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Enzymatic Hydrolysis of Used-Frying Oil Using *Candida Rugosa* Lipase in Solvent-Free System

Roslina Rashid[a*], Nor Athirah Zaharudin[a], Ani Idris[a]

[a]*Department of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia*

*Corresponding author: roslina@cheme.utm.my

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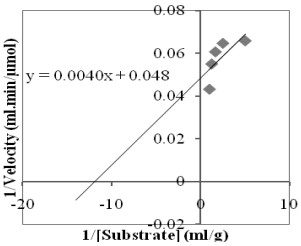
Graphical abstract



Abstract

The effects of enzymatic hydrolysis of used-frying oil were carried out using an immobilized lipase from *Candida rugosa* in solvent-free system. Used-frying oil was used as a substrate in this study due to abundance amount of used-frying oil present in Malaysia as its disposal problem become a very serious environmental issue. The high free fatty acids (FFA) content in used-frying oil has raised the interest for the utilization of this waste into valuable products. Even though used-frying oil is not suitable for human consumption and being extensively used for biodiesel production, FFA from used-frying oil could be utilized to produce various types of non-edible products. Effects of enzyme loading, water content, reaction temperature, buffer pH and agitation speed on the degree of hydrolysis were investigated. The experiments were conducted at constant 3 hours reaction time. It was found that the effect of variables were very significant by influencing the hydrolysis process. The hydrolysis process achieved the highest yield of fatty acids at enzyme concentration of 1.5% (w/v), buffer volume to oil volume ratio of 3:1, temperature of 40 ˚C, pH of 7 and agitation speed of 220 rpm. Under these described conditions, nearly 98.1% degree of hydrolysis was achieved. A kinetic model based on Michaelis-Menten equation was used to determine the rate constant of $V_{max}$and $K_m$and it was found that the values are20.8333µmol/ml.min and0.0833g/ml respectively which were gained from Lineweaver-Burk plot.

Keywords: Used frying oil; candida rugosa lipase; enzyme loading; hydrolysis; fatty acids

1.0 INTRODUCTION

Management of used-frying oil poses a significant challenge due toits disposal problems and possible contamination towards water and land resources. Commonly, used-frying oil exists in a liquid or semi-solid form, and solid waste regulations forbid any liquid waste to be disposed in landfills. The most common and simplest disposal method used especially by food industry is pouring the waste into drain or watercourse. In the end, this waste will results in pipes clogging and destruction towards wastewater and septic used-frying oils will be discarded inappropriately. Recycle of this oil into new product such as a feedstock for production of biodiesel or soap is one of an alternative way to ensure sustainable development for this planet.

Even though fatty acids acquire from the used-frying oil are not suitable for human consumption, it can be utilized and undergone intensive purification for the production of non-food or non-edible products including paints, lubricants, plasticizers, detergents, surfactant and anti-slip agents. Used-frying oil also known for its high fatty acids content compared to fresh cooking oil [1]. Hence, used-frying oil utilization is very useful in meeting the demand from both domestic and global fatty acids production. The utilization of waste cooking oil would be desirable since this approach would not divert resource from the food supply [2].

Hydrolysis of oils and fat is an important industrial operation. The products, fatty acids and glycerol are basic raw materials for a wide range of applications including as a raw material for the production of oleo chemicals such as fatty alcohol [3]. Existing methods for production of fatty acids are based on chemical and physical methods [4]. Under these methods, polymerization of fat and by-products take place resulting in dark fatty acids and discoloured aqueous glycerol solution which required physical refining (distillation) under high temperature and vacuum. The process extremely consumed high energy. Enzymatic hydrolysis is chosen as it can be performed at low temperature to save energy, and exhibits high selectivity, leading to product with high purity and fewer side products [5]. Thus, with the aid of lipase, used-frying oil undergoes enzymatic hydrolysis and converted into fatty acids and glycerol. This method particularly save a lot of energy and reduce environmental pollution where the reaction performed at ambient pressure and mild reaction temperature.

Many literatures on enzymatic hydrolysis of fresh cooking oil for the production of fatty acids have been well documented. The use of used-frying oil as substrate for biodiesel production has also been explored. However, only limited literatures are available

concerning on the production of fatty acids from used-frying oil using lipase. Therefore, this study investigates the hydrolysis of used-frying oil using lipase at different operating conditions. The effects of operational parameters such as enzyme loading, water content, temperature, pH, and agitation speed on used-frying oil hydrolysis were investigated using lipase from *C. rugosa*. The use of immobilized lipase offers many benefits such as, high stability of enzyme, enzyme reusability, less downstream process and predicted production yield. This study strives to provide basic data for making full use of used-frying oil in a harmless manner.

■2.0 EXPERIMENTAL

2.1 Materials

Used-frying oil was obtained from university cafeteria, UniversitiTeknologi Malaysia (UTM). The lipase enzyme from *Candida rugosa* immobilized on Immobead 150 (activity of 100 U/g) was purchased from Sigma-Aldrich (M) Sdn. Bhd. Phenolphthalein indicator, sodium phosphate monobasic and dibasic (0.1 M), ethanol (95% v/v), iso-octane (99.8% purity), toluene, potassium hydroxide (KOH) 1.0 M, and sodium hydroxide (NaOH) 1.0 M were purchased from Merck (Malaysia) Sdn. Bhd. All chemicals are analytical grade and were used without further purification.

2.2 Method

Method proposed by Pinsirodom and Parkin[5] was used for the determination of fatty acids concentration. Method used for pre-treatment procedure was conducted according to the method proposed by author Pazouki*et al*. [6].

2.3 Pre-treatment of Used-frying Oil

100 g of used–frying oil was filtered by applying a reduce pressure system using a filter paper (Whatman 42). The filtered used-frying oil was stirred and heated to 100ºC for 15 minutes. This procedure is important as to eliminate excess water content in the oil.

2.4 Preparation of Titration Cocktail

10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) thymolphthalein indicator were placed in seven 25 ml Erlenmeyer flasks. These titration cocktails were used to determine the reactivity of subsamples of the reaction mixture [6].

2.5 Enzymatic Hydrolysis

10 ml used-frying oil was filled in 50 ml conical flask. An appropriate amount of buffer solution was added to a total volume of 40 ml with desired ratio of oil to buffer solution (v/v). Then, 0.2 g of *C. rugosa*lipase was added for final enzyme concentration of 0.5% (w/v). Two layers mixture was observed. The mixture was agitated in the orbital shaker at 40ºC at 200 rpm. At six reaction intervals (30, 60, 90, 120, 150 and 180 min), 2 ml of the reaction mixture was removed and each sample was transferred to a separate flask containing titration cocktail prepared earlier. The contents were swirled immediately as to stop the reaction.

2.6 Varying the Reaction Parameters

The effect of enzyme loading was studied by varying the mass of enzyme added to the reaction mixture where the total working volume for each experiment was set as 40 ml. The enzyme concentration was expressed in the form of percentage, w/v and varied from 0.5 to 2.5%, while other parameters remain constant (buffer volume to oil volume ratio (1:1), reaction temperature (40 ºC), buffer pH (7), and agitation speed (200 rpm)).

For the effect of water content, the buffer volume to oil volume ratio was varied from ratio of 1:1 to 5:1 at fixed condition (enzyme loading (1.5% w/v), reaction temperature (40 ºC), buffer pH (7), and agitation speed (200 rpm)).

In order to optimize the reaction temperature, the temperature was varied from 35 to 60 ºC at fixed condition (enzyme loading (1.5% w/v), buffer pH (7), water to substrate ratio (3:1), and agitation speed (200 rpm)).

The effect of buffer pH was studied by varying the pH of the buffer solution from pH of 5 to pH of 9. Other parameters were set constant (enzyme loading (1.5% w/v), water to substrate ratio (3:1), reaction temperature (40 ºC), and agitation speed (200 rpm)).

Optimization of agitation speeds was done by varying the agitation speed of orbital shaker from 160 to 260 rpm at fixed condition (enzyme loading (1.5% w/v), water to substrate ratio (3:1), buffer pH (7), and reaction temperature (40 ºC)).

For each manipulated parameters, previous optimum value gained was used for the next experimental set**.**

2.7 Analysis Method

Degree of hydrolysis was determined by titration method using 1.0 M sodium hydroxide (NaOH). Each sample from six different time intervals were titrated with 1.0 M NaOH using burette until a light blue colour appear. Into the last titration cocktail, 5 ml of sodium phosphate buffer and substrate mixture was added. This mixture wasperformed as a blank titration and was titrated with 1.0 M NaOH.

The degree of hydrolysis was calculated using equation 1 as reported by Noor *et al*. [3]:

$$X\ \% = \frac{[V_i - V_o] \times N \times 10^{-3} \times AMW}{W \times f_o} \times 100\% \quad (1)$$

X is degree of hydrolysis (%), *Vi* is volume of NaOH for sample to reach the end point (ml), *Vo* is volume of NaOH for the blank to reach the end point (ml), *N* is normality of NaOH (mol/L), 1.0 M for this experiment, *W* is weight of sample (g), *AMW* is average molecular weight of fatty acid (268.4347 g/mol), and $f_o$is the volume fraction of oil at the start of reaction.

2.8 Enzyme Kinetics

The effect of substrate concentration on the rate of reaction was evaluated to determine the Michaelis-Menten kinetic parameters, K_m and V_{max}. Lineweaver-Burk double reciprocal plot was constructed by fitting a graph of enzyme velocity versus substrate concentration. Different concentrations of oil were prepared using iso-ocatane as a solvent. Other parameters were set constant including enzyme loading = 1.5%, 3:1 water to substrate ratio, buffer pH = 7, reaction temperature = 40 ºC, agitation speed = 220 rpm and 3 hours reaction time.

■3.0 RESULTS AND DISCUSSION

3.1 Effect of Enzyme Loading

Appraisal on enzyme optimum concentration is crucial in avoiding enzyme wastage. In order to increase the degree of hydrolysis, it is necessary to add successively high amounts of lipase. However,

high lipase concentration would make the oil-water-lipase mixture to be paste and leads to difficulty in separation processes [7]. The effect of enzyme loading was varied from 0.5 to 2.5% (w/v). The experimental result demonstrates the increased in degree of hydrolysis as the enzyme concentration increase from 0.5 to 1.5% (w/v). For an ideal condition, an increase of enzyme concentration would proportionally increase the degree of hydrolysis. However, this proportional relationship was not observed in this study.

Figure 1 shows the effect of enzyme concentration on the degree of hydrolysis. The increase of enzyme loading increased the degree of hydrolysis up to 1.5% (w/v) of enzyme concentration. Beyond this value, further increase of enzyme concentration did not increase the degree of hydrolysis, but reduced it significantly. This trend also reported in the study of virgin coconut oil hydrolysis using immobilized lipase [8]. The decrease in degree of hydrolysis has directly influence the decrease in the amount of fatty acids liberated.

The optimum degree of hydrolysis was recorded at 1.5% (w/v) of enzyme concentration with 34% degree of hydrolysis. The decreasing pattern after the optimum enzyme concentration might appeared due to enzyme saturation at the interface which favouring the reaction to produce less amount of product [9]. Lipases are enzymes that hydrolysed ester of long chain aliphatic acids from glycerol at oil-water interface. Saturation of enzymes at the interface has limited the surface area for hydrolysis to occur. As a result, fewer amounts of fatty acids were produced. This mainly explains by the limitation of interfacial area for catalysis due to the saturation of enzymes in the bulk phase, reducing the flexibility of enzymes during catalysis [8, 10].

Hence, enzyme concentration has a strong impact on the catalytic process. As lipase concentration increase, lipase moves from the aqueous phase to the interface at increasing rate, making the interaction with the substrate increases and resulting towards enhanced hydrolysis. However, as the concentration of lipase is sufficiently high to saturate the available interface, the extant of hydrolysis begins to fall due to limitation of area for catalysis.

3.2 Effect of Water Content

Water reacts as a reactant in the hydrolysis and as a modifier for lipase functionality during the reaction. Effect of water content towards degree of hydrolysis is determined by varying the amount of buffer at constant pH. Generally, degree of hydrolysis increase as the volume of buffer is increased until the optimum value is achieved. Increase in buffer volume afterwards will result to the decrease in degree of hydrolysis.Several studies have demonstrated that enzymatic behaviour is related to the nature of lipase that reacts at the oil-water interface [11, 12]. Figure 2 shows the effect of buffer volume towards the total percentage of hydrolysis degree for different ratio ranging from 1:1 to 5:1 at constant 3 hours of reaction time. In enzyme-catalysed hydrolysis reaction, the buffer volume to substrate volume ratio directly responds toward the degree of hydrolysis to achieve equilibrium.

The product formation will be decreased with the increased in buffer volume due to the competition of fatty acids and lipase towards the interface. The availability of water for biocatalyst and maintaining the enzymatic activity is different depending on water partitioning system [13]. This also may attribute to the dilution effect of lipase concentration in the water phase.

The increase in water content promotes the balance in the reaction, which increase the degree of hydrolysis, promotes the reaction to produce high amount of fatty acids [7]. For the cases of high water content, the hydrolysis rate began to slow down due to the decrease in contact between the lipase and the oil. The excess water will generate a thicker water layer around the enzyme surface and cause a diffusivity problem of substrate and product from the enzyme active sites [18]. Higher water content also leads to aggregation of enzyme cause by the surface tension effect. Hence, 3:1 ratio believes to provide an adequate interfacial area for the hydrolysis to occur with 94.4% degree of hydrolysis.

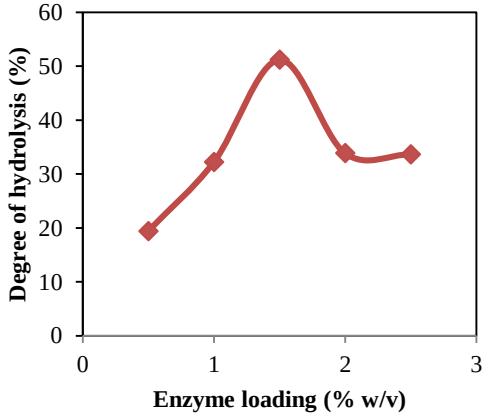


Figure 1 Effect of enzyme loading on the rate of hydrolysis (Temperature = 40ºC; pH = 7; Agitation speed = 200 rpm; Buffer volume to oil volume ratio =1:1 (v/v); Reaction time = 3 hours)

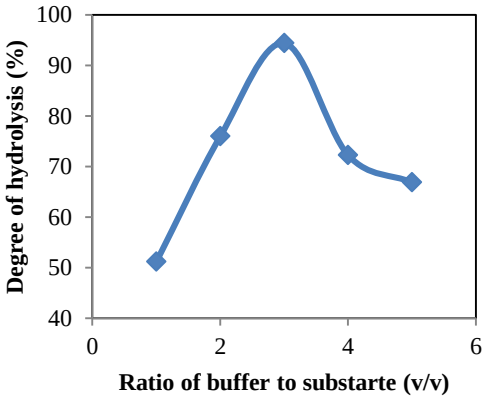


Figure 2 Effect of buffer volume to oil volume ratio on the degree of hydrolysis (Temperature = 40ºC; pH = 7; Agitation speed = 200 rpm; Enzyme concentration = 1.5% (w/v); Reaction time = 3 hours)

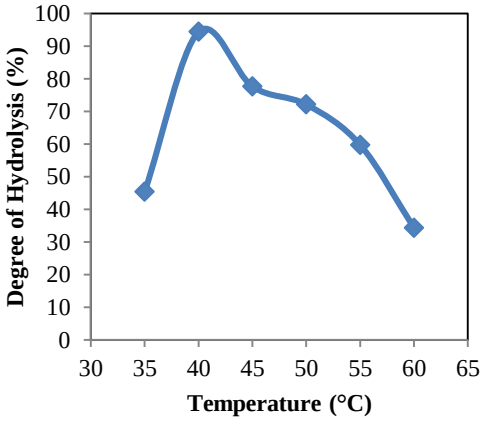


Figure 3 Effect of temperature on the degree of hydrolysis (Enzyme concentration = 1.5% (w/v); Buffer volume to oil volume ratio= 3:1 (v/v); pH= 7; Agitation speed = 200 rpm; Reaction time = 3 hours)

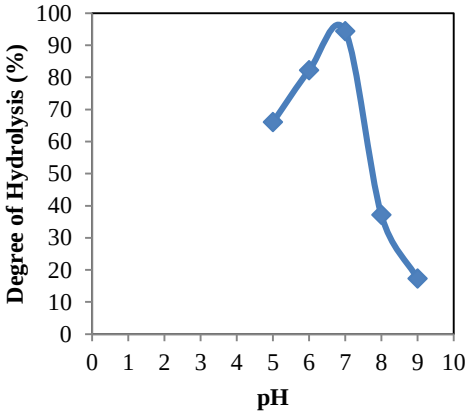


Figure 4 Effect of pH on the degree of hydrolysis (Enzyme concentration = 1.5% (w/v); Buffer volume to oil volume ratio = 3:1; Temperature = 40 °C; Agitation speed = 200 rpm; Reaction time = 3 hours)

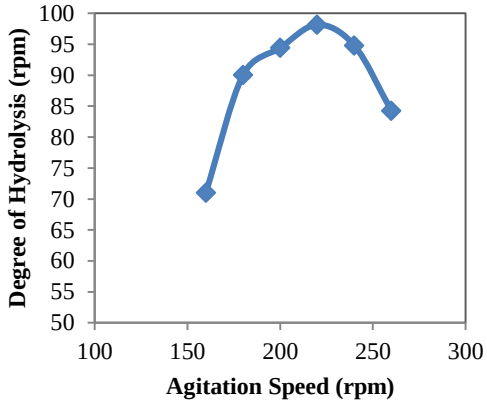


Figure 5 Effect of agitation speed on the degree of hydrolysis (Enzyme concentration = 1.5% (w/v); Buffer volume to oil volume ratio = 3:1 (v/v); Temperature = 40°C; pH = 7; Reaction time = 3 hours)

3.3 Effect of Temperature

Temperature has a variety effects on enzyme activity. The raise in temperature has accelerated the mobility of substrate and product. However, the parallel increase of temperature and catalytic rate are considerable up to a point where denaturation becomes significant. Later, small increase in temperature will cause great increase in denaturation resulting in the decrease of product formation. Effect of temperature on degree of hydrolysis is shown in Figure 3. The temperature was varied from 35to 60°C. Figure 3 initially shows the increased in degree of hydrolysis with the increased in temperature. The degree of hydrolysis increased from 19.4% at temperature of 35 °C to 94.4% at temperature of 40°C. This is mainly due to the increase in the rate constant and partly due to increase in interfacial area with increase in temperature. Al-Zuhair*et al.* [10] have considered this condition on the hydrolysis of fresh palm oil by lipase. However, the degree of hydrolysis decreased sharply after temperature of 40°C. The decreasing trend may appear from the deactivation of enzyme. Theoretically, enzyme tertiary structure will disrupts and denature at high reaction temperature.

At temperature of 40 °C, the highest degree of hydrolysis (94.4%) was achieved. Later, degree of hydrolysis starts to decrease to 34.3% at 60 °C. These values agree with the phenomena of enzyme denaturation at high temperature. It is known that most protein tend to denatured at temperature of 50 °C [10]. In addition, the optimum temperature of microbial lipase lies between 37 to 55 °C. Ting *et al*. [13] has found the optimum temperature for immobilized *C. rugosa*lipase on the hydrolysis of soybean oil was in the range of 40 to 42 °C. Yigitoglu and Temocin [27] found the optimum temperature for olive oil hydrolysis using *C. rugosa*lipase at the temperature of 40 °C. Therefore, previously reported values supported the finding of this study.

3.4 Effect of pH

pH plays a vital role in the hydrolysis reaction in order to achieved optimum production of fatty acids [4]. Therefore, effect of buffer pH was investigated in the range of 5 to 9. The resulting effect of varying the initial buffer pH on the degree of hydrolysis is presented in Figure 4. From the figure, the degree of hydrolysis initially increases to a maximum (optimum) pH and then decreases afterwards. The increasing in degree of hydrolysis was observed from pH of 5 to pH 7 with 66.08% to 94.4% degree of hydrolysis. The sharp drop on the degree of hydrolysis from 37.18 to 17.34% was observed with the increase in pH from 8 to 9. The sharp fall in the degree of hydrolysis after the optimum pH has clearly explained how explicitly pH can affect the hydrolysis rate.

Change of pH affects the ionization of free substrate and enzyme. Lipase activity is affected by pH due to its effect on ionization of lipase-substrate complex. Extreme pH will contribute to the denaturation of lipase and substrate begins to breakdown as its concentration decreased [11]. As a result, product inhibition become more significant and leads to a decreased in the rate of reaction. Garcia *et al.* [17] has obtained an optimum pH of 7 for *C rugosa*lipase in the hydrolysis of milk fat triglycerides. Kang and Rhee [19] and Yigitoglu and Temocin [27] also reported the same optimum pH on the hydrolysis of olive oil using *C. rugosa*lipase. On the other hand, Ting *et al*. [16] has obtained the optimum pH for *C. rugosa*lipase on soybean oil hydrolysis lies between pH of 7 to pH of 8. For the hydrolysis of palm olein, the optimum pH has found at the pH of 7.7 [4]. All the values gained from the previous literatures are mostly based on the hydrolysis of fresh edible oil. None of the studies has used the used -frying oil as the main substrate. Our findings show no different in the optimum pH values for used frying oil hydrolysis as well as fresh edible oil hydrolysis. Again, the optimum pH value found in this study complies with the values gained by several literatures. The optimum pH for used frying oil hydrolysis was found at pH of 7 with 94.4% degree of hydrolysis.

3.5 Effect of Agitation Speed

Lipase is an interfacial enzyme.The rate of hydrolysis increases with the increase in interfacial area. Agitation speed influence the degree of hydrolysis as it reduces the droplet size, increasing the specific interfacial area between oil and the aqueous phase [4].High agitation speed provide high shear in the medium, generating a fine

dispersion and offer large interfacial area for the hydrolysis reaction to occur [12].The effect of agitation speed on the degree of hydrolysis is presented in Figure 5. The agitation speeds were varied from 160 to 260 rpm.It is clear from the figure that the degree of hydrolysis increased (71.03 to 98.17%)with the increasing in the agitation speed until agitation speed of 220 rpm.After that, the decreased in the degree of hydrolysis was observed from agitation speed of 240 to 260 rpm (94.80 to 84.23%). The highest degree of hydrolysis (98.17%) was recorded at agitation speed of 220 rpm.

Since lipase hydrolysed oil at the interface, the increase in interfacial area prompts the increase in degree of hydrolysis [21]. However, as speed of agitation increases, lipase is exposed towards higher shear stress and resulting in rapid unfolding of lipase. This rapid process causes a raise in denaturation leading to a decrease in degree of hydrolysis. Increasing interfacial area increase the reaction rate by the occurrence of special fit between the respective geometries of lipase active site and aggregates of substrate which leads to high activation effect [22, 23]. At low agitation speed, the effect of enhancement of interfacial area on hydrolysis is greater compared to the effect of lipase deactivation. Thus, degree of hydrolysis increases. The same trend as the present study was also observed in the castor oil hydrolysis [14] and palm olein hydrolysis [4] using *C. rugosa* lipase.

The optimum agitation speed value found in the present study was higher compared to the reported values [14, 4, 24, 25]. All the previous studies used fresh edible oil for the hydrolysis. Used-frying oil has higher viscosity compared to the usual fresh cooking oil [26]. Thus, higher agitation speed is required to provide more shear stress and reduce the droplet size for rapid contact between oil and enzymes. In this study, agitation speed of 220 rpm has been considered as an optimum agitation speed.

3.6 Enzyme Kinetic Parameters

The effect of substrate concentration on the rate of reaction was evaluated to determine the Michaelis-Menten kinetic parameters, K_m and V_{max}. Lineweaver-Burk double reciprocal plot was constructed by fitting a graph of enzyme velocity versus substrate concentration. K_m and V_{max} values were calculated using linear regression analysis. Figure 6 shows the Lineweaver-Burk plot for used-frying oil hydrolysis at different substrate concentration (0.2 to 1 g/ml). The hydrolysis shows the low K_m value (0.0833 g/ml) and higher V_{max} value (20.833 µmol/ml.min) than other *C. rugosa* lipase hydrolysis reported in literatures [27, 4, 16, 17]. A low K_m value represents a high affinity of lipase towards the substrate. It only takes a very little or low concentration of substrate to be half saturated. High V_{max} value is much favourable as it shows the high conversion of substrate to product per unit time when the enzyme saturated with substrate making its maximal velocity higher.

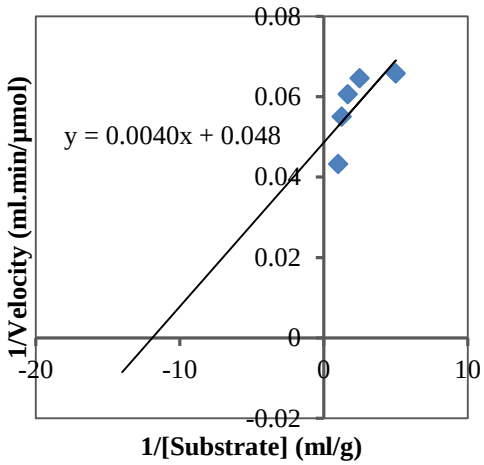


Figure 6 Lineweaver-Burk plots for used-frying oil hydrolysis (1.5% (w/v) enzyme concentration; 3:1 buffer volume to oil volume ratio; 40 °C; 220 rpm; 3 hours)

■4.0 CONCLUSION

Enzymatic hydrolysis of used-frying with lipase is one of the promising methods to convert used-frying oil into valuable fatty acids. Enzymatic hydrolysis using lipase does depend on variety of parameters including enzyme concentration, water content, temperature, pH, and agitation speed. The hydrolysis process achieved the highest degree of hydrolysis at enzyme concentration of 1.5% (w/v), buffer volume to oil volume ratio of 3:1, temperature of 40 ˚C, pH of 7 and agitation speed of 220 rpm under constant 180 minute reaction time. Under these described conditions, it was found that nearly 98.1% of hydrolysis degree was achieved. Through the optimization process, lipase has experienced the increasing and decreasing pattern in degree of hydrolysis depending on the manipulated variables introduced into the system. The profile for each manipulated parameters shows a bell shape curve, where initial degree of hydrolysis increased towards the critical (optimum) value and decreased afterwards due to unfavourable effects of extreme reaction parameters. A kinetic model based on Michaelis-Menten equation was used to determine the rate constant of V_{max} and K_m and it was found to be 20.83µmol/ml.min and0.08 g/ml, respectively.

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