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Enzymatic Hydrolysis of Used-Frying Oil Using *Candida rugosa* Lipase

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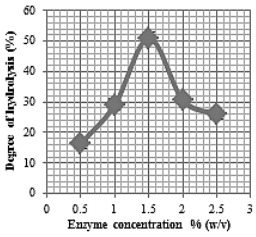
Graphical abstract



Abstract

Hydrolysis of used-frying oil had been carried out by using an immobilized lipase from *Candida rugosa* in solvent-free system. Used-frying oil was considered as the substrate in this study due to abundance amount of used-frying oil present in Malaysia as its disposal problem has 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 the 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 speeds on the hydrolysis process were investigated. The experiments were conducted at constant reaction time of 3 hours. It was found that the effect of variables were very significant on 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, it was found that nearly 97.15±1.31% of hydrolysis degree was achieved with 2533.33±26.67 µmol/ml of fatty acids was produced.

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

Abstrak

Hidrolisis minyak menggoreng terpakai telah dijalankan dengan menggunakan lipase pegun daripada *Candida rugosa* dalam system bebas pelarut. Minyak menggoreng terpakai dipilih sebagai substrat dalam kajian ini kerana jumlahnya yang banyak di Malaysia serta masalah pelupusannya menjadi salah satu isu alam sekitar yang serius. Kandungan asid lemak bebas (FFA) yang tinggi dalam minyak menggoreng terpakai telah membuka peluang memanfaatkan bahan buangan ini untuk penghasilan pelbagai jenis produk. Walaupun minyak menggoreng terpakai tidak sesuai untuk kegunaan manusia dan secara meluas digunakan untuk penghasilan biodiesel, asid lemak bebas daripada minyak menggoreng terpakai boleh digunakan untuk penghasilkan pelbagai jenis produk bukan makanan. Kesan pemuatan enzim, kandungan air, suhu, pH penampan, dan kelajuan pengadukan terhadap proses hidrolisis telah diselidiki. Kajian ini telah dijalankan pada masa tindak balas 3 jam. Didapati bahawa kesan pemboleh ubah memberi impak yang signifikan ke atas proses hidrolisis. Proses hidrolisis minyak menggoreng terpakai mencapai kandungan asid lemak bebas tertinggi pada pemuatan enzim 1.5 U/ml, nisbah penampan kepada jumlah minyak 3:1 (v/v), suhu 40˚C, pH 7, dan kelajuan pengadukan 220 rpm. Sebanyak 97.15 ± 1.31 % takat hidrolisis telah dicapai dengan dengan 2533.33 ± 26.67 µmol /ml µmol asid lemak telah dihasilkan.

Kata kunci: Minyak menggoreng terpakai; *Candida rugosa lipase*; pemuatan enzim; asid lemak

1.0 INTRODUCTION

Developed countries are the major worldwide contributors to the increasing amount of used-frying oil. Management of such oil poses a significant challenge because of their disposal problems and possible contamination towards water and land resources. Disposal of used-frying oil is relatively difficult due to their physical form. Usually used-frying oil exists in a liquid or semi-solid form and solid waste regulations prohibit any liquid waste to be disposed in landfills. The most common and simplest disposal method use especially by food industry is pouring the waste into drain. In the end, this waste will results in pipes clogging and destruction towards wastewater and septic system.

About 74,000,000 litres of waste olive oil collected every year around the world and discarded inappropriately.[1] The Energy Information Administration in United State estimated that 100 million gallons of waste cooking oil were produced per day in USA.[2] With the mushrooming of fast food centres and restaurants in Malaysia, it is expected that extensive amounts of 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. Abundant amount of used-frying oil from food industry and

household draw an attention that this particular waste could be utilized and recycled to produce valuable products. Generally enzymatic hydrolysis of oil and fat with lipase produced fatty acid and glycerol. Enzymatic hydrolysis of lipase is one of the promising methods to convert used-frying oil into valuable fatty acid.[3] Glycerol and fatty acids are widely used as raw materials in foods, cosmetics, and pharmaceutical industries.

Even though fatty acids gained from the used-frying oil are not suitable for human consumption, it can be utilize and undergo 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 has been known for its high fatty acids content compared to fresh cooking oil.[4] Hence, used-frying oil utilization is very useful in meeting the demand from both domestic and global production of fatty acids. The utilization of used-frying oil would be desirable since this approach would not divert resource from the food supply.[5] Most of seed oil such as sunflower, rapeseed, soybean, and palm oil has the carbon chain length ranging from C16 to C18, which lack functionalities that contribute to wider industrial application such as hydroxyl and epoxy groups.[6]

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 basic material for the production of oleo chemicals such as fatty alcohol.[7] Existing methods for production of fattyare based on chemicals and physical methods.[8] Under these method, polymerization of fat and by-products take place resulting in dark fatty acids and discoloured aqueous glycerol solution that required physical refining (distillation) under high temperature and vacuum whereby the process extremely consumed high energy. Enzymatic hydrolysis is chosen in this research because it can be performed at low temperature to save energy, and exhibits high selectivity, leading to product with high purity and fewer side products.[7]

Therefore, with the aid of lipase, used-frying oil was undergo enzymatic hydrolysis and converted into fatty acids and glycerol. This method on the other hand could save a lot of energy and reduce environmental pollution as 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.[7-12] Many literatures also reported the use of used-frying oil as substrate for biodiesel production.[4,13,14] However, only limited literatures are available regarding the production of fatty acids from used-frying oil using lipase. Hence, this study aims to investigate 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 speeds on used-frying oil hydrolysis as well as fatty acids yield were investigated. Using immobilized 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 the cognizance of 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 with pH value of 4.69, acid value of 8.94±0.29 mg KOH/g, oleic acid concentration of 41.85±0.53% wt. and palmitic acid concentration of 39.12±0.98% wt. The enzyme *C. rugosa*immobilized on Immobead 150 (2370 U/g), thymolphthalein indicator, sodium monobasic and dibasic 0.1 M were purchased from Atama Tech Sdn. Bhd. Ethanol with 95% (v/v), iso-octane with 99.8% purity and sodium hydroxide 1.0 M were purchased from Xcellent Trading & Marketing. All chemicals are of analytical grade and were used without further purification.

2.2 Method

Method proposed by Pinsirodom and Parkin[15] was used for the determination of fatty acids concentration. Method used for pre-treatment procedure was conducted according to the method proposed by Pazouki*et al*.[16]

2.3 Pre-treatment of Used-frying Oil

Used–frying oil contains lot of impurities resulting from continuous cooking and oxidation process. Therefore, pre-treatment is required to eliminate the indiscerptible impurities. Firstly, 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

Before conducting the enzymatic reaction, 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.[16]

2.5 Enzymatic Hydrolysis

Initially, 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=40ºC, 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%; reaction temperature = 40ºC; buffer pH = 7; agitation speed = 200 rpm).

In order to optimize the reaction temperature, the temperature was varied from 35 to 60ºC at fixed condition (enzyme loading = 1.5%; buffer pH = 7; 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%; reaction temperature = 40ºC; agitation speed = 200 rpm).

In order to optimize the agitation speed, the agitation speeds were varied from 160 to 260 rpm fixed condition (enzyme loading = 1.5%; reaction temperature = 40ºC; buffer pH = 7).

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

2.7 Analysis Method

The degree of hydrolysis was determined by titration the samples 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 appears. Into the last titration cocktail, 2 ml of sodium phosphate buffer and substrate mixture was added. This mixture was performed as a blank titration and was titrated with 1.0 M NaOH. The quantity of fatty acids liberated in each subsample is based on the equivalents amount of NaOH used to reach the end-point of the titration. The final unit for the following equation is µmol fatty acid/ ml subsample. Concentration of fatty acids for each sample was calculated using equation 1 as reported by Pinsirodom and Parkin.[15]

$$C_o = \frac{[V_i - V_o] \times N \times 1000}{v \times f_o} \quad (1)$$

C_o is concentration of fatty acids, V_i is volume of NaOH for sample to reach the end point (ml), V_o 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, v is volume of sample, 2 ml and f_o is the volume fraction of oil at the start of reaction.

The degree of hydrolysis was calculated using Equation 2 as reported by Noor *et al.*[7]:

$$X\,\% = \frac{[V_i - V_o] \times N \times 10^{-3} \times AMW}{W \times f_o} \times 100\% \quad (2)$$

X is degree of hydrolysis (%), V_i is volume of NaOH for sample to reach the end point (ml), V_o 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, and 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.

■3.0 RESULTS AND DISCUSSION

3.1 Effect of Enzyme Loading

Assessment on enzyme optimum concentration is crucial in avoiding enzyme wastage. In order to increase the degree of hydrolysis, it was necessary to add successively high amounts of lipase. However, high amount of lipase concentration would make the oil, water, lipase, to be paste and leads to difficulty in separation processes.[17] In this study, the effect of enzyme loading was varied from 0.5 to 2.5% (w/v). The experimental result shows 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 here.

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 a critical 1.5% (w/v) enzyme concentration. Beyond this value, further increase of enzyme concentration did not increase the degree of hydrolysis, butreduced it significantly. This trend also reported in the study of virgin coconut oil hydrolysis using immobilized lipase.[9] On the other hand, the fall in degree of hydrolysis has directly influence the decrease in the amount of fatty acids liberated. The effect of enzyme concentration upon fatty acids (FA) liberated is shown by Figure 2. As shown in Figure 2, the fatty acids concentration was increased with the time for enzyme concentration of 0.5 to 1.5% (w/v). However for the enzyme concentration of 2 and 2.5 % (w/v), the increasing amount of fatty acids only last for the first 90 to 150 minutes, where the decreasing amount of fatty acids are vivid towards the end of reaction.

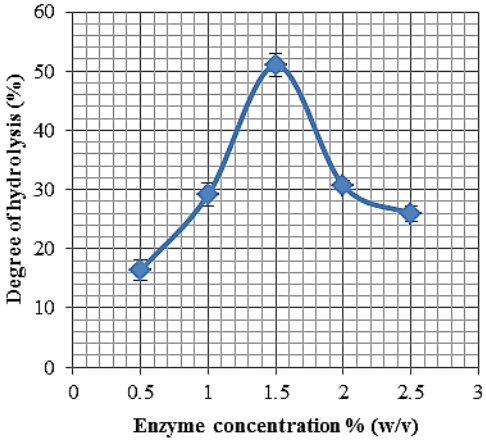


Figure 1 Effect of enzyme loading on the rate of hydrolysis (Temperature 40 ºC; pH 7; 200 rpm, 1:1 water: oil, 3 hours)

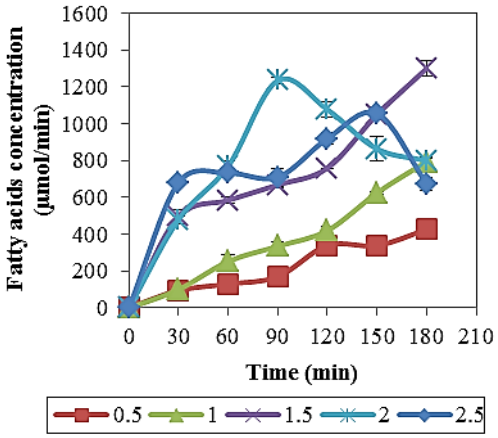


Figure 2 Effect of enzyme concentration (% w/v) upon extent of 3 hours of reaction time

The optimum degree of hydrolysis was recorded at 1.5% (w/v) enzyme concentration with 51.0±1.92% degree of hydrolysis and 1030±50 µmol/ml concentration of fatty acids at 180 minutes reaction time. The decreasing trend after the optimum enzyme concentration (2 to 2.5% w/v) might appeared to enzyme saturation at the interface which favouring the reaction to produce less amount of product.[18] Lipases are enzyme that hydrolysed ester of long chain aliphatic acids from glycerol at oil-water interface. Saturation of enzyme 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.[9,19]

3.2 Effect of Water Content

Water reacts as a reactant in the hydrolysis and the modifier for lipase functionality throughout the reaction. Effect of water content towards degree of hydrolysis was generally determined by varying the amount of buffer at constant pH. Generally, degree of hydrolysis increase as the volume of buffer is increase until the optimum buffer volume is achieved. Increase in buffer volume afterwards will result to a decrease in degree of hydrolysis. Several studies have shown the enzymatic behaviour is related to the nature of lipase that reacts at the oil-water interface.[10, 20]. Figure 3 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 reaction time.

In enzyme-catalysed hydrolysis reaction, the buffer volume to the substrate volume ratio directly responds towards the degree of hydrolysis and the time required to achieve equilibrium. Ratio of water and oil is crucial factors to regulate the chemical equilibrium.[17] With the increase of water content, the hydrolysis rate is directly increased. When the amount of water content reached ratio of 3:1, the hydrolysis achieved the highest degree of hydrolysis. Too high or low water content causes a decrease in the hydrolysis rate. The volume of water present in the system will affect the reversibility of reaction towards hydrolysis or esterification.

Figure 4 illustrates the concentration of fatty acids increased with time as for the ratio of 1:1 to 3:1. For the ratio of 4:1 and 5:1, the concentration of fatty acids increased until 120 minutes, and then gradual decrease in fatty acids was observed afterwards. The optimum ratio of buffer volume to oil volume ratio for castor oil hydrolysis was found at ratio of 3:1,[20] which particularly support the finding from this study. The study also proves that as the buffer volume increased, the percentage of fatty acids produced also increased until the optimum buffer volume is achieved. Yet, the percentage of fatty acids produced decreased due to the competition of fatty acids and lipase toward the interface. In general, the availability of water for biocatalyst and maintain the enzymatic activity is varied depending on the water partitioning in system.[21] This also may attribute to the dilution effect of lipase concentration in the water phase.

The increase in water content promotes the balance in positive reaction, which increased the degree of hydrolysis making the reaction to produce high amount of fatty acids.[17] 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 create a thicker water layer around the enzyme surface and cause a diffusivity problem of substrate and product from the enzyme active sites.[9] Higher water content also leads to aggregation of enzyme cause by the surface tension effect. Excessive water content in the reaction also might denature the enzyme protein content particles permanently.

Hence, 3:1 ratio is believes to provide adequate interfacial area for the hydrolysis to occur with 91.4±0.90% degree of hydrolysis and 2383.33±23.33 µmol/ml of fatty acids concentration.

3.3 Effect of Temperature

Temperature has a variety of effects on enzyme activity. The raise in temperature has accelerated the mobility of substrate and the product. However, the parallel increase of temperature and catalytic rate are considerable up to a point where denaturation becomes significant. Afterwards, small increases in temperature cause great increases in denaturation with result in the decrease of product formation. Increasing the reaction temperature has affected the production of fatty acids, which clearly showed an increase in conversion. Effect of temperature on degree of hydrolysis is shown in Figure 5. The temperature was varied from 35 to 60°C.

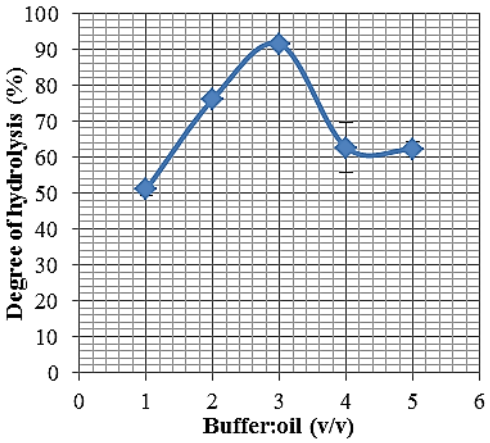


Figure 3 Effect of water content on the degree of hydrolysis (Enzyme concentration 1.5% (w/v), Temperature 40^{0}C; pH 7; 200 rpm, 3hours)

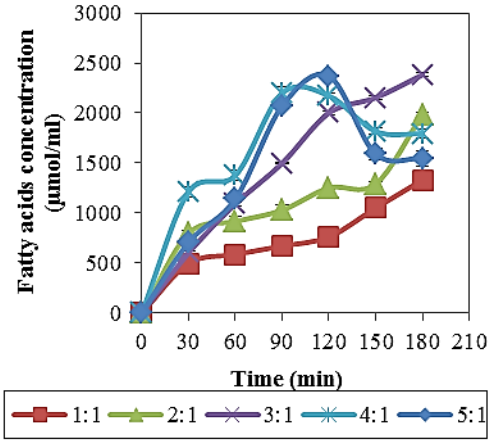


Figure 4 Effect of water content (v/v)upon extent of 3 hours of reaction time

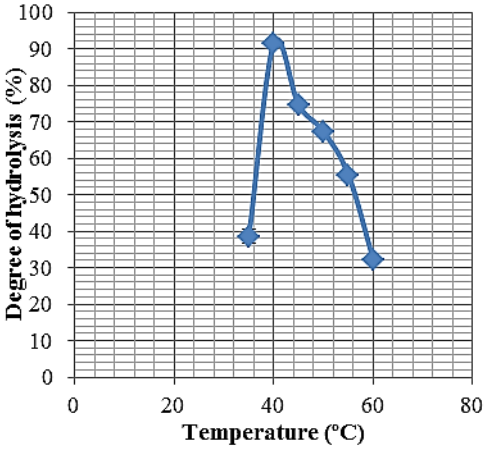


Figure 5 Effect of temperature on the degree of hydrolysis (Enzyme concentration 1.5% (w/v), 3:1 buffer volume to oil volume ratio; pH 7; 200 rpm, 3 hours)

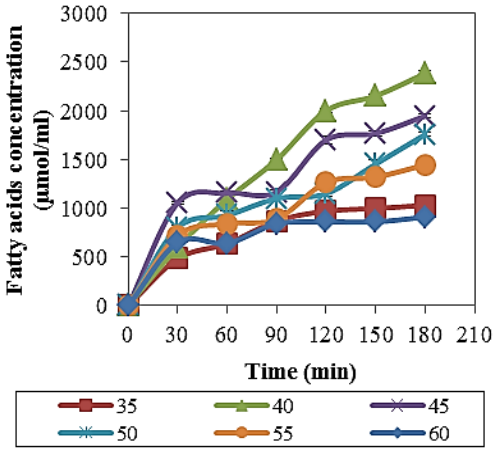


Figure 6 Effect of temperature upon extent of 3 hours of reaction time

From Figure 5, initially the degree of hydrolysis increased with the increase in temperature. The degree of hydrolysis increased from 38.60±1.79% at temperature of 35°C to 91.4±0.90% at temperature of 40°C. This is mainly due to the increase in the rate constant with the temperature and partly due to the increase in interfacial area with temperature. Al-Zuhair*et al.*[19] have discussed 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. Temperature of 40°C represents the highest degree of hydrolysis up to 91.4±0.90%. Later, degree of hydrolysis start to decreased to 74.65±0.19% at 45°C, 67.36±0.24% at 50°C and 32.21±0.80% at 60°C. These values again agree with the phenomena of enzyme denaturation at high temperature.

It is known that most protein tend to denatured at temperature of 50°C.[19] In addition, the optimum temperature of microbial lipase lies between 37 to 55°C. Ting *et al.*[17] 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[3] found the optimum temperature for olive oil hydrolysis using *C. rugosa* lipase at temperature of 40°C. Therefore, previously reported values supported the finding of this study. Enzyme shows the highest activity at 30 to 40ºC and quickly denatured at temperature above 50ºC.[22]

The effect of varying the temperature on the fatty acids production for 3 hours profile is provided in Figure 6. Here, the temperature changes affect the amount of fatty acids produced. As the temperature increases, the total concentration of fatty acids for 3 hours has increased from temperature of 35 and 40°C. However, the concentration of fatty acids produced showed a significant decreased with an increase in temperature from 45 to 60°C. On the other hand, Figure 6 represents the proportional relationship between the amounts of fatty acids produced with the increase in time. Generally, varying the temperature affects the catalysed reaction and thermal activation of the enzyme. In the low temperature range, rate of inactivation is negligible compared with the rate of catalysed reaction. Inactivation becomes more significant at higher temperatures causing protein to denature from conformational changes. This can be associated with the broken of hydrogen bonds and the unfold molecule ceases to function.[23]

The presence of deactivated enzyme at the interface would also hinder the active enzymes from penetrating the interface. The same trend for the production of fatty acids at various temperatures within 0 to 8 hours of reaction were demonstrated in the hydrolysis of crude palm olein by lipase from *C. rugosa*[11] and hydrolysis of virgin coconut oil using immobilized reactor.[9]Hence, *C. rugosa* lipase produced high yield of fatty acids at the temperature of 40°C with fatty acids concentration of 2383.33±23.33 µmol/ml. Beyond this temperature, *C. rugosa* lipase lost its activity due to denaturation.

3.4 Effect of pH

The behavior of an enzyme molecule may modified by its immediate microenvironment. pH plays a crucial role in hydrolysis reaction in order to achieved optimum production of fatty acids.[8] 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 7. From Figure 7, degree of hydrolysis initially increased to a maximum (optimum) pH and then decreased afterwards.

The increase in degree of hydrolysis was observed from pH of 5 to pH 7 with 65.67±0.67% to 91.4±0.90% degree of hydrolysis respectively. The sharp decrease on degree of hydrolysis from 27.80±0.49 to 15.34±0.14 % was then observed with the increase in pH from 8 to 9. The sharp fall in degree of hydrolysis after the optimum pH has clearly explained how explicitly pH can affect the hydrolysis rate. The enzyme optimized its performance in an alkaline medium but approximately neutral rather than a very acidic or alkaline medium.

Change in pH affects the ionization of free substrate and enzyme. Hence, 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.[20] As a result, product inhibition become significant and leads to a decreased in the rate of reaction.

The fatty acids concentration profile for 3 hours reaction on various buffer pH is presented by Figure 8. Mostly, all buffer pH have experienced the increase in fatty acids concentration as the time increase. Fatty acids concentration achieved its highest concentration at buffer pH of 7 with 2383.33±23.33 µmol/ml of fatty acids concentration. Hence, lipases certainly needed an optimum pH for the hydrolytic reaction to take place in order to produce high yield of fatty acids.

Garcia *et al.*[24] obtained an optimum pH of 7 for *C rugosa* lipase in the hydrolysis of milk fat triglycerides. Kang and Rhee[12] and Yigitoglu and Temocin[3] also reported the same optimum pH on the hydrolysis of olive oil using C. rugosa lipase. Ting *et al.*[17]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.[8]

All the values gained from the previous literatures are based on the hydrolysis of fresh edible oil. None of the studies had used the used-frying oil as the main substrate. There is no different in 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. Theoptimum pH for used frying oil hydrolysis is at pH of 7 with the fatty acids yield of 2383.33±23.33 µmol/ml.

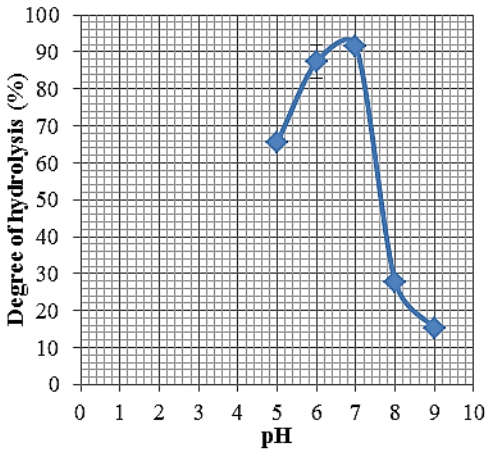


Figure 7 Effect of pH on the degree of hydrolysis (Enzyme concentration 1.5% (w/v); 3:1 buffer volume to oil volume ratio; 40 °C; 200 rpm; 3hours)

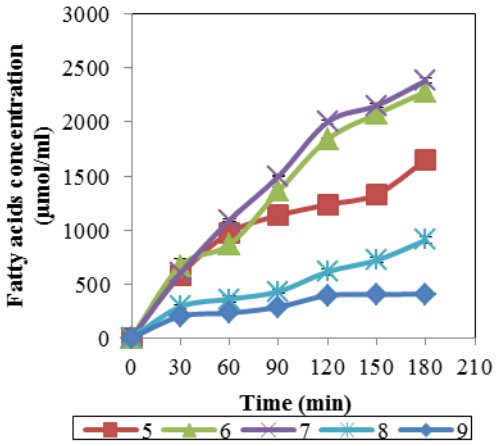


Figure 8 Effect of pH upon extent of 3 hours of reaction time

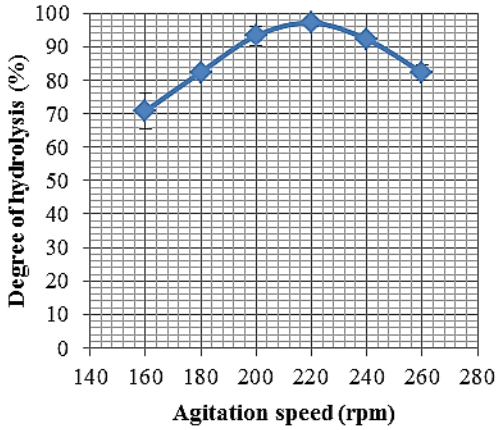


Figure 9 Effect of agitation speed on the degree of hydrolysis (Enzyme concentration 1.5% (w/v); 3:1 buffer volume to oil volume ratio; 40 °C; pH 7; 3hours)

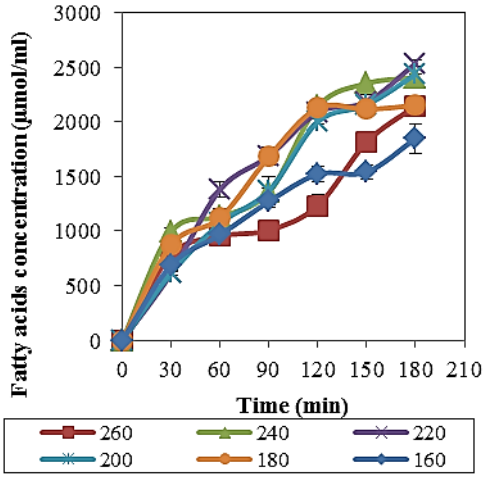


Figure 10 Effect of agitation speeds upon extent of 3 hours of reaction time

3.5 Effect of Agitation Speed

Lipase is an interfacial enzyme. Agitation speed has influenced the degree of hydrolysis by increasing the specific interfacial area between oil and enzymes by reducing the droplet size.[5] Putli*et al.*[10]defined high agitation speed provides high shear in the medium, generating a fine dispersion and provide large interfacial area for the hydrolysis reaction to occur. Goswami*et al.*[20] have explained the effect of higher agitation speed towards hydrolysis degree of castor oil using *C. rugosa* lipase which is proportional with the increase in the shear stress, giving the unfolding of lipase to occur more rapidly, leading to possibility of denaturation. In this study, the agitation speeds were varied from 160 to 260 rpm. Other parameters such as enzyme concentration (1.5% v/w), buffer volume to oil volume ratio (3:1), temperature (40 ºC), and buffer pH (7) were set constant. The resulting effect of varying the agitation speeds on the degree of hydrolysis is presented in Figure 9.

As shown in Figure 9, the degree of hydrolysis increased with the increase in agitation speed until agitation speed of 160 to 220 rpm (70.82±5.19 to 97.15±1.31). However, degree of hydrolysis decreased from agitation speed of 240 to 260 rpm (92.24±1.31 to 82.32±2.44). The highest degree of hydrolysis was recorded at agitation speed of 220 rpm with 97.15±1.31degree of hydrolysis.Generally, lipase hydrolysed oil at the interface, the increase in interfacial area accelerates the increased in degree of hydrolysis.[7] Conversely, as the agitation speed increases, lipase is exposed towards higher shear stress, resulting in rapid unfolding of lipase. This rapid process causes a raise in denaturation leading to a decrease in degree of hydrolysis. The same trend as the present study was also observed in the castor oil hydrolysis[20] and palm olein hydrolysis[8] using *C. rugosa* lipase.

Fatty acids concentration profile for 3 hours reaction on various agitation speeds is shown by Figure 10. Result shows that fatty acids concentration for all varied agitation speeds increased as the time increases. Fatty acids concentration achieved its highest concentration at agitation speed of 220 rpm with 2533.33±26.67 µmol/ml of fatty acids. Thus, fatty acids yield at the end of 3 hours reaction time is related to the availability of interfacial area for the reaction to occur. Too low or too high agitation speed would contribute to the decrease in fatty acids yield due to less contact between the aqueous phase and oil.

Many literatures have investigated the effect of agitation speed on lipase catalysed hydrolysis with broad agitation speed

range (up to 1300 rpm). Higher working volume particularly required more shear stress to unfold lipase for rapid reaction to occur. Serri*et al.*[8] found the optimum agitation speed of palm olein hydrolysis at 200 rpm. Other literatures[4, 25, 26] also found and used agitation speed of 200 rpm for lipase catalysed hydrolysis for different types fresh of edible oil. The optimum agitation speed value found in the present study was higher compared to the reported value. The differences might appear from the source of substrate and enzyme used. Nevertheless, all the previous studies have been used fresh edible oil for the hydrolysis. The optimum agitation speed for used frying oil hydrolysis is 220 rpm with the fatty acids yield of 2383.33±23.33 µmol/ml and degree of hydrolysis of 97.15±1.31.

■4.0 CONCLUSION

Used-frying oil hydrolysis using immobilized *C. rugosa* lipase had been investigated by varying the reaction parameters including enzyme concentration, water content, reaction temperature and buffer pH. The profile for each manipulated parameters showed a bell shape curve, where initial degree of hydrolysis was increased towards a critical (optimum) value and decreased afterwards due to unfavourable effects of extreme reaction parameters. On the other hand, production of fatty increased with the time. However, for the effect of enzyme concentration, production of fatty acids was decreased after 120 to 150 minutes of reaction for enzymes concentration of 2 and 2.5%. This trend also appeared for the effect of water content at ratio of 4:1 and 5:1. In this study, the optimal hydrolysis conditions are at 1.5% (w/v) enzyme concentration, 3:1 buffer volume to oil volume ratio, temperature of 40ºC, pH of 7 and agitation speed of 220 rpm for 3 hours reaction. Under these conditions, the hydrolysis degree achieved 97.15±1.31% with fatty acids yield of 2533.33±26.67 µmol/ml.

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References

[1] M. Canakci. 2007. The Potential of Restaurant Waste Lipids as Biodiesel Feedstock. *Bioresource Technology*. 98(1): 183–190.
[2] A. Radich. 2006. Biodiesel Performance, Costs and Use. *US Energy Information Administration*. 1–7.
[3] M. Yigitoglu, Z. Temocin. 2009. Immobilization of *C. rugosa*Lipase on Glutaraldehyde-Activated Polyester Fiber and Its Application for Hydrolysis of Some Vegetable Oils. *J. of Mol. Catal. B: Enzym*. 66(2010): 130–135.
[4] S. F. Abdul Halim, A. H. Kamaruddin. 2008. Catalytic Studies of Lipase on FAME Production from Waste Cooking Palm Oil in a Tert-Butanol System. *Process Biochemistry*. 43(12): 1436–1439.
[5] N. Taufiqurrahmi, A. R. Mohamed, S. Bhatia. 2011. Production of Biofuel from Waste Cooking Palm Oil using Nanocrystalline Zeolite as Catalyst: Process Optimization Studies. *Bioresource Technology.* 102(22): 10686–10694.
[6] B. B., Marvey, C. K., Segakweng, and H. C. M, Vosloo. 2008. Ruthenium Carbene Mediated Metathesis of Oleate-type Fatty Compounds. *International. Journal of Molecular Science*. 9(4): 615–625.
[7] M. Noor, M. Hasan, and K. B. Ramachandran. 2003. Effect of Operating Variables on the Hydrolysis Rate of Palm Oil by Lipase. *Process Biochemistry*. 39(1): 13–20.
[8] B. B. Marvey, C. K. Segakweng, H. C. M. Vosloo. 2008. Ruthenium Carbene Mediated Metathesis of Oleate-type Fatty Compounds. *Int. J. Mol. Sci*. 9(4): 615–625.
[9] L. S. Chua, M. Alitabarimansor, C. H. Lee, R. Mat. 2012. Hydrolysis of Virgin Coconut Oil using Immobilized Lipase in a Batch Reactor. *Enzyme Research*. 2012(2012): 1–5.
[10] M. S. Puthli, V. K. Rathod, A. B. Pandit. 2006. Enzymatic Hydrolysis of Castor Oil: Process Intensification Studies. *Biochemical Engineering Journal*. 31(1): 31–41.
[11] L. L. You, B. S. Baharin. 2006. Effects of Enzymatic Hydrolysis on Crude Palm Olein by Lipase from *Candida rugosa*. *Journal of Food Lipids.* 13(1): 73–87.
[12[] H. S. Garcia, F. X. Malcata, C. G. Hill, C. H. Amundson. 1992. Use of *C. rugosa* Lipase Immobilized in a Spiral Wound Membrane Reactor for the Hydrolysis of Milk Fat. *Enzyme and Microbial Technology*. 14(7): 535–545.
[13] J. Cvengros, Z. Cvengrosova. 2004. Used Frying Oils and Fats and Their Utilization in the Production of Methyl Esters of Higher Fatty Acids. *Biomass and Bioenergy*. 27(2): 173–181.
[14] G. Knothe, and K. R. Steidly. 2009. A Comparison of Used Cooking Oils: A Very Heterogeneous Feedstock for Biodiesel. *Bioresource Technology*. 100(23): 5796–580.
[15] P. Pinsirodom, K. L. Parkin. 2001. Current Protocols in Food Analytical Chemistry. 3rd Ed. USA: John Wiley & Sons, Inc. C3.1.1–C3.1.13.
[16] M. Pazouki, F. Zamani, A. H. Zamzamian, M. Fahar, G. Najafpour. 2010. Esterification of Free Fatty Acids by *Rhizopus oryzae* as Cell-Catalyzed from Used Cooking Oil for Biodiesel Production. *World Applied Sciences Journal*. 8(6): 719–724.
[17] C. Wang, X. Zhou, W. Zhang, , Y. Chen, A. Zeng, F. Yin, J. Li, R. Xu, S. Liu. 2011. Study on preparing fatty acids by lipase hydrolysis waste oil from restaurants. *Power and Energy Engineering Conference (APPEEC), 2011 Asia-Pasific*. March 25-28 2011. Wuhan, China: IEEE. 2011. 978–980.
[18] D. Rooney, L. R. Weatherley. 2001. The Effect of Reaction Condition Upon Lipase Catalysed Hydrolysis of High Oleate Sunflower Oil in a Stirred Liquid-Liquid Reactor. *Process Biochemistry*. 36(10): 947–953.
[19] S. Al-Zuhair, M. Hasan, K. B. Ramachandran. 2003. Kinetic Hydrolysis of Palm Oil using Lipase. *Process Biochemistry*. 38(8): 1155–1163.
[20] D. Goswami, J. K. Basu, S. De. 2009. Optimization of Process Variables In Castor Oil Hydrolysis by *Candida Rugosa* Lipase with Buffer as Dispersion Medium. *Biotechnology and Bioprocess Engineering*. 14(2): 220–224.
[21] W. J. Ting, K. Y. Tung, R. Giridhar, W. T. Wu. 2006. Application of Binary Immobilized *Candida rugosa* Lipase for Hydrolysis of Soybean Oil. *Journal of Molecular Catalysis B, Enzymatic*. 42(1–2): 32–38.
[22] L. Gubicza, K. Székely, O. Ulbert, K. Bélafi-Bakó. 2000. Enhancement of the Thermostability of a Lipase by Medium Engineering. *Chem. Papers.* 54(6): 351–354.
[23] K .J. Laidler, B. F. Peterman. 1979. Temperature Effects in Enzyme Kinetics. D.L. Purich, (ED). *Methods in Enzymology*, Vol. 63. *Enzyme Kinetics and Mechanism. Part A: Initial Rate Inhibitor Methods*. New York, NY: Academic Press. 234–257.
[24] S. T. Kang J.S. Rhee. 1989. Characteristics of Immobilized Lipase-Catalyzed Hydrolysis of Olive Oil of High Concentration in Reverse Phase System. *Biotechnology and Bioengineering*. 33(11): 1469–1476.
[25] Y. H. Chew, L. S. Chua, K. K. Cheng, M. R. Sarmidi, R. A. Aziz, C. T. Lee. 2008. Kinetic Study on the Hydrolysis of Palm Olein using Immobilized Lipase. *Biochemical Engineering Journal*. 39(3): 516–520.
[26] C. F. Torres, A. M. Toré, T. Fornari, F. J. Senorans, G. Reglero. 2007. Ethanolysis of Waste Materials from Olive Oil Distillation Catalysed by Three Different Commercial Lipases: A Kinetic Study. *Biochemical Engineering Journal*. 34(2): 165–171.