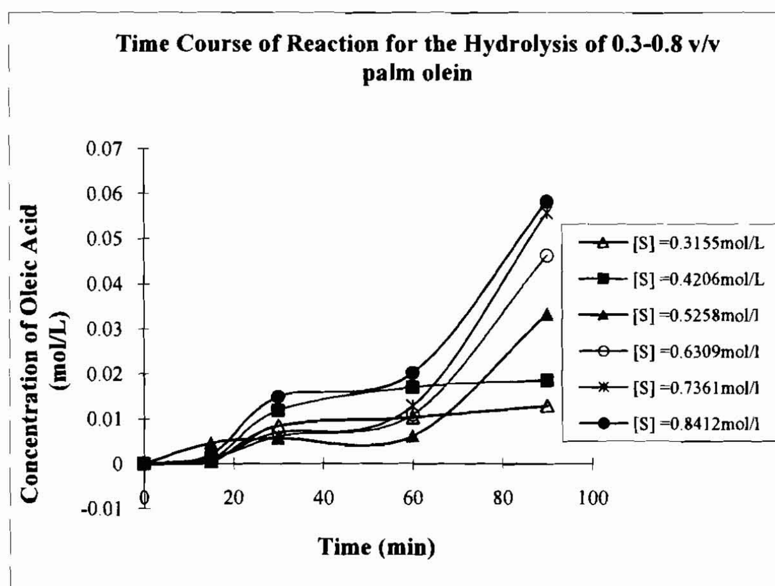

Figure 4 Concentrations of oleic acid produced versus time for the hydrolysis of 0.3155 mol.L^{-1} to 0.8412 mol.L^{-1} palm olein

Each time course of reactions was followed by gas chromatography. Reactions were stopped at 90 minutes in order to save time because many repetitive experiments at various initial concentrations of substrate had to be performed in order to determine the K_m and V_{max}.

The real enzymatic reaction mechanism is complex anddifficult to decipher. This is mainly due to the interactive nature of biological processes. According to Figure 4, it is observed that for concentration ranging from 0.5258 mol.L^{-1} to 0.8412 mol.L^{-1}, the rates of reactions were increased for up to 90 minutes while for substrates concentration ranging from 0.3155 mol.L^{-1} to 0.4206 mol.L^{-1}, the plateau had been reached at 30 minutes indicating that the reaction had reached its equilibrium state.

3.3 Data Analysis for Kinetics Studies

The kinetics studies were conducted based on the initial velocity analysis. From the hydrolysis profiles, the initial rate of hydrolysis for each substrate concentration was determined using the slope of the linear portion of their progress curves. The initial reaction rates, v_0 at different palm olein concentration, [S] and all the rearrangement values of v_0 and [S], namely $1/v_0$, 1/[S], v_0/[S] and [S]/v_0 are summarized in Table 1. The initial rate of hydrolysis was expressed in moles (oleic acid produced) per L per min. The data in Table 1 were used to determine the value of V_{max} and K_m by using the Lineweaver-Burk plot, the Eadie-Hofstee plot and the Hanes-Woolf plot.

Initially, six data points (option 1) from Table 1 were used to plot the three plots. However, in order to get the more precise values of V_{max} and K_m, two data points have been eliminated. In other words, only four data points (option 2) were considered and shown in Figure 5, 6 and 7.

Table 1 Initial reaction rates and other rearrangement values for the hydrolysis of 0.3155 mol.L^{-1} to 0.8412 mol.L^{-1} palm olein.

[S] (mol.L^{-1})	v_0 (mol.L^{-1}.s^{-1})	$1/v_0$ (L.s.mol.$^{-1}$)	1/[S] (L.mol^{-1})	[S]/v_0 (s)	v_0/[S] (s^{-1})
0.3155	0.0005	2000	3.17	0.0016	631
0.4206	0.0007	1429	2.38	0.0017	601
0.5258	0.0009	1111	1.90	0.0017	584
0.6309	0.001	1000	1.58	0.0016	631
0.7361	0.001	1000	1.36	0.0014	736
0.8412	0.001	1000	1.19	0.0012	841

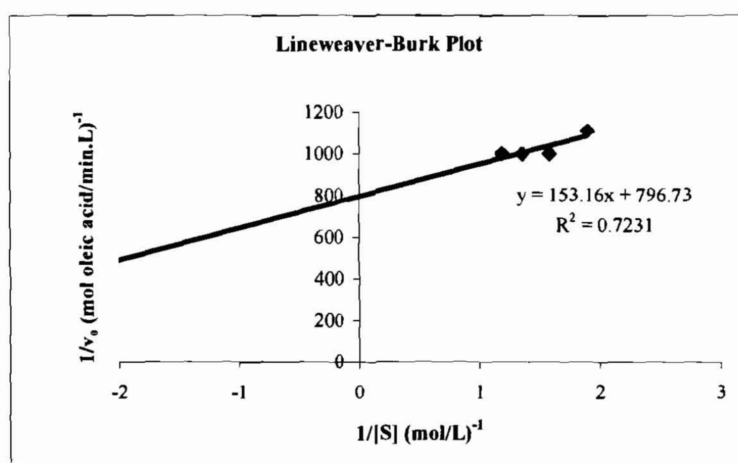

Figure 5 Lineweaver-Burk Plot

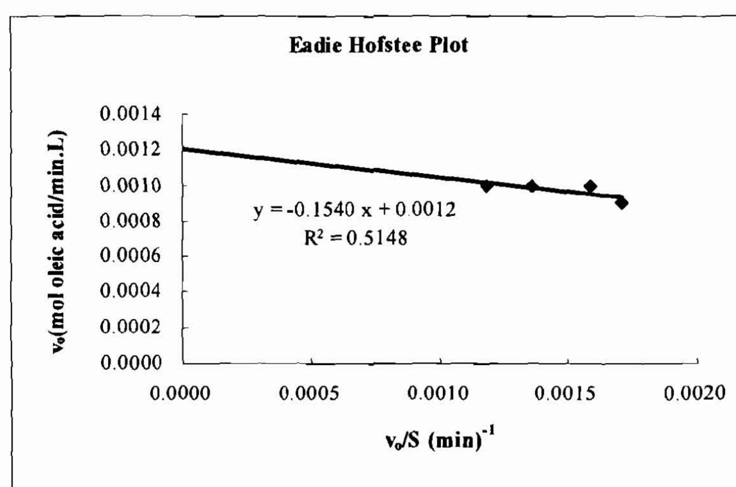

Figure 6 Eadie-Hofstee plot

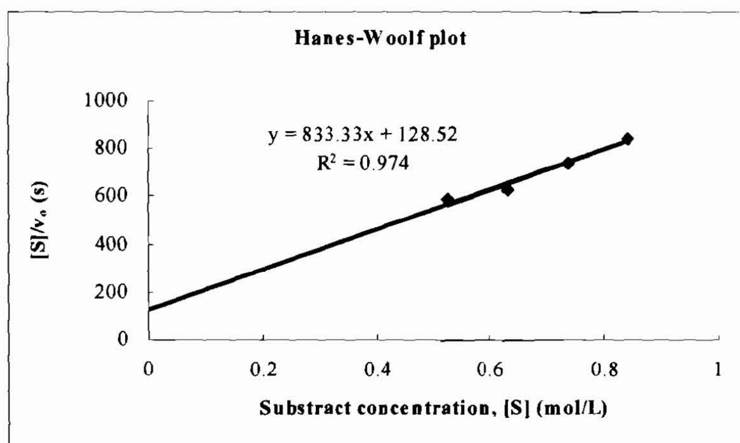

Figure 7 Hanes-Woolf plot

The apparent kinetic parameters including the maximum reaction rate (V_{max}) and the Michaelis constants (K_m) of immobilized lipases obtained from the three types of plots are summarised in Table 2.

Table 2 Summary of the kinetic parameters

Type of Plots	V_{max} (mol.L^{-1}.min^{-1})	K_m (mol.L^{-1})
Lineweaver-Burk plot	0.00126	0.192
Eadie-Hofstee plot	0.00120	0.154
Hanes-Woolf plot	0.00120	0.154
Average	**0.00122**	**0.167**

An average value of V_{max} and K_m was obtained from Lineweaver-Burk Plot, Eadie-Hofstee Plot and Hanes-Woolf plot. The value was 0.00122 mol.L^{-1}.min^{-1} for V_{max} and 0.167 mol.L^{-1} for K_m.

It is quite difficult to determine the best method of data plotting to be used in order to determine the value of V_{max} and K_m. In any case it should be emphasized that the data should be as good as possible. The lineweaver-Burk plot is still the most common method being used and it has the advantages that the variables v and [S] are plotted on separate axes. However, an analysis of the errors involved in the collection of the data (and hence in the determination of the parameters K_m and V_{max}) showsx that there was a highly non-uniform distribution of errors over the range of values of 1/v and 1/[S] in the lineweaver-Burk plot. For this reason, the plots from Eadie-Hofstee and Hanes-Woolf have been recommended as the distribution of errors in these plots were more uniform.

According to Steven and Lee (2005), who used the same substrate and the same loading of the same enzyme but different reactor which was a batch stirred tank reactor and without the addition of water. The values of V_{max} and K_m they reported were 0.00114

mol.L^{-1}.min^{-1} and 2.53 mol.L^{-1} , respectively. Their V_{max} compared reasonably well with the values obtained in this study whereas their K_m was significantly larger compared to 0.167 mol.L^{-1} obtained in this study. It is known that a small value of K_m indicating a high affinity of enzyme towards the substrate, hence it was likely that the addition of small amount of water in this study (2 mL water over 40 mL total reaction mixture) was favorable to improve the affinity between substrate-enzyme due.

3.4 Effect of Substrate Concentration on Rate of Reaction

Based on the data from Table 1, a Michaelis-Menten Plot is plotted as Figure 8 which shows the effect of palm olein concentration on the initial rate of reaction.

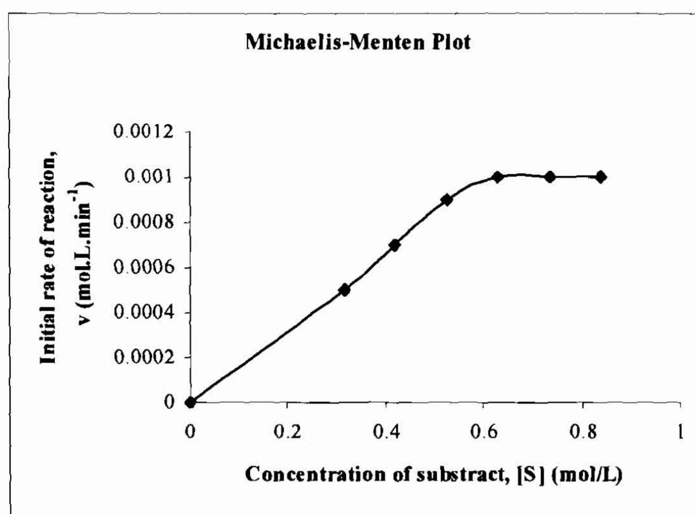

Figure 8 Michaelis-Menten plot

Initially, the oil concentration was varied from 0.3155 mol.L^{-1} to 0.6309 mol.L^{-1}. In this range of substrate concentration, the initial rate of hydrolysis varied linearly with substrate concentration. Noor et al. (2003) also observed that the rate of hydrolysis of tallow, coconut oil and olive oil by lipase from *Candida rugosa* varied linearly with oil concentration. Experiments were also conducted at high oil concentrations, up to 0.8412 mol.L^{-1} to check the substrate inhibition. However, these experiments showed that no substrate inhibition was observed for oil concentration up to 8412 mol.L^{-1}.

4.0 CONCLUSIONS

The hydrolytic activity of immobilised Lipase was found to be 8 kU.g^{-1} and this activity was assumed constant throughout the whole study. The kinetics studies were carried out using initial velocity analysis. $V_{max,}$ the maximum rate of reaction and K_m, the Michaelis-Menten constant were determined based on Michaelis-Menten model and their values were derived from the Lineweaver-Burk plot, Eadie-Hofstee plot and Hanes-Woolf plot. The average values of V_{max} and K_m obtained from these three plots were 0.00122 mol. L^{-1}.min^{-1} and 0.167 mol.L^{-1} respectively. The investigated range of substrate

concentration was varied from 0.3155 $mol.L^{-1}$ to 0.8412 $mol.L^{-1}$. No substrate inhibition was observed for palm olein concentration of up to 0.8412 $mol.L^{-1}$.

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