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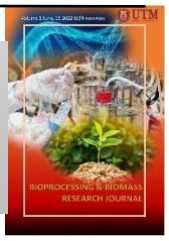

Research Article

Enzyme Kinetic of Lipase Catalysed Acidolysis of Used Cooking Palm Oil

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ABSTRACT

Enzymatic acidolysis of various fats and oils has successfully shown a promising ability to alter specific positions of lipids and incorporate desirable fatty acids at specific positions. However, used cooking oil utilization for enzymatic acidolysis is still lacking scientific investigation. Hence, this study aims to utilize used cooking palm oil (UCPO) by enhancing the oleic acid content via enzymatic acidolysis using immobilized *C. rugosa* lipase. The optimum substrate molar ratio (mol/mol) was identified based on the analysis of the peroxide, iodine, and acid values. Then, kinetic parameters, K_m and V_{max} for the enzymatic acidolysis of UCPO were calculated. Substrate molar ratio of 1:1, 1:2, 1:3, 1:4, and 1:5 mol/mol (oil: acid) was varied to evaluate the peroxide, acid, and iodine values. The reaction conditions such as reaction temperature (50 °C), reaction time (24 hours), enzyme concentration (0.05 g), agitation speed (250 rpm), and pH (7) were fixed throughout the experiment. The Michaelis-Menten kinetic model was selected to describe the kinetic of enzymatic acidolysis of UCPO. The result showed that incorporation of oleic acid in UCPO has been successfully achieved with the increased in IV value from 39.47 I_2/g to 110.53 I_2/g. The optimum substrate molar ratio obtained was 1:3 mol/mol with the highest iodine value of 110.53 I_2/g. The best linear regression approach is Lineweaver-Burk plot with the values of V_{max} and K_m were 34.5658 µmol/ml.min and 0.06 mol/L, respectively. The enzyme activity for *C. rugosa* lipase was obtained at 0.5804 µmol/min.ml.

INTRODUCTION

Palm oil is the world's most consumed edible oil. Malaysia is the world's second-biggest producer, accounting for 39% of global palm oil output in the year 2010/2011 (Zakaria, 2018). The increase in the human population has increased the quantity of used cooking oil produced (UCO). UCO was produced mainly from a variety of sources such as households, restaurants, catering establishments, hotel chains, fast food outlets, and industrial kitchens. An approximate 50,000 tons of UCO are reportedly disposed of without proper treatment in Malaysia (Zaharudin *et al*., 2018). Since UCO can block collection pipes and create sanitary sewage overflow, they cannot be released directly into the environment or wastewater as waste (Moazeni *et al*., 2019).

The demand for vegetable oil increases each year and the most widely used vegetable oil is palm oil, which is commonly used in Africa, South America, and South East Asia countries. Palm oil is also widely used in almost all food industries, cosmetic formulations, and biodiesel productions. However, the utilization of UCO for various industrial applications is still limited especially revolving around biodiesel productions. It is known that the utilization of UCO in the production of biodiesel improves the economic feasibility due to the price of feedstock that primarily hindered biodiesel production (Chhetri et al., 2008). In addition, UCO also contains a high concentration of free fatty acids which can be used in the production of soap (Heater *et al*., 2019).

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Hence, enzymatic acidolysis offers a promising utilization and enhancement for UCO by incorporating the desired acyl group/ fatty acids at a specific position of triacylglycerol (TAG). Enzymatic acidolysis involves the substitution of fatty acids with lipids acyl. The replacement of fatty acids residue occurs at the 1,3-positions of the TAGs, as *sn-1,3* regiospecific lipases are responsible to attain the 2-position. According to De Santi *et al.* (2021), the acidolysis reaction comprises two steps, where the source of TAG is hydrolysed into diacylglycerols and monoacylglycerols. The second step involved the esterification of fatty acids from the glycerol backbone to yield a new structured TAG. It is also a quick and easy way to incorporate particular fatty acids into triacylglycerols and achieved the required functionality compared to the conventional time and cost-consuming genetic engineering (Hamam and Shahidi, 2004). In addition, Kuo *et al*. (2020) produced docosahexaenoic (DHA) acid and eicosapentaenoic (EPA) acid ethyl ester through lipase (Novozym® 435) catalysed. Wei *et al.* (2021) produced the human milk substitute through enzymatic acidolysis of palm stearin with oleic and linoleic acids.

The objectives of the research are to determine the optimum molar substrate ratio of UCPO enzymatic acidolysis based on the analysis of the peroxide, iodine, and acid values. Then, the kinetic parameters for the enzymatic acidolysis of UCPO were determined. Pre-treatment of used cooking palm oil was conducted before the enzymatic acidolysis procedure using filtration and heating method to eliminate impurities and excess water. Immobilized lipase from *C. rugosa* was used throughout the study. Different substrate molar ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 mol/mol (oil: acid) were used to study the peroxide, acid, and iodine values. Other reaction parameters such as reaction temperature (50 °C), reaction time (24 hours), enzyme concentration (0.05 g), agitation speed (250 rpm), and pH (7) were fixed throughout the experiment. The K_m and V_{max} values were determined based on Michaelis-Menten kinetic model. Three different linear regression approaches were plotted and the average values of K_m and V_{max} were calculated.

MATERIALS AND METHOD

Materials

5L of used cooking palm oil (UCPO) was obtained from Meranti Food Court at University Technology Malaysia (UTM). Fresh palm oil (Buruh) and pure olive oil (Naturel) were purchased from grocery store. *C. rugosa*, thymolphthalein, phenolphthalein in 1% ethanol, thyodene indicator, pure oleic acid, gum arabic, n-hexane, 95% ethanol, acetone, Hanus solution, carbon tetrachloride, glacial acetic acid, isooctane, sodium hydroxide, potassium iodide, and potassium hydroxide were purchased from Sigma-Aldrich (M) Sdn. Bhd. All chemicals are analytical grade and were used without further purification.

Pre-treatment of UCPO

3.5 L of UCPO was filtered using a sieve batch by batch of 250 ml (Przybylski *et al.*,2013). The filtered UCPO was heated and stirred at 100 °C for 15 minutes to remove excess water.

Determination of enzyme activity

The enzyme activity of *C. rugosa* lipase was determined using olive oil and gum arabic as substrates (Zaharudin *et al*., 2018; Ifeduba and Akoh, 2014). 5 g of olive oil and 1 g of gum arabic were stirred continuously in a 250 ml conical flask containing 100 ml of sodium phosphate buffer. 0.05g of *C. rugosa* lipase were weighed and added to the mixture. Then, the mixtures were incubated using an incubator shaker at 37 °C with an agitation speed of 200 rpm for 20 minutes. 5ml of the sample was collected every 5 minutes throughout the incubation period. Each sample taken was added into a conical flask containing 10 ml of ethanol and 2 drops of thymolphthalein. The mixtures were titrated with 0.1 N NaOH until the end (light blue colour). The quantity of free fatty acids released by *C. rugosa* lipase was calculated according to the equation below:

$$F = \frac{(V_1 - V_0) \times 0.1\ N \times 1000}{V_t} \quad (1)$$

where:

F	=	Amount of FFA (µmol/mL)
N	=	Sodium thiosulfate normality (0.1 N)
V_1	=	Volume of sodium hydroxide used to neutralize the sample (ml)
V_0	=	Volume of sodium hydroxide needed to neutralize the blank solution (ml)
V_t	=	Volume of the sample (ml)

The amount of FFA released was plotted against the reaction time, and the hydrolytic activity was calculated using the slope of the linear section of the curve. One unit (U) of activity was defined as the quantity of lipase required to generate 1µmol of FFA in a minute under the assay conditions.

Determination of Peroxide Value

10ml of UCPO and 50 ml of glacial acetic acid-isooctane with a ratio of 3:2 v/v were mixed in a 250 ml conical flask and stirred at a low speed. Next, 1 ml of 10% potassium iodide was added and stirred continuously for 60 seconds. 0.1 g of thyodene indicator was dissolved in 100 ml distilled water and then added to the mixture. The mixture was mixed vigorously and titrated immediately with 0.1 N sodium thiosulphate. The peroxide value was calculated using the equation as shown below:

$$POV = \frac{(V_1 - V_0) \times N \times 1000 \times T}{V_S \times 2} \quad (2)$$

where:

POV	=	Peroxide value of UCPO (mmol/L)
V_1	=	Volume of sodium thiosulfate needed to neutralize UCPO (ml)
V_0	=	Volume of sodium thiosulfate needed to neutralize the blank solution (ml)
N	=	Sodium thiosulfate normality (0.1 N)
T	=	Sodium thiosulfate titre
V_S	=	Volume portion of substance (ml)

Determination of Iodine Value

An approximate 0.1g of UCPO and 20 ml of carbon tetrachloride (CCl_3) were poured into a 250 ml conical flask. The solution was dissolved by applying a high agitation speed. Next, 25 ml of Hanus solution was added and stirred for a minute. It was sealed and left in a dark room at 20 °C

for 30 minutes. 10ml of 10% potassium iodide solution was added with 100 ml of water and sealed before it was shaken for 30 seconds. Finally, the sample mixture was titrated with 0.1 N sodium thiosulfate. The endpoint of the titration was from brownish red to colourless (Zaharudin *et al.*, 2018). The iodine value was calculated using the equation below:

$$IV = \frac{(V_0 - V_1) \times N \times 12.69}{W} \quad (3)$$

where:

IV = The iodine value of UCPO (I_2/g)
V_1 = Volume of sodium thiosulfate needed to neutralize UCPO (ml)
V_0 = The volume of sodium thiosulfate needed to neutralize the blank solution (ml)
N = Sodium thiosulfate normality (0.1 N)
W = Weight of UCPO (g)

Determination of Acid Value

Approximately 100 ml of neutralized ethanol-toluene mixture (1:1 v/v) and 4 g of treated UCPO were inserted into a conical flask. The mixture was titrated with standard potassium hydroxide (KOH) with three drops of phenolphthalein as an indicator. The endpoint of the titration was from pink to cloudy or colourless (Zaharudin *et al.*, 2018). The acid value was calculated using the equation as shown below:

$$AV = \frac{MW \times N \times V_1}{W \times 1000} \quad (4)$$

where,

AV = The acid value of UCPO (mg KOH/g)
MW = The molecular weight of KOH (56.1 g/mol)
V_1 = The volume of KOH used to neutralize the sample solution (ml)
N = KOH normality (1.0 N)
W = Weight of UCPO (g)

Enzymatic Acidolysis

UCPO was mixed with oleic acid at a variety of substrate molar ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 (oil: acid) with a working volume of 50 ml in 100 ml conical flasks (Abed *et al.*, 2018). Water (1% w/w) and 0.05g of immobilized *C. rugosa* lipase were added to it. The acidolysis was established at a temperature of 50 °C for 24 hours with an agitation speed of 250 rpm. The peroxide, iodine, and acid values of the samples were determined. All reactions were performed in triplicate value and the average mean value was taken.

TAG Separation

10 ml of the acetone-ethanol mixture (1:1 v/v) was added into a 50 ml conical flask. 3 drops of phenolphthalein indicator were added and titrated with 0.5 N NaOH. Next, 25 ml of hexane was added to the mixture. The mixture was transferred into a separatory funnel and left for about a day to separate (Liu *et al.*, 2022). Both layers were collected in a beaker.

Kinetic Study

Titration values obtained from the enzymatic acidolysis (Elias *et al.*, 2021) were used to calculate the mass of oleic acid present in each sample using the equation:

$$Mass\ of\ Oleic\ Acid = \frac{(V_1 - V_0) \times N \times MW}{1000} \quad (5)$$

where,

V_1 = Volume of KOH used to neutralize the sample solution (ml)
V_0 = Volume of KOH needed to neutralize blank solution (ml)
N = KOH normality (1.0 N)
MW = Molecular weight of Oleic Acid (g)

Based on the results gained, the reaction progress graph for each molar ratio was constructed by plotting the mass of oleic acid (product) against time. The initial velocity, v_0, for each molar ratio were calculated using equation below. Lastly, 3 linear regression graphs of Lineweaver-Burk, Eadie-Hosftee, and Hanes Woolf were plotted to determine the V_{max} and K_m values. The equation as shown as below:

$$v_0 = \frac{Oleic\ acid\ mass\ at\ 2\ hr - Oleic\ acid\ mass\ at\ 6\ hr}{Time\ (6\ hr - 2\ hr)} \quad (6)$$

RESULTS AND DISCUSSION

Enzyme Activity

The hydrolytic activity of *C. rugosa* lipase was obtained in the presence of olive oil and gum Arabic as a substrate is 0.5804 µmol/ml.min. **Figure 1** shows the amount of fatty acid released for 20 minutes. The enzyme activity was obtained from the generated straight-line equation.

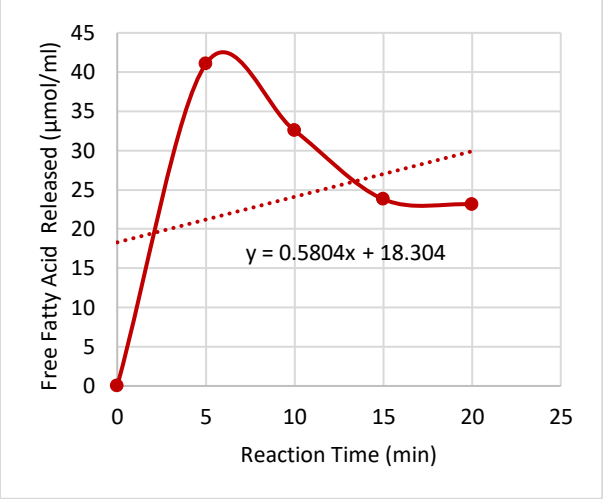

Figure 1 Enzyme activity for *C. rugosa* lipase (free fatty acid released at 5 minutes interval for 20 minutes incubation time).

Chemical Properties of UCPO and Fresh Cooking Palm Oil

Peroxide (PV), iodine (IV), and acid (AV) values of UCPO were determined before the acidolysis reaction. The same chemical properties were also compared for fresh cooking palm oil (FPO). **Figure 2** shows the measured peroxide, iodine, and acid values of UCPO and FPO. The results showed that the PV for UCPO (92.16 meq/kg) was higher compared to FPO (12.32 meq/kPV). A high peroxide value indicates a high oxidation degree (Jurid *et al.*, 2020).

The double bonds oxidation of unsaturated fat causes the primary oxidation product, peroxide to form.

On the other hand, IV of UCPO is lower compared to FPO. The iodine values obtained for UCPO and FPO were 52.16 I_2/g and 39.47 I_2/g respectively. The decrease in the IV after the frying process is due to the changes in fatty acids composition during the frying duration. Oxidation and polymerization consume double bonds, resulting in the decrease of IV. The measured AV for UCPO and FPO was attained at 13.88 mg KOH/g and 5.31 mg KOH/g respectively. The higher AV of UCPO compared to FPO was attributed to an increase in fatty acid content during the frying process (Yusuff *et al.*, 2021). The frying process generally raptures the TAG double bond structures and initiates oxidative alteration (Zaharudin *et al.*, 2018). Manzoor *et al.* (2022) specified the increase in the decomposition of double bonds associated with the length of frying time.

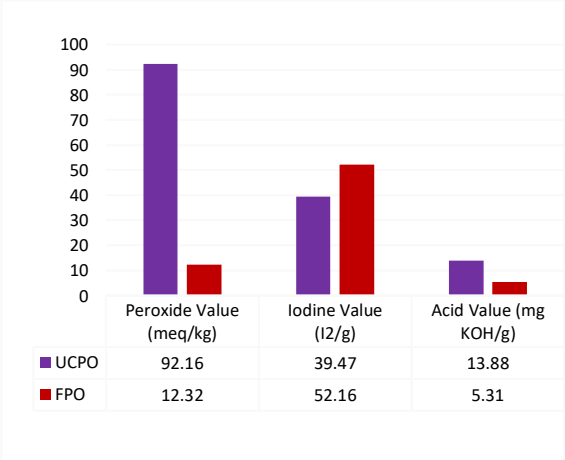

Figure 2 UCPO and FPO properties before enzymatic acidolysis

The overall result shows that UCPO has higher PV and AV compared to FPO. However, IV for FPO is higher compared to UCPO. The frying process has influenced the PV, IV, and AV of the oil. Cooking oil that experienced continuous and repeated frying process at higher temperatures (above 180 °C) as well as constant contact with air and water caused complex degradation reactions in the oil and produced numerous compounds (Mariana *et al.*, 2020). According to Jurid *et al.* (2020), frying decrease the unsaturated value of the oil as specified by a decrease in IV and AV. In the study, PV does increase after the 1st frying cycle but decreased from the 2nd to 5th frying cycle. The increasing PV in 1st frying cycle shows the occurrence of primary oxidation and the decreasing trend afterwards is due to the instability of peroxide at a higher temperature

Enzymatic Acidolysis

The substrate molar ratios (oil:oleic acid) were varied from 1:1 to 1:5. Effect of PV, IV, and AV on the acidolysis reaction were observed for each varied ratio. **Figure 3** shows the effect on PV for each substrate molar ratio. From **Figure 3**, the PV dropped as the substrate molar load rose. A low PV suggests a high degree of unsaturation, which results in autoxidation (oxidative rancidity) (Tiefenbacher, 2017). Lowest PV were found at substrate molar ratio of 1:5 with a PV of 11.06 meq/L. The double bond in fats and oils is responsible for autoxidation. As a result, oil with higher degree of unsaturation is more prone to autoxidation. Furthermore, when the molar substrate ratio increases, so does the proportion of unsaturated fatty acids, resulting in more C=C links in the oil.

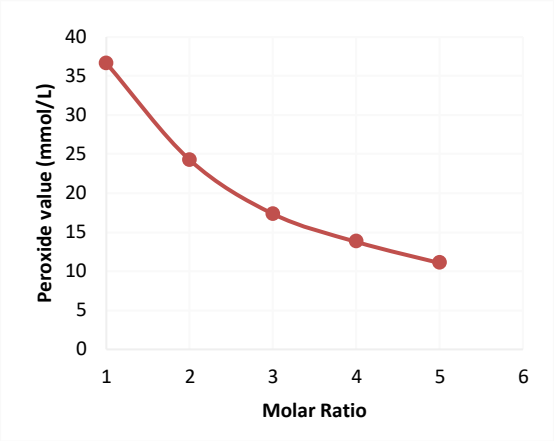

Figure 3 Effects on the peroxide value (mmol/L) for different substrate molar ratio.

Figure 4 shows the effect of iodine value for different substrate molar ratio. As for IV, a distinct pattern was seen as shown in **Figure 4**. The IV increased until it reached the substrate molar ratio of 1:3 and then steadily reduced afterwards. At substrate molar ratio of 1:3, a highest IV was recorded (110.53 I_2/g), indicating an optimum substrate molar ratio. The rise in iodine content indicates that oleic acid has been successfully incorporated into triacylglycerol (TAG) (Mohd Fazel *et al.*, 2021). Oleic acid in palm oil is mostly found at the *sn*-2 position in TAG (Bronsky *et al.*, 2019). This trend showed that the greater the IV, the higher the oleic acid incorporation. However, there was a significant reduction in IV at a molar substrate ratio of 1:4 and 1:5. An excessive quantity of water, on the other hand, might result in inadequate oleic acid incorporation in UCPO. The large amount of water in the oil favours hydrolysis rather than acidolysis (Ren *et al.*, 2019).

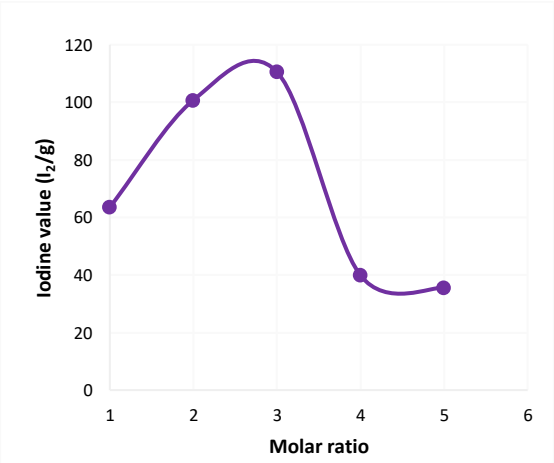

Figure 4 Effects on the iodine value (I_2/g) for different substrate molar ratio.

Figure 5 shows the effects on the AV for different substrate molar ratio. The result represents the AV decreased with the increased number of substrates. There

was only a slight decrease in AV between substrate molar ratio of 1:4 and 1:5 at 7.58 and 6.28 mg KOH/g respectively. This specifies that the amount of ester in the oil represents the amount of FFA in the oil. The increasing amount of acyl donor promotes a positive effect on oleic acid incorporation. Therefore, increasing the substrate molar ratio can maximise the amount of unsaturated fatty acids resulting a decrease in the AV (Wang et al., 2019).

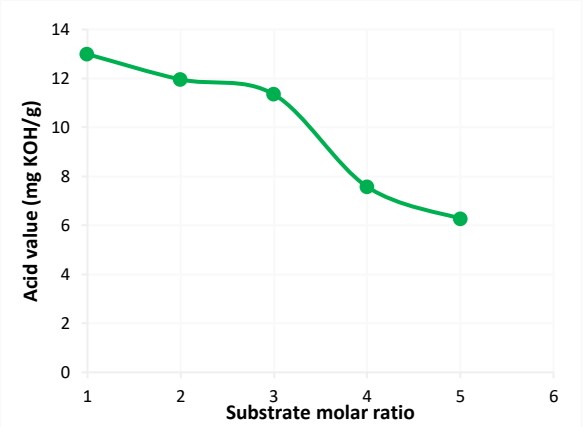

Figure 5 Effects on the acid value (mg KOH/g) for different substrate molar ratio.

Kinetic Study

K_m and V_{max} values were determined using the Lineweaver-Burk plot of 1/initial velocity (v_0) vs 1/substrate concentration (1/[S]). **Figure 6** illustrates the oleic acid concentration profile against reaction time with varied substrate concentrations. The concentration of oleic acid in each sample was calculated. **Equation 6** was opted to estimate the initial velocity (v_0). **Table 1** shows the initial velocity for each different initial substrate concentration, [S], and rearrangement values, $1/v_0$, 1/[S], $[S]/v_0$, and $v_0/[S]$, as calculated using the methods presented by Wanyonyi *et al.* (2017). 2 alternatives linear regression approaches, such Eadie-Hofstee and Hanes-Woolf plots, were also constructed to determine K_m and V_{max} values.

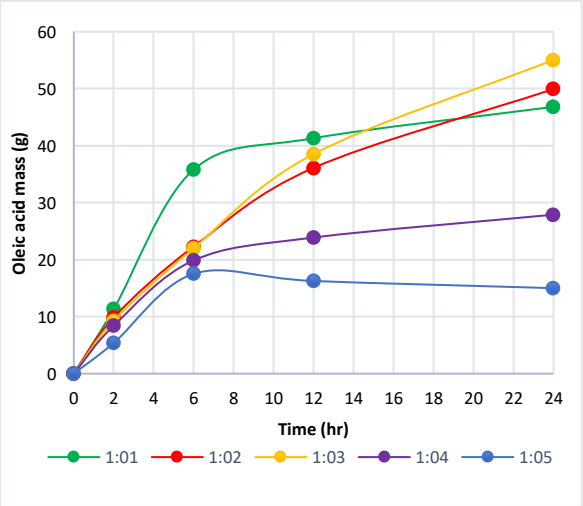

Figure 6 Effect of substrate molar ratio on mass of oleic acid (g) for 24 hours reaction time

Table 1 Initial reaction rates and other rearrangement values for each varied substrate molar ratio.

Substrate molar ratio	v_0 (g/hr)	$1/v_0$ (hr/g)	[S] (g)	1/S (g^{-1})	S/v_0 (hr)	v_0/S (hr^{-1})
1:1	6.125	0.163	0.50	2.000	0.082	12.250
1:2	3.133	0.319	0.33	3.000	0.106	9.398
1:3	3.185	0.314	0.25	4.000	0.078	12.740
1:4	2.905	0.344	0.20	5.000	0.069	14.525
1:5	3.038	0.329	0.17	6.000	0.055	18.225

A low K_m value indicates that lipase has a strong affinity towards the substrate (Badoei-dalfard *et al.*, 2019). Half saturation can be achieved with a very little or low concentration of substrate. When the enzyme is exceptionally saturated with substrate, a high V_{max} value indicates a high conversion of substrate to product per unit time, resulting in a greater peak velocity. K_m value for acidolysis of UCPO with oleic acid obviously proposed that a stable UCPO/oleic acid substrate complex able to rapidly convert to product, the greater *C. rugosa* lipase affinity towards the substrate. This is in line with the finding from Elias *et al.* (2021).

The Lineweaver-Burk plot for the acidolysis of UCPO at varied substrate concentrations of 0.5 to 0.17 g is shown in **Figure 7**. The Michaelis-Menten equation was used to fit the kinetic data. The obtained V_{max} and K_m values were 34.5658 µmol/mg.min (U/mg) and 0.06 mol/L (M) respectively. **Table 2** summaries the values of K_m and V_{max} in various units. The combination of a low K_m value and a high V_{max} value is the most favourable, indicating a high enzyme-substrate affinity (Peng *et al.*, 2022). K_m and V_{max} values of lipases vary greatly (Kuo *et al.*, 2020). On the other hand, K_m value of 0.746 M and V_{max} value of 28.61 µmol/mg.min were obtained from transesterification synthesis of ethyl butyrate in presence of *C. rugosa* lipase (Devi *et al.*, 2017).

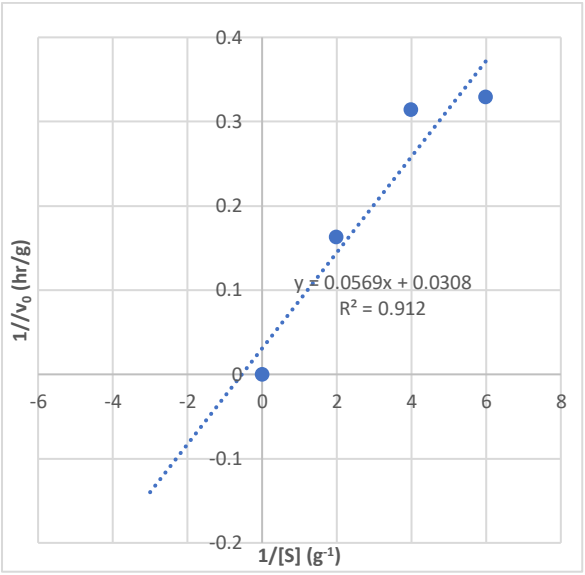

Figure 7 Lineweaver-Burk plot for acidolysis of UCPO.

Table 2 K_m and V_{max} values (Lineweaver-Burk plot) in various units.

Unit	mol/L (M)	g/ml	µmol/ml.min (U/ml)	µµmol/ mg.min (U/mg)	mol/ L.min
K_m	0.06	0.0181			
V_{max}			34.5658	20.7395	0.3457

Figures 8 and **Figure 9** shows the Eadie-Hofstee and the Hanes-Woolf plots which were generated for comparative purposes in this study. The K_m and V_{max} values derived from the Eadie-Hofstee plot (**Figure 8**) were 1.7311 mol/L and 6.4690 µmol/ml.min, respectively. The Hanes-Woolf plot (**Figure 9**) attained K_m value of 0.0132mol/L and a V_{max} value of 12.5114 mol/ml.min. **Table 3** summarises the kinetics characteristics from these three figures, including Michaelis-Menten constants, K_m, maximal reaction rate, V_{max} and the R^2 values. The lowest K_m value was obtained from Hanes-Woolf plot. However, the R^2 and V_{max} values from this plot was the lowest. On the other hand, Lineweaver-Burk plot delivered the highest V_{max} and R^2 values. Thus, the best linear regression approach is Lineweaver-Burk plot due to high R^2 value with highest V_{max} and lower K_m values.

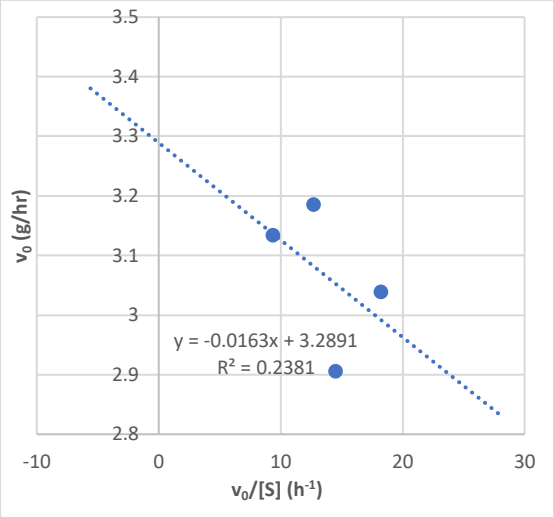

Figure 8 Eadie-Hofstee plot for acidolysis of UCPO.

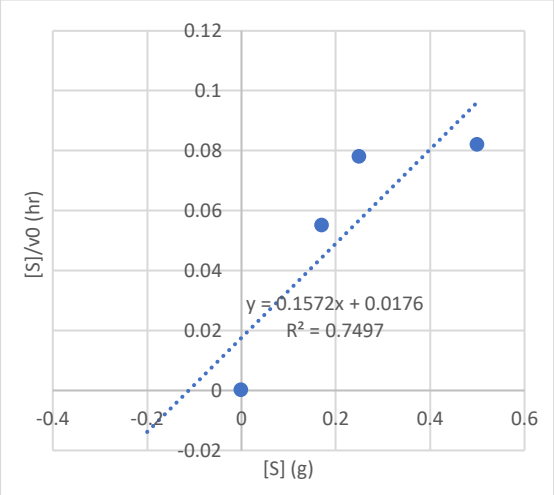

Figure 9 Hanes-Woolf plot for acidolysis of UCPO.

Table 3 Kinetic parameters for the three types of plots

Types of plot	R^2 Value	K_m (mol/L)	V_{max} (µmol/ml.min)
Lineweaver-Burk	0.912	0.06	34.5658
Eadie-Hofstee	0.2381	1.7311	6.4690
Hanes-Woolf	0.7497	0.0132	12.5114

CONCLUSION

Enzymatic acidolysis is one of the most promising techniques for converting UCPO into enhanced TAG. PV and AV of UCPO are higher than FPO. Meanwhile, UCPO has a lower iodine value than FPO. Enzymatic acidolysis of UCPO was conducted by varying the molar substrate ratio. Determination of optimum value was based on PV, IV, and AV analysis. The optimum substrate molar ratio was found at 1:3 mol/mol with the highest iodine value of 110.53 I_2/g. The increased in IV from 39.47 I2/g (before acidolysis) to 110.53 I_2/g (after acidolysis) indicated that incorporation of oleic acid in UCPO has successfully achieved, where the content of unsaturated fatty acid is at most. Lineweaver plot provided the best linear regression method with V_{max} and K_m values of 0.06 mol/L and 34.5658 µmol/ml.min respectively with the R^2 value of 0.912. The obtained enzyme activity for *C.rugosa* lipase was 0.5804 µmol/min.ml.

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