HYDROLYSIS OF WASTE COOKING OIL BY FREE *CANDIDA RUGOSA* LIPASE

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UNIVERSITI SAINS MALAYSIA

2022

HYDROLYSIS OF WASTE COOKING OIL BY FREE *CANDIDA RUGOSA* LIPASE

by

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Project report submitted in partial fulfilment of the requirement for the degree of Bachelor of Chemical Engineering

2022

ACKNOWLEDGEMENT

I would like to express my deepest appreciation to all those who provided me the possibility to complete this report. A special gratitude I give to my final year project supervisor, Professor Datin Dr. Azlina Harun @ Kamaruddin, whose contribution in stimulating suggestions and encouragement, helped me to coordinate my project especially in writing this report. This progress report would not have been possible without her direction and constant participation in every step. Sincere appreciation to her for imparting her expertise, wisdom, and sincere advice as well as additional motivations.

Next, a sincere thank you to Universiti Sains Malaysia (USM), in particular, the School of Chemical Engineering, for providing the facilities, engineering expertise, and skills needed for the project. I would like to extend my sincere gratitude to all of the lecturers who led seminars and helped us prepare progress reports. Special thanks to Mr. Jackson Robinson, a doctoral student working under Prof. Azlina, who is always willing to assist me in resolving issues relating to the design of the experiment.

Additionally, I would like to take this opportunity to deeply thank my family for supporting me, for their prayers, and for their encouragement to successfully complete the progress report. I want to thank all of my friends as well as everyone who helped, encouraged, and helped to complete this report.

Last but not least, I would love to reach out and thank those who directly or indirectly, have lent their hand in completing this progress report.

A Helan
July 2022

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LIST OF ABBREVIATIONS

ABR	Anaerobic baffled reactor
AF	Anaerobic filter
ASP	Activated sludge process
CCD	Central composite design
CCFC	Central composite face centered design
CCID	Central composite inscribed design
CCRD	Central composite rotatable design
df	Dilution factor
DOE	Design of Experiment
EAB	Enzyme-substrate complex
EPQ	Enzyme-product complex
FFA	Free fatty acid
MBBR	Moving bed bioreactor
OFAT	One-Factor-at-A-Time
OG	Oil and grease
PPL	Porcine pancreatic lipase
p-NP	p-nitrophenyl
p-NPB	p-nitrophenyl butyrate
RBC	Rotating biological contactor
RSM	Response Surface Methodology
rpm	Revolution per minute
SBR	Sequencing batch reactor
UASB	Upflow anaerobic sludge blanket

HIDROLISIS MINYAK MASAK SISA OLEH *CANDIDA RUGOSA LIPASE* BEBAS

ABSTRAK

Penyingkiran minyak dan gris daripada rawatan air sisa merupakan isu yang mencabar apabila melibatkan kaedah fizikal dan kimia kerana penggunaan bahan kimia berbahaya dan mempunyai masalah alam sekitar. Oleh itu, lipase adalah kaedah baru yang terbukti untuk membuang atau merendahkan kandungan lipid dalam sistem air kerana ia mempunyai potensi bertindak sebagai biomangkin. Minyak masak sisa telah dipilih sebagai substrat untuk kajian ini kerana Malaysia menghasilkan sejumlah besar minyak masak dan kerana pelupusan telah menjadi masalah alam sekitar yang besar. Ujian enzimatik aktiviti lipase bebas telah dilakukan dan aktiviti pemangkin ialah 970.0 U/mg enzim. Kesan tindak balas masa, suhu, kandungan air, dan pemuatan enzim ke atas proses hidrolisis telah disiasat. Masa tindak balas optimum untuk proses hidrolisis ialah 120 minit Diperhatikan bahawa pembolehubah mempunyai kesan yang ketara ke atas proses hidrolisis. Proses hidrolisis mencapai hasil tertinggi asid lemak pada suhu 45 ^{o}C, nisbah isipadu kepada isipadu minyak 4:1, pH 7, kelajuan pengadukan 140 rpm dan kepekatan enzim 0.025 (w/v) %. Di bawah keadaan ini, lemak acid bebas penukaran didapati hampir 41.7% ± 0.253 .

HYDROLYSIS OF WASTE COOKING OIL BY FREE *CANDIDA RUGOSA* LIPASE

ABSTRACT

The removal of oil and grease from the wastewater treatment is a challenging issue when involving physical and chemical method due to the usage of hazardous chemical and having environmental problems. Thus, lipase is a proven novel method to remove or degrade lipid content in water system as it has a potential acting as biocatalyst. Waste cooking oil was chosen as the substrate for this study because Malaysia produces a large amount of it and because disposing of it has become a major environmental problem. Enzymatic assay of free lipase activity has been performed and the catalytic activity was 970.0 U/mg enzyme. Effect of reaction of time, temperature, water content, and enzyme loading on the hydrolysis process were investigated. The optimum reaction time for the hydrolysis process is 120 minutes It was observed that variables had a significant impact on the hydrolysis process. The hydrolysis process achieved the highest yield of fatty acids at the temperature of 45^{o}C, buffer volume to oil volume ratio of 4:1, pH of 7, agitation speed of 140 rpm and enzyme concentration of 0.025 (w/v) %. Under these conditions, it was found that nearly 41.7% ± 0.253 of the free fatty acid conversion.

CHAPTER 1: INTRODUCTION

1.1 Research Background

“Oil and grease’’ are one of the pollutants regularly found in water and wastewater that refer to a class of organic compounds with a low affinity for water as their defining characteristic. Hydrocarbons, fatty acids, soaps, lipids, and waxes are the compounds classified into “oil and grease”. Since such a diverse spectrum of compounds makes up a complex class, it is not surprising that “oil and grease” is considered one of the most difficult contaminants to remove in oil processing wastewater (Pintor et al.,2016).

According to studies, a significant amount of oily wastewater is produced from household sources such as human and kitchen waste (Ahmad et al.,2020). Repetitively used cooking oil could be discovered in large portion amid other types of oily materials that are responsible by population explosion and rapid urbanisation. Despite the tiny volume ratio, a single litre of oil can theoretically contaminate one million litres of water. Because of the greatly reduces long chain fatty acids in these wastes, they have a very high biological oxygen demand (Kumar et al., 2015). Wastewater containing a high concentration of oily material should be avoided while performing biological treatment systems (Sanghamitra et al.,2021). High strength of oily material has an adverse impact on oxygen transfer efficiency in aerobic treatment system. Meanwhile, long chain fatty acids, for instance oleic acid, the product of lipid hydrolysis well-known inhibitors for anaerobic systems (Nakhla et al., 2003).

Various approaches have been employed to separate oily material from wastewater such as chemical, physical, physico-chemical, biological, combined methods, treatment using bio-surfactant and biological treatment involving enzymatic activity. Concentration, droplet size, and the physical character of oily substances includes in processing factors which are the most important regulating parameters for the extent of removal of oil. Some methods for treating

oily wastewater have already been implemented, including conventional physico-chemical methods such as sedimentation, coagulation, filtration, and adsorption, as well as biological methods such as the activated sludge process (ASP), moving bed bioreactor (MBBR), rotating biological contactor (RBC), sequencing batch reactor (SBR), anaerobic baffled reactor (ABR), upflow anaerobic sludge blanket (UASB), hybrid reactor, anaerobic filter (AF) and many more (Sanghamitra et al., 2021).

Physical and chemical approaches of oily wastewater treatment have drawbacks, which includes formation of chemical sludge, energy requirement for dosing, high operating cost, and time-consuming process for separation of oil using gravitational force (Sanghamitra et al., 2021). Aside from the downsides of conventional methods, inhibiting biological activity in activated sludge reactors and causing clogging and fouling of pumps and piping are also categorize under effect from operating conventional wastewater treatment plants. Moreover, the presence of oily particles in wastewater is frequently indicates of the occurrence of toxic micro pollutants, particularly those that are hydrophobic in nature (Pintor et al., 2016). Oily materials are not truly susceptible to chemical bonding in the case of chemical treatment due to their non-ionic nature. Likewise, there seems to be a challenge with recovering oil from wastewater and disposing of it. The biggest limitation of physico-chemical approaches is that oily particles do not remain permanently stabilised in terms of carbonaceous materials (Sanghamitra et al., 2021).

Enzymes have found a wide range of applications, from a vast variety of enzyme-catalysed industrial processes to use within the molecular biology toolbox. Because of the specificity of enzymes, it is possible to remove extremely refractory contaminants from hard industrial waste and highly diluted effluents. Enzymes are also exploited in the final polishing of municipal and industrial effluents after conventional treatment, as well as in the initial phase of anaerobic digestion to increase the hydrolytic capability of the in microbial consortia.

Enzymes are effective catalysts, with enzyme stability being a key challenge in biocatalysts and a prerequisite for the majority of their uses. Based on researcher (Jeganathan et al., 2007), lipases are enzymes or biocatalysts, which have the capability to catalyse the hydrolysis of fats, oils, and grease (triacyclglycerols) to liberate long-chain fatty acids (LCFAs) and glycerol.

1.2 Problem Statement

Waste cooking oil is typically generated when various edible vegetable oils are used to prepare meals in homes, hotels, restaurants, and other catering outlets. In urban areas, disposal of waste cooking oil into sewers, drains and rivers contributes to environmental contamination, clogs drain, and contaminates land and aquatic habitats, while its consumption has a detrimental effect on human and animal health. Furthermore, inappropriate waste cooking oil is disposed results in foam development, improve the organic load on water sources, hinder wastewater treatment, reduced dissolved oxygen concentration, and change ecosystem balance (Awogbemi et al., 2021). Moreover, current practises for oily particles removal typically rely on physical and chemical pre-treatment using processes like dissolved air flotation and skimming to overcome this issue. Yet not only are these generally utilized methods expensive, but they also fail to effectively remove colloidal and emulsified particles (Sarac at al., 2015).

Enzyme-based processes typically have minimal capital and energy costs, and enzymes are biodegradable. Enzymes provides advantages such as high selectivity, moderate environments, and less waste. In that context, lipases (triacylglycerol ester acylhydrolases, EC 3.1.1.3) are enzymes that catalyse the hydrolysis of triglycerides to glycerol and free fatty acids (Silva et al., 2014). Lipases are one of the most commonly employed enzymes in biotechnology field. Microbial lipases have piqued the interest of enormous industry due to their adaptability, greater productivity, and season independence production compared to their animal and plant counterparts (Mateos et al., 2021).Lipase, like other enzymes have several limitations in their

free form. Free lipase has limited operating stability, high costs, and cannot be easily recovered or reused at the end of the reaction, causing product separation to difficult. Immobilizing enzymes on solid supports is one way to overcome these constraints. In recent years, these has been a significant growth in the usage of enzyme immobilisation technologies, primarily as an approach to enhance biocatalyst stability and enable recovery/reuse stages (Almeida et al., 2021).

1.3 Research Objective

The study's purpose is to successfully eliminate waste cooking oil using an enzymatic technique in a batch system employing a hydrolysis process with free *Candida rugosa* lipase. The following objective are then prioritised:

I. To determine the enzymatic assay of free *Candida rugosa*.
II. To analyse the hydrolysis reaction of lipase and oil with the effects of various reaction parameters.

1.4 Scope of Study

Application of enzyme as biocatalyst has been a substitution for the conventional removal of waste cooking oil method due to its ability to minimize the capital cost, deduct energy consumption, and most importantly biodegradable. Therefore, comprehension of the parameter that influence the performance of the hydrolysis reaction is imperative. The scope of this project is to determine the optimum parameter such as the reaction time, temperature, enzyme loading, speed of agitation, water content. In this research, free Candida *rugosa* was used as biocatalyst and enzymatic assay was determined.

Temperature, pH value, enzyme loading, agitation speed, and water content are five parameters being manipulated for the optimising process using OFAT method to observe the

effect on enzymatic hydrolysis of waste cooking oil. Each variable's range varies from 30 minutes to 180 minutes , 25 to 100 degrees Celsius, 2 to 12 milligrams, 50 to 350 revolutions per minute, and 1:1 to 6:1.

CHAPTER 2: LITERATURE REVIEW

2.1 Enzyme as Biocatalyst

Organic toxic waste (oil and grease (O&G)) causes ecological damage to aquatic organisms (Islam et al., 2013) plants, animals, and humans (Lan et al., 2009) as well as being mutagenic and carcinogenic. They discharge from a variety of sources, forming a layer on the water's surface that reduces dissolved oxygen levels. The presence of an O&G layer reduces the biological activity of the treatment process, which involves the formation of an oil film around microbes in suspended matter and water. As a result, the water's dissolved oxygen levels dropped. The oxygen molecules are then difficult for microbial to oxidise on hydrocarbon molecules, resulting in ecological damage to water bodies (Alade et al., 2011:Facchin et al., 2013). Traditional techniques use skimming tanks and oil and grease traps in treatment plants to remove oil and grease, but the main disadvantage of these methods is their low removal efficiency. Thus, green technology employing enzyme as biocatalyst could be a solution to overcome the drawback of oil removal from wastewater treatment.

2.2 Enzymatic Hydrolysis

Lipases catalyse the cleavage of triacylglycerol ester bonds while also consuming water molecules, a process known as hydrolysis. The Colgate-Emery process is used in the American fatty acid industry to split fats. Superheated steam is sprayed into the fat during this process. Pressures of 700 psig and temperatures of 240-260 ^{o}C or higher are typical conditions for splitting. Nitrogen is sprayed over the entire fat splitting operation. Polyunsaturated fats, on the other hand, degrade rapidly and must be extensively purified by distillation for most applications. Furthermore, the Colgate-Emery process consumes a lot of energy, about 340 Btu per pound of oil split. As a result, using the lipase enzyme as a catalyst, an efficient and low-cost method of rapidly hydrolysing oleaginous materials of all types into their constituent fatty

acids and glycerol has been developed. A colourless, non-oxidized material can be obtained by hydrolysis in an organic solvent at room temperature. The fatty acid is produced as a free acid rather than an acid salt, and it can be washed out of the lipase with organic solvents. Yields of more than 97 percent are possible (Akoh and Min, 1998).

Therefore, using lipases for enzymatic splitting of fats in the presence of excess water is more appealing because the reaction proceeds under mild pressure and temperature conditions with high specificity, yield, and waste reduction. Currently, this technology is used to make fatty acids, diglycerides, monoglycerides, flavouring agents for dairy products, and laundry and household detergents. Table 1.1 summarizes the enzymatic hydrolysis with free and immobilized lipase with different substrate from various literatures.

Table 1.1 A literature survey of various lipase-catalyzed hydrolysis process.

Substrate (Triglycerides)	Enzyme Origin	Method	Operating conditions	Rection Media	Fatty Acids	Analysis	References
Crude and waste soybean oil	Orange lipase	Free	Hydrolysis of soybean oil using 5% lipase extract over the reaction medium volume		Methyl palmitate, methyl linoleate, methyl oleate and methyl stearate.	-analyze the bioremediation of lipase catalyzed cooking oil waste -assess the toxicity of soybean oil and its waste (with and without enzymatic treatment)	(Okino-Delgado C.H., Zanoni do Prado D., Facanali R., Ortiz Marques M.M., Nascimento A.S., Fernandes C.J.C., Zambuzzi W.F., Fleuri L.F.,2017)
Canola, olive, and soybean oil	Lipase extract powder from dormant castor bean seeds	Free	Performed at 25^{o}C under atmospheric pressure (3h period) and agitated with mechanical stirrer at a constant speed of 1000 rpm.	10g of vegetable oils (canola, olive, and soybean)/90 ml buffer acetate (100 mmolL^{-1}) (pH 4.5)/2g of lipase extract powder		-determination of hydrolysis activity on different vegetable oils -enzymatic extract showed a higher activity on oils rich on linoleic ($C_{18:2}$) and linolenic acid ($C_{18:3}$) - the influence of the independent variables (mass ratio oil:buffer, temperature and $CaCl_2$ concentration)	(Avelar M.H.M., Cassimiro D.M.J., Santos K.C., Domingues R.C.C, F. de Castro H., Mendes A.A., 2013)
Virgin coconut oil (VCO)	*Candida rugosa* lipase (CRL) and	Free	Carried out under four different parameters:	Hydrolysis of VCO by CRL	47.23% of lauric acid	-effects of VCO to buffer ratio	(Nguyen T.A.V., Truong D.Le.,

	porcine pancreas lipase (PPL)		VCO to buffer ratio/lipase concentration/pH/temperature	used phosphate buffer/ PPL used borate buffer and dissolving VCO into isooctane with 1:1 (VCO: Solvent) ratio.	with CRL while 44.23% lauric acid with PPL	-effects of lipase concentration -effects of pH -effects of temperature -degree of hydrolysis	Phan N.H., Tran L.B., 2018)
Waste cooking oil	Lipase produced by *Penicillium chrysogenum* through solid state fermentation	Free	A ratio of 1:1 (emulsified cooking oil waste to lipase) was mixed		17% oleic acid and 5% of stearic acid	-characterize the waste cooking oil by the occurrence of high molecular weight hydrocarbons and polymerized derivative of esters. -identify the lipase hydrolyzed products	(Kumar S., Negi S., 2015)
Lubricating oil	Triacylglycerin lipase (*A. niger* lipase)	Immobilized by adsorption onto chitosan, celite and cellulose acetate as support	Carried at room temperature and at pH 7.2 and kept in an orbital shaker at 150 rpm for 3 days.	Only oil, oil/0.1g bead, oil/0.2g bead and oil/0.3g bead at pH 7.2 using sodium bicarbonate		-characterize of different supports - effects of time influencing protein binding in the support - effects of rpm on residual protein concentration -immobilized study	(Dumore N.S., Mukhopadhyay M., 2012)

					-removal efficiency of oil and grease	
Fish oil	Lipase B from *Candida antarctica* and *Rhizomucormiehei*	Immobilized by adsorption on butyl, octyl and dodecyl chitosan	The mixture was agitated at 500 rpm in a flat bottom reactor and incubated at 35^{o}C.	Mixture contained 10ml of 20mM phosphate buffer at pH 7	-an integral study of the use of hydrophobic chitosan as a low-cost support for immobilizing lipase -analyze application in the selectice hydrolysis of fish oil.	(Urrutia p., Arrieta R., Alvarez L., Cardenas C., Mesa M., Wilson., 2018)
Palm oil	Lipase from *Candida cylindracea*	Immobilized on hydrophobuc zeolite type Y	Carried at different temperature, at pH 7 and stirring rate v = 150rpm.	Microaqueous two-phase reaction system containing isooctane as organic medium and lecithin as surfactant.	-analyse time-course of lipase immobilization on zeolite -application of KEKAM equation in the kinetics of lipase immobilization -study the hydrolysis of palm oil by zeolite-immobilized lipase	(Knezevic Z., Mojovic L., Adnadjevic B., 1998)
Palm oil	*Candida rugosa and Yarrowia lipolytica*	Free	Centrifuged at 4000rpm for 15 min to eliminate solid and then diluted two-fold in water.		-characteristics of the POME used -analyze COD removal under a	(Monnat T., Phattrapan T., Warawut.C, 2017)

				non-sterile condition -study undiluted POME pretreatment by crude extracellular enzyme preparations under a sterile condition -study undiluted POME pretreatment by culture with free yeast cells under sterile condition	
Palm olein	Commercial immobilized lipase (Lipozyme TL IM)	Immobilized lipase (bead size 0.3-1.0 mm, wet bulk density 415 kg/m^3)	Batch stirred tank reactor consisted of a water jacketed vessel (at 55^oC and 200rpm) and repeat different flow rates	-develop mass transfer limitations is significant in immobilized enzymes packed bed reactors	(Chew Y.H., Chua L.S., Sarmidi M.R., Aziz R.A., Lee.C.T, 2008)

2.3 Reaction System in Enzymatic Hydrolysis

Waste cooking oil alternatively can be removed through enzymatic reaction to sustain green environment. Even though used frying oil is unsafe for consumption and is widely used in biodiesel production, FFA from used frying oil could be used to make a variety of non-edible products (Rashid et al., 2014). The hydrolysis was performed by lipase with reactions parameter such as temperature, pH, enzyme loading, agitation speed and water content being studied.

2.3.1 Effect of Reaction Time

Reaction time is certainly crucial factor to determine the correct incubation period of the reaction in order to manage the time. From a study of enzymatic of castor oil, the trend was showed a proportionally increase in the percentage of hydrolysis and at one stage the trend beginning to show a plateau curve. The initial 5 min of hydrolysis reaction, the yield produced was 13.7%, and the next 30 min the yield increase to 31.39%. As the reaction further extended, at 60 min of reaction 40.14% of yield was obtained, while at 75 and 90 min, the yield of 42.08% and 43.46% was obtained, respectively. The hydrolysis reaction slowed down after 1 hour of reaction (Puthli et al., 2006)

This phenomenon is an indication of the rate of enzymatic castor oil hydrolysis was not linear with time. According to a previous study, fatty acids have higher interfacial activity than enzymes, indicating that they have a higher affinity for the aqueous-organic interface. Although no experiments regarding the interaction of the enzyme and fatty acids have been carried out, time study has supported this aspect of product inhibition. Thus, it can be inferred that the initial stage of the reaction involves more interaction between the enzyme and the oil, which results in a faster rate of hydrolysis. However, as time passes, a significant amount of fatty acids created during hydrolysis accumulate at the boundary between the aqueous and non-

aqueous phases, pushing the enzyme away from the boundary. Due to the continuous reduction in the amount of conjugated lipase enzyme contact with the castor oil during the hydrolysis process, the rate of hydrolysis decreased, especially after one hour (Puthli et al., 2006).

2.3.2 Effect of Temperature

The rate of reaction increases as the temperature rises at initial moment. This is because the rate constant increase with temperature and increase in interfacial area with temperature. In addition to interfacial area, the mean drop diameter decreases as the temperature increases. Mainly caused by the reduction in viscosity of the oil and the surface tension at the interface between water and oil, with increase in temperature. However, the initial rate dropped dramatically after 50^{o}C due enzyme deactivation. Since the presence of the inactive enzyme at the interface would prevent the active enzyme from penetrating the interface, it might contribute to enzyme deactivation, which would further reduce the reaction rate (Al-Zuhair et al., 2003).

Other studies performed by (Silva et al., 2014), investigate the effect of the temperature on the hydrolytic activity of the extract and immobilized of porcine pancreatic lipase (PPL) in the range of 30 to 70^{o}C. The optimal temperature for lipase activity is seen to shift from 45^{o}C for crude PPL extract to 50^{o}C for immobilized PPL. Between 50 and 55^{o}C, immobilized PPL outperformed crude PPL extract in term of thermal denaturation. This finding could be explained by the formation of conformational constraint on enzyme movements as result of hydrophobic interaction between the enzyme and the support. Although the optimum temperature increased after PPL immobilization on PHB particles, crude PPL extract was more thermo-stable than immobilized lipase at temperatures above 60^{o}C. These results could be attributed to a decrease in the concentration of stabilizing agents such as salts, polyols, and sugars in crude PPL extract following immobilization. These compounds are preferentially

retained in the supernatant, lowering their concentrations in the immobilized lipase's microenvironment. It has previously been reported that the presence of stabilizing agents such as polyols and sugar increase the catalytic activity and stability of PPL at high temperatures.

2.3.3 Effect of Enzyme Loading

The amount of enzyme loading influences the lipase hydrolysis reaction significantly. In (Hasan et al., 2015) study, the enzyme loading was adjusted between 96.43U/mL and 385.73U/mL, corresponding to 2g to 8g of immobilised beads. Generally, as the enzyme concentration increased while the substrate concentration remained constant, the reaction rate increased as well. According to the findings, increasing enzyme loading resulted in an increase in fatty acid production and hydrolysis conversion. Under the conditions of pH 7.5, temperature 40^{o}C, and agitation at 200 rpm, the best enzyme loading was found to be 385.73 U/mL, which is equivalent to 8g of immobilized beads. Whereas (Ozturk et al., 2001) proposed that enzyme activity reached its peak when C.*rugosa* lipase was immobilised on chitosan beads at concentration of 160 U/mL. In addition, the optimal enzyme loading required for the reaction is also determined by the interaction between the support and the enzyme.

Based on (Zaharudin et al., 2018) estimating appropriate enzyme loading is critical to minimize enzyme wastage. The maximum percentage of hydrolysis degree 63.43%, was obtained with enzyme loading of 0.8 percent and 1.0 percent (w/w), as reported by (Rashid et al., 2014). In considering economic aspect, enzyme loading of 0.8% (w/w) was chosen as the best enzyme loading in this study over 1.0% (w/w) since the high concentration of enzyme utilised in the reaction will contribute to an increase in overall process cost. According to (Goswami et al., 2009), a continuous high amount of lipase was required to enhance the degree of hydrolysis. Yet, a high concentration of lipase causes the oil, water, and lipase mixture to form a paste-like structure, making separation processes problematic (Wang et al., 2011).

2.3.4 Effect of Water Content

Throughout the hydrolysis, water acts as both reactant and a modulator for lipase functionality. Generally, the effect of water content on the degree of hydrolysis was investigated by altering the amount of buffer while maintaining a constant pH. In overall, as the buffer volume is increased, the degree of hydrolysis increases until the optimum buffer volume is reached. After then, increasing the buffer volume reduces the degree of hydrolysis. In this investigation, the buffer volume to oil volume ratio was varied from ratio 1:1 to 5:1 at fixed conditions of enzyme loading (1.5 %), reaction temperature (40^{o}C), buffer pH 7, agitation speed at 200 rpm and 3 hours reaction time (Rashid et al., 2014).

The buffer volume to substrate volume ratio immediately relates to the degree of hydrolysis and time required to attain equilibrium in an enzyme-catalyzed hydrolysis reaction (Rashid et al., 2014). The fat hydrolysis reaction is a common thermodynamic balancing control. The water-oil ratio is a crucial component in determining chemical equilibrium (Wang et al., 2011). The hydrolysis achieved the highest degree of hydrolysis when the amount of water content reached a ratio of 3:1. The hydrolysis rate is reduced when the water content is too high or too low. The amount of water in the solution influences the reversibility of the reaction toward hydrolysis or esterification (Rashid et al., 2014).

The increase in water content favours the balance in positive reaction, which increases the degree of hydrolysis, causing the reaction to produce a large number of fatty acids. Because of the decreased interaction between the lipase and the oil in circumstances of high-water content, the hydrolysis rate began to slow down. The additional water will form a thicker water layer surrounding the enzyme active sites. Higher water content also causes enzyme agglomeration due to the surface tension effect. Excessive water content in the process may permanently denature the enzyme protein content particles (Rashid et al., 2014).

2.4 One-Factor-At-A-Time (OFAT)

Engineers and scientists often perform One-Factor-At a-Time (OFAT) to solve problem that uses a pool of potential causes to identify the critical causes of an effect. The strategy used is to alter one cause while keeping everything else constant. Optimization is a technique for reducing or maximizing an objective function that connects the variable to be optimized with the design and operating variables. OFAT is a fundamental optimization process for screening the best candidate for a particular factor (Ayinla et al., 2017). This approach is easy and simple; however, it is time consuming.

CHAPTER 3: METHODOLOGY

In this chapter, the details on chemicals used and the experimental work of the hydrolysis of waste cooking oil are reported. The experimental work includes the enzymatic assay of lipase, optimization of hydrolysis reaction and kinetic study of the enzymatic hydrolysis.

3.1 Materials and Chemicals

The chemicals required for enzymatic hydrolysis of waste cooking oil (used palm oil) are listed in Table 3.1. All the materials and chemicals were obtained in reagent grade purity and were used as receive without any modification.

Table 3.1 List of materials and chemicals in this study

Materials and Chemicals	**Purity**	**Supplier**
Lipase from *Candida rugosa*, Type VII (Enz-no:3.1.1.3)	1176 U/mg	Sigma-Aldrich, USA
4-nitrophenyl butyrate ($C_{10}H_{11}NO_4$)	98%	Sigma-Aldrich, USA
Phenolphthalein		Fisher Scientific, UK
Ethanol	99.4%	Fisher Scientific, UK
Sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$)		Fisher Scientific, UK
di-Sodium hydrogen phosphate (Na_2HPO_4)	99%	Fisher Scientific, UK
Sodium hydroxide (NaOH)	99%	Fisher Scientific, UK

3.2 Experimental Procedures

Figure 3.1 shows the flow diagram of the research project for hydrolysis of waste cooking oil. Enzymatic assay and optimization of hydrolytic reaction will be carried out using methodologies that will be described later.

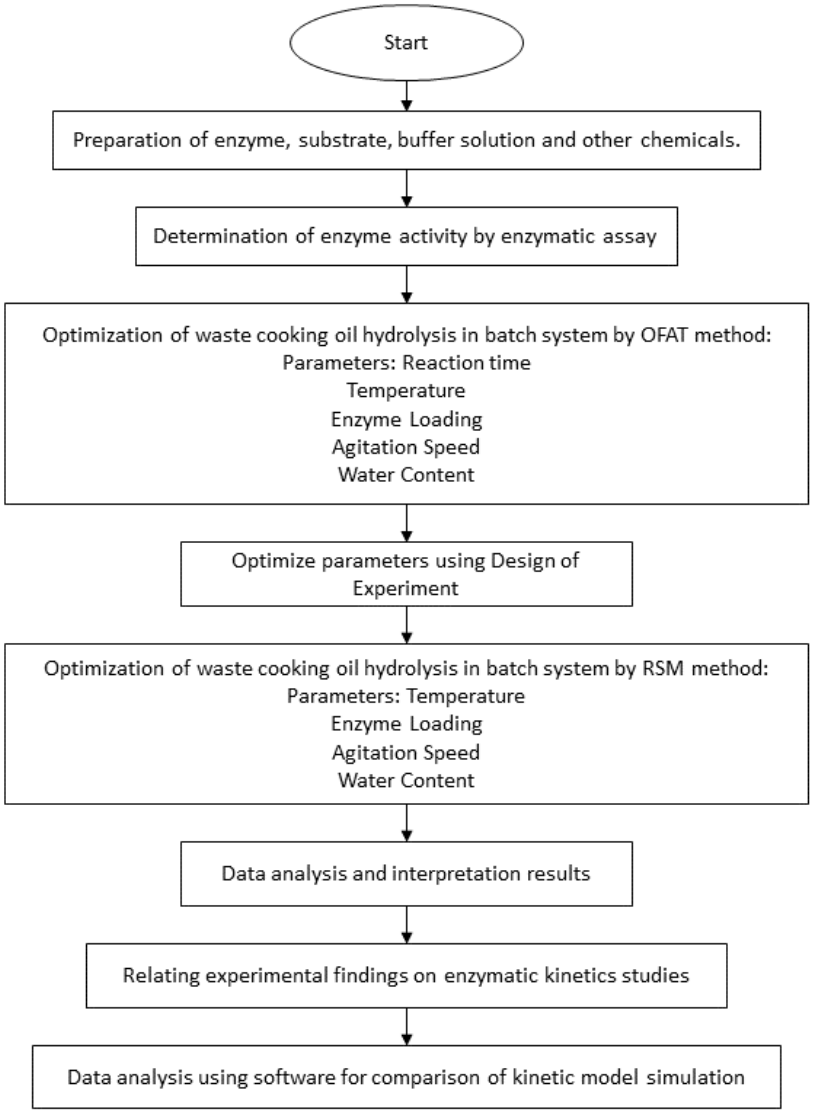

Figure 3.1 Flow Diagram of Research Project

3.2.1 Phosphate Buffer Solution Preparation

Buffers serve to adjust and stabilize the desired pH during the enzyme assay. They consist of a weak acid and a strong basic component. The hydrogen ion regulation in biological systems highlights the importance of buffering systems. Amino acids present in proteins in cells and tissues contain functional groups that act as weak acid and bases. Nucleotides and several other low molecular weight metabolites that undergo ionization also contribute effectively to buffering in the cell. However, phosphate and bicarbonate buffer systems are most predominant in biological systems. The phosphate buffer has a pK_a of 6.86. Hence, it provides effective buffering in the pH range of 6.4 to 7.4 which applicable in enzymatic reactions. (Mohan et al., 2006)

In the study of enzymatic reaction, sodium phosphate buffer was used as buffering medium. Meanwhile, di-Sodium hydrogen phosphate (Na_2HPO_4) and Sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$) were used to prepare phosphate buffer solution. The ratio of acid to base was determined with the Henderson-Hasselbalch equation as shown below to prepare a buffer of the desired pH. The optimum buffering capacity of the pair occurs at a pH corresponding to its pKa, which is 7.21 from (Haynes, 2014).

The Henderson-Hasselbach equation:

$$pH = pKa + log\left(\frac{[Base]}{[Acid]}\right) \tag{3.1}$$

Further calculations of the ratio of chemicals required for the phosphate buffer solution preparation at specific pH is attached in Appendix.

3.2.2 Enzymatic Assay

The free lipase activity was determined spectrophotometrically using p-nitrophenyl butyrate (*p*-NPB) as the substrate as previously reported (Theerachat et al., 2017). One unit (U) of enzyme activity was defined as the amount of enzyme needed to release 1μmole of butyric acid from *p*-NPB per minute at 25°C and pH=7.0. (Theerachat et al., 2017). Based on this method, a yellow compound quantified by the absorbance at 400 nm indicate the hydrolysis of p-nitrophenyl butyrate (*p*-NPB) convert into p-nitrophenyl (*p*-NP). The reaction medium consists of 4μL of *p*-NPB and 2000μL phosphate buffer at pH 7.0 and 5.4 mg of free lipase enzyme was incubated for 5 minutes. The reaction was stopped by adding few droplets of 10 pH buffer solution. Meanwhile, a blank reaction was prepared by replacing *p*-NPB with deionizing water. The blank and assay then was pipetted into right cuvettes to observe the absorbance of the solution by setting the wavelength at 400 nm using UVis spectrophotometer.

Calculation of extinction coefficient

Beer-Lambert Law Equation:

$$A_{400nm} = \varepsilon CL \tag{3.2}$$

A_{400nm} = Absorbance of sample at 400 nm

ε = Extinction coefficient, $\mu M^{-1}\ cm^{-1}$

C = Concentration, μM

L = path length, cm

Calculation of enzyme activity

$$A = Units/mg\ enzyme\ = \frac{(\Delta A_{400nm}/min\ Test\ -\ \Delta A_{400nm}/min\ Blank)(V)(df)}{\varepsilon m} \tag{3.3}$$

V = volume of assay

df = dilution factor

ε = Micromolar extinction coefficient of p-nitrophenol at 400 nm

m = mass of free enzyme

3.2.3 Pre-treatment of Waste Cooking Oil

Waste cooking oil contains a lot of impurities resulting from continuous cooking and oxidation process. Therefore, a pre-treatment is required to eliminate the indistinguishable impurities. The collected waste cooking oil was filtered using a filter paper (Whatman 42), the filtered oil then was heated to 100^{o}C and swirled with magnetic bar for 15 minutes using magnetic stirrer. This procedure is necessary as to eradicate excess water content in the oil. (Rashid et al., 2014)

3.2.4 Preparation of 1 % Phenolphthalein Indicator

Before conducting the enzymatic hydrolysis reaction, 10 ml of 1% (w/v) phenolphthalein solution was prepared composed of 0.1 g of phenolphthalein dissolved in 50% of ethanol (10 ml) and 50% of water to prepare 10 ml of indicator solution.

3.2.5 Enzymatic Hydrolysis

The hydrolysis of waste cooking oil is a batch operation study. The substrates (waste cooking oil and water), the water content is from the buffer solution with 7.0 pH were varying at different ratios. Then, starting from 0.001g of free C. *rugosa* lipase was added into the mixture of substrates and obtain a final enzyme concentration of 0.005 % (w/v) and a blank was prepared without adding free lipase. From here, two layers mixture was observed. This reaction was carried out in a 50 ml conical flask under a specific speed of agitation of 140 rpm while, the temperature of the batch process varying in the orbital shaker. At six reaction

intervals with (30, 60, 90, 120, 150, and 180 min) and determine the free fatty acid concentration using titration method.

3.2.6 Analysis Method

The degree of hydrolysis was determined by the titration of the samples using 0.25 M sodium hydroxide (NaOH). In each experiment, there are six samples from different time of intervals were titrated with 0.25 M of NaOH using burette until the samples turn into pale pink colour. The mixture contain substrates only was performed as a blank titration and was titrated with 0.25M NaOH. The quantity of fatty acids liberated in each sample is based on the equivalents amount of NaOH used to reach the end-point of the titration. (Rashid et al.,2014) The degree of hydrolysis was estimated using equation shown below as reported by (Rashid et al.,2014):

$$X\% = \frac{(V_i - V_o)(Nx10^{-3})(AMW)}{W}\ x\ 100\% \qquad (3.4)$$

V_i = volume of NaOH for sample to reach end point (ml)

V_o = volume of NaOH for the blank to reach the end point (ml)

N = normality of NaOH (mol/L)

W = weight of sample (palm oil)

AMW = average molecular weight of fatty acid (268.4347 g/mol)

3.2.7 Effect of Reaction Time

The effect of time in the hydrolysis reaction was investigated by varying the reaction time with the interval of 30 minutes until 3 hours, whereas the other parameters kept as constant such as buffer volume to oil volume ratio is about 3:1, enzyme loading of 0.01%(w/v), agitation speed of 140 rpm and pH 7.

3.2.8 Effect of Temperature

The effect of temperature on the reaction was investigated by varying the temperature from 35°C to 60°C for each trial with a 5°C increment. The other parameters, such as pH 7, enzyme loading of0.01%(w/v), agitation speed of 140 rpm, and buffer volume to oil volume ratio of about 3:1, are kept constant and at optimum reaction time.

3.2.9 Effect of Water Content

The effect of water content can be conducted by varying the buffer volume to oil volume ratio. The selected buffer volume to oil volume ratio range is 1:1 to 6:1 and the other variables employed from the previous run.

3.2.10 Effect of Enzyme Loading

The effect of enzyme loading is investigated by varying the enzyme loading from 1 to 6 mg in 1 mg increments, to obtain a final enzyme concentration of 0.005%(w/v) up to 0.03%(w/v). The optimum temperature and pH are used, while the other parameters are kept constant from previous runs.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Enzymatic Assay of Lipase Activity

The hydrolytic of free lipase activity was measured using spectrophotometric rate determination method at the wavelength of 400nm to observe the absorbance of *p*-nitrophenyl butyrate to *p*-nitrophenyl which formed a yellow solution indicates the release of butyric acid from *p*-NPB.. The analysis was performed in triplicate and the activity of the free lipase was calculated using equation (3.3) and tabulated the values in the Table 4.1 below.

Table 4.1 Averaged enzymatic activity of Candida rugosa lipase

Media	**Enzyme activity, A (U/mg enzyme)**
Sample 1	779.79
Sample 2	1128.83
Sample 3	1001.41
Average	970.00

The hydrolytic activity of free lipase enzyme has an average of 970.00 U/mg enzyme, which one unit (U) of enzyme activity was defined as the enzyme hydrolyze 1μmol of substrate in one minute under assay conditions. However, the result obtained was lower than the actual enzyme activity *C.rugosa* lipase (1176 U/mg enzyme) labelled in the container. In this case, enzymes display their highest activity at their respective optimum conditions, deviation from the optimum cause a reduction of the activity, depending on the degree of the deviation. Enzymes are sensitive substances present in small amount (Bisswanger, 2014), thus this factor might the cause reduction of activity of enzyme.