IMMOBILIZED LIPASE CATALYSED ESTERIFICATION OF FORMIC ACID AND METHANOL

By

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Maisara Adleena binti Mohd Ali

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LIST OF SYMBOLS

Symbol	Description	Unit
[A]	Concentration of A	mmol
[B]	Concentration of B	mmol
K_{mA}	Steady state Michaelis constant for substrate A	mmol
K_{mB}	Steady state Michaelis constant for substrate B	mmol
K_{mO}	Steady state Michaelis constant for oleic acid	mmol
K_{mM}	Steady state Michaelis constant for methanol	mmol
K_{iA}	Inhibition constant for substrates A	mmol
K_{iB}	Inhibition constant for substrates B	mmol
K_{iO}	Inhibition constant for oleic acid	mmol
K_{iM}	Inhibition constant for methanol	mmol
v	Initial rate of reaction	mmol/min/g
Vmax	Maximum reaction rate	mmol/min/g
R^2	Coefficient of determination	
V	Volume of assay	ml
C	Micromolar extinction coefficient of p-Nitrophenol at 400 nm	ml/mmol cm
df	dilution factor	
m	mass of enzyme	
C_{Ai}	Initial reaction acid concentration	M
C_{Af}	Final reaction acid concentration	M

LIST OF ABBREVIATION

Symbol	Description
OFAT	One-factor-at-a-time
p-NPB	P-nitrophenyl butyrate
p-NP	P-nitrophenyl
SDG	Sustainable development goals
RSM	Response surface methodology
A	First substrate
B	Second substrate
E	Enzyme
P	1^{st} product
Q	2^{nd} product
EA	Enzyme-substrate complex
EAB	Enzyme-acyl-substrate complex
EAP	Enzyme-acyl-product
EP	Enzyme-product
EQ	Enzyme-product

PENGESTERIFIKASI ASID FORMIK DAN METANOL DIMANGKINKAN OLEH LIPASE TERSEKATGERAK

ABSTRAK

Metil format merupakan salah satu ester mudah dengan mempunyai pelbagai kegunaan dalam aplikasi industri. Pengesteran Fischer adalah salah satu pengesteran konvensional yang menggunakan mangkin asid dan memberi kesan negatif seperti isu alam sekitar. Penggunaan biomangkin merupakan kaedah alternatif untuk tindak balas pengesteran dan memberikan impak yang baik dari segi aspek kemampanan dan mesra alam. Tesis ini menerangkan pengoptimuman pengesteran enzimatik dengan menggunakan teknik satu-faktor-pada-satu-masa. Tindak balas pengesteran enzimatik terdiri daripada asid formik dan metanol yang dimangkin oleh lipase *Candida rugosa* tersekatgerak dalam pelarut n-heksana untuk menghasilkan metil format. 62.24 U/mg lipase *C. rugosa* bebas telah disekatgerak oleh penjerapan fizikal pada sokongan Celite 545 dan menghasilkan aktiviti lipase sebanyak 50 U/mg. Pengaruh masa tindak balas, pemuatan enzim dan nisbah molar substrat telah dikaji. Pembetukan metil format tertinggi sebanyak 30.22% telah diperolehi pada 40°C, nisbah 1:4 molar asid formik kepada metanol, 4 g/L pemuatan enzim dengan kelajuan pengadukan 150 rpm dalam 1.5 mL n-heksana selama 60 minit masa tindak balas. Data sekunder daripada artikel kajian telah digunakan untuk mengkaji dan membandingkan model kinetik melalui analisis regresi bukan linear menggunakan persamaan kadar untuk setiap model. Ping Pong Bi-Bi dengan perencatan oleh kedua-dua substrat merupakan model paling sesuai dengan data eksperimen yang bernilai 0.98 bagi R^2. Parameter kinetik yang diperolehi ialah, V_{max}= 5.98 mmol/min/g, K_{mM} = 0.24 mmol, K_{mO} = 1.16 mmol, K_{iO} = 0.68 mmol and K_{iM} = 0.19 mmol.

IMMOBILIZED LIPASE CATALYSED ESTERIFICATION OF FORMIC ACID AND METHANOL

ABSTRACT

Methyl formate is the simplest ester with various uses in industrial applications. Fischer esterification is a conventional esterification that uses acid catalysts, giving drawbacks such as environmental issues. The utilization of biocatalyst is an alternative method for esterification reaction, which provides better sustainability and eco-friendly aspect. This thesis describes the optimization of enzymatic esterification by using the OFAT technique. The reaction of enzymatic esterification involved formic acid and methanol catalyzed by immobilized *Candida rugosa* lipase in n-hexane solvent to produce methyl formate. 62.24 U/mg of free *C. rugosa* lipase was immobilized by physical adsorption on Celite 545 support, which resulted in 50 U/mg support in a specific activity. The effect of reaction time, enzyme loading and substrate molar ratio were studied. The highest conversion of 30.22% was obtained at 40℃, with a 1:4 molar ratio of formic acid to methanol, 4 g/L of enzyme loading with an agitation speed of 150 rpm in 1.5mL n-hexane for 60 minutes reaction. Secondary data from the research article was used to study and compare the kinetic model via non-linear regression analysis through rate equation. The best fit with the experimental data was Ping Pong Bi-Bi with inhibition by both substrates with an R^2 value of 0.98. The kinetic parameter obtained were, V_{max}= 5.98 mmol/min/g, K_{mM} = 0.24 mmol, K_{mO} = 1.16 mmol, K_{iO} = 0.68 mmol and K_{iM} = 0.19 mmol.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Generally, an ester is produced through catalysed esterification reaction between a carboxylic acid and alcohol. Furthermore, the reactions generate water as part of the ester production, which is shown in Figure 1.1. Esterification is a reversible process, and equilibrium conditions limit the degree of conversion. In order to prevent the reverse reaction from happening, water formed as a by-product in a reaction must be consistently removed from the reaction mixture. Hence, the formation of the wanted product, an ester, can be shifted beyond the thermodynamic equilibrium, and complete conversion of the reactants can be obtained. Traditionally, the esterification reaction is identified as Fischer esterification as it uses an acid catalyst, typically in the form of sulphuric acid, to increase the reaction rate and act as a dehydrating agent (Khan et al., 2021).

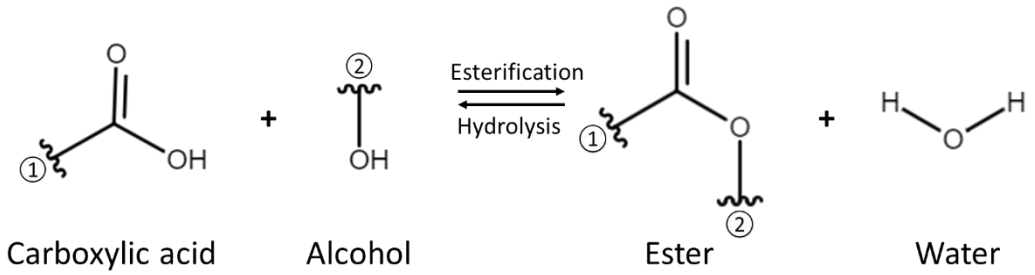

Figure 1.1 Esterification reaction between carboxylic acid and alcohol

During the 1990s, the biocatalyst activity of enzymes started to call attention in esterification reaction and is known as enzymatic esterification. It is an alternative way to use in the reaction in order to meet the sustainability process. This is due to its significant activity and selectivity, mild operating conditions, ability to speed up the reaction by lowering the activation energy, and maintained in its organic media (Zappaterra et al., 2020). Lipase such as *Candida antartica* lipase A, *Candida antartica* lipase B, *Novozym* 435, *Burkholderia*

cepacian, *Bacillus subtilis* and *Candida rugosa* are utilized in the fine chemical industry application. This is because lipase is a non-toxic chemical as it comes from a natural resource, extracted from plants, microorganisms and animals through the purification process (Chandra et al., 2020; Beslin, 2018). Furthermore, lipase enzymes are sustainable and eco-friendly as it capable of being reused multiple times in the reaction compared to being wasted for one reaction. Resulting in low environmental issues and logistical liability (Chandra et al., 2020). This thesis describes the synthesis of methyl formate via enzymatic esterification from formic acid and methanol catalyzed by immobilized *Candida rugosa* lipase.

Methyl formate or methyl methanoate is a methyl ester of formic acid. It is the simplest example of an ester with the characteristics of low boiling point, 31.5°C, colourless liquid, flammable and delicate odour (Reutemann & Kieczka, 2000). The industrial uses of methyl formate included the manufacture of formamide, dimethylformamide or other formic acid derivatives as building blocks. Due to its high vapour pressure, methyl formate can be used as a blowing agent for foams and a quick-drying finish. It also can be a replacement for CFCs, HCFCs, and HFCs as methyl formate has zero potential for global warming and ozone depletion. Furthermore, it is formerly used as a polar aprotic solvent, a fumigant, a refrigerant and an insecticide (Reutemann & Kieczka, 2000).

1.2 Methyl Formate Market

Esters have become prominent as components in lubricants, flavouring agents, and surfactants, among other things. The automobile, cosmetics, and food sectors all utilise them. The simplest formate ester is methyl formate, which is frequently used in the foundry and plastics industries. It is also used as a precursor in the production of formic acid, which is a critical raw ingredient in the organic chemical industry and is used in animal feed, silage preservatives, and leather tanning (Methyl Formate Market Share & Trends Analysis, 2020)

The chemical company has had great growth in recent years and is likely to continue to grow steadily in the foreseeable future. Socioeconomic dynamics, such as high population expansion and the emergence of the wealthy middle class in developing nations, are expected to fuel the market's long-term growth. It has resulted in an increase in infrastructure investment in developing nations like China and India. Consistent economic development in emerging nations like China, India, Brazil, and ASEAN countries, as well as increased per capita disposable income and spending, are likely to fuel demand for pharmaceutical goods, building and construction, and agriculture which accelerates the expansion of the methyl formate in the market (Methyl Formate Market, 2022).

The global situation of rising disease burden and intensive R&D efforts for medication innovation are the primary drivers of the pharmaceutical industry's growth. Methyl formate is used as an agent in drug delivery to regulate the release of medications in the human body. Methyl formate is able to increase the resistance of anti-cancer medications used to treat tumours. As people are aware more health-conscious, the demand for preventive pharmaceuticals is increasing across the globe (Methyl Formate Market Share & Trends Analysis, 2020)

Methyl formate contributes to a zero-ozone depletion layer and zero global warming. As a result, it is frequently employed in the production of polyurethane foams. The ability of polyurethane foams to offer insulation in building and construction materials, reduce energy

usage and control interior temperature is predicted to raise the demand for polyurethane foams in the near future (Methyl Formate Market, 2022).

The demand for plant-based diets is predicted to rise as the world's population grows and people's disposable income rises. This is expected to boost demand for fumigants and insecticides, which will boost the worldwide methyl formate market (Methyl Formate Market Share & Trends Analysis, 2020)

The major producer of methyl formate in North America, Europe, Asia Pacific, Latin America, Middle East and Africa, as the market of methyl formate is highly concentrated across the globe. Due to the increases demand for methyl formate for various application such as pharmaceutical industry, construction and building, and agricultural industry, the global market intend to increase per year in order to meet the demand, which is shown in Figure 1.2. In spite of methyl formate production being affected due to COVID-19 during 2019 and 2020, however, the market is expected to pick up the pace post-COVID-19. The global methyl formate market was analysed by huge manufacturers such as BASF, Eastmans, Triveni Chemicals and Mitsubishi Gas Chemical (Methyl Formate Market Size, Share, Trend and Forecast, 2022). In addition, India's methyl formate market demand which depicts in Figure 1.3, stood at 9.43k tonnes in 2021 and is forecast to achieve 15.56k tonnes in 2030, which grows a CAGR of 5.72% (ChemAnalyst, 2022).

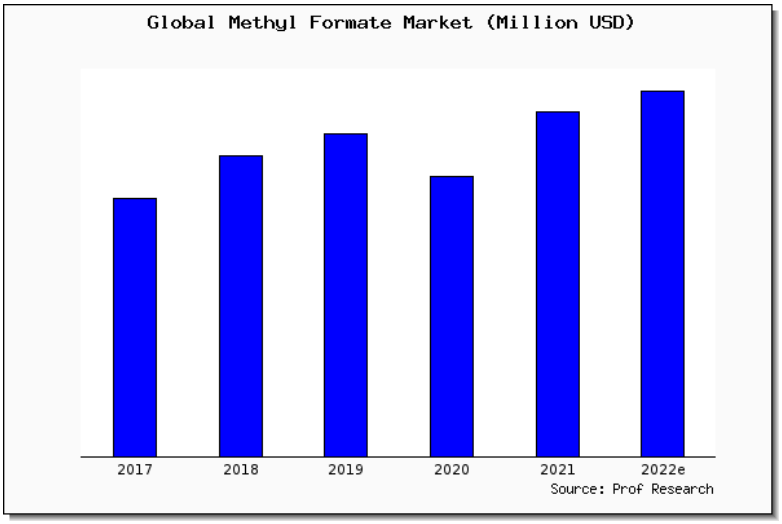

Figure 1.2 The trend of the global methyl formate market (Methyl Formate Market Size, Share, Trend and Forecast, 2022)

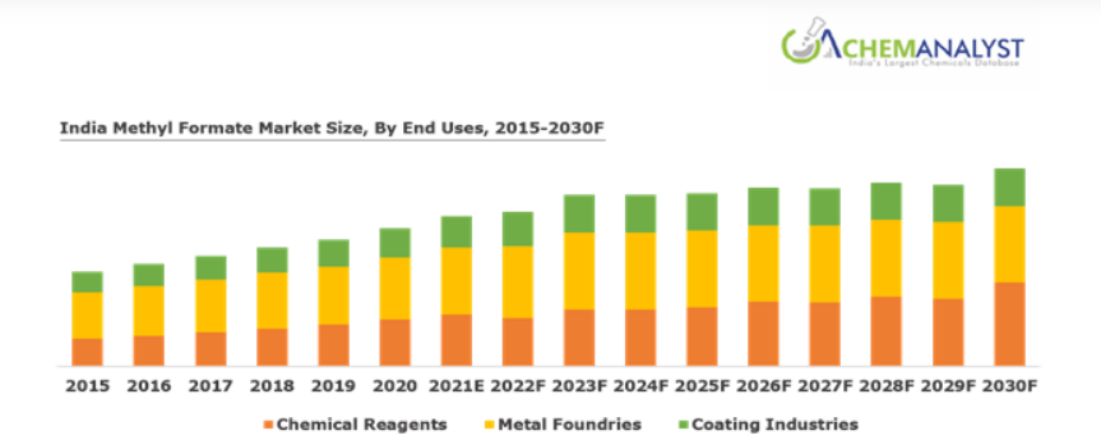

Figure 1.3 A forecast of India methyl formate market from 2022 to 2030 (ChemAnalyst, 2022)

1.3 Problem Statement

A catalyst speeds up the reaction yet remains chemically unchanged. They are able to be reused and enhance the efficiency of the reaction in a short time. Therefore, the different catalyst used has a different effect on the reaction. Conventionally, an esterification reaction is an organic reaction which converts carboxylic acid in the presence of excess alcohol with a strong acid catalyst to form ester and water (Khan et al., 2021). The reaction is required to use a large amount of strong acid, such as sulphuric acid, with a high operating condition that includes temperature and pressure. This is because a large amount of strong acid catalyst is able to remove substantial water to prevent a reversible reaction. In spite of this, the reaction with a strong acid tends to produce a side product which requires an additional process such as separation and purification process (Lam & Lee, 2011). The most important aspect that should be acknowledged for every used or produced of reactant, catalyst or product are the environmental issue and industry standards. For this case, as a strong acid catalyst is required a high amount in the reaction, it causes an environmental issue. This is because acid catalysts can become poisoned by waste products which pollute the environment and are harmful if exposed to human as acid catalysts contain high toxicity. Besides, serious corrosion to reactor walls, valves and pipelines may occur due to strong acidic properties (Lam & Lee, 2011). This method does not meet the industry standard. Hence, a better replacement for an acid catalyst in an esterification reaction is an enzyme catalyst.

The function of an enzyme catalyst is the same as an acid catalyst, where it able to increase the rate of reaction by lowering the energy activation of reaction. Therefore, an enzyme catalyst is more environmentally friendly and sustainable for a reaction because it requires relatively low operating conditions such as temperature and pressure. The formation of undesired products reduces as enzyme catalyst gives high selectivity to the substrate, resulting in an ease of purification process. Besides, enzyme catalyst can be evolved into immobilized enzyme where it enhances the ability to reuse multiple times in the reaction.

This is because the immobilized enzyme may not suffer aggregation due to the dispersion of the enzyme on the support surface, resulting in slight decreases in enzyme activity (Rodrigues et al., 2013). Hence, the immobilized enzyme will be used as a catalyst for enzymatic esterification between formic acid and methanol to form methyl formate.

1.4 Research Objectives

i. To immobilize free lipase *Candida rugosa* using Celite 545 support material via adsorption technique.

ii. To study the effect of various reaction parameters and determine the optimum reaction conditions of formic acid and methanol using one-factor-at-a-time (OFAT) method. The parameters are of reaction time, enzyme loading and molar ratio of substrates.

iii. To compare the esterification kinetics between bi-substrate kinetic models of enzyme catalysed esterification by correlating experimental findings from secondary data.

1.5 Scope of Research

To achieve the objectives of study, three major scopes are covered in this research which includes immobilizing enzymes, optimization of enzymatic esterification through effect of reaction parameter and comparing kinetic mechanism.

Candida rugosa lipase is used for immobilization supported by Celite 545 with cold acetone as solvent. Three parameters, reaction time, the effect of enzyme loading and the effect of molar ratio of substrates, are conducted to obtain the optimization of enzymatic esterification. Secondary data from Ramamurthi and McCurdy's (1994) article is extracted via PlotDigitizer to compare the three models of the Bi-Bi substrates mechanism to justify the suitable mechanism. The kinetic parameter calculation for each model is done using Polymath software.

CHAPTER 2

LITERATURE REVIEW

2.1 Biocatalyst

The biocatalyst is a protein which drives non-spontaneous chemical reactions within biological systems. Enzymes are one of the biocatalysts which are very sensitive to temperature and pH compared to inorganic catalysts. The activities of enzymes can be modulated and controlled. Enzymes have been extracted, manipulated, and employed in a variety of industries because they are active in vitro and organic in nature (Sutipatanasomboon, 2022). Inorganic catalyst is made from ions, metal atoms or solid surfaces which cannot regulate like enzymes. Even though both enzyme and inorganic catalyst are able to speed up the rate of a chemical reaction by several orders and magnitude and reduce the activation energy, yet inorganic catalyst has a higher turnover rate and works differently in reaction mechanism compared to the enzyme (Buxbaum, 2015). This is because enzymes are proteins that operate in living organisms. The majority of their actions are best performed in the aqueous phase, in a state that closely reflects the organism's natural state, which is sensitive to temperature and pH variations (van Schie et al., 2021).

For the esterification reaction, lipase is used as an enzyme known as a hydrolytic enzyme. A hydrolytic enzyme is a function of splitting a different biomolecule, including esters, peptides and glycosides. It also breaks down fat into the simplest unit, which is lipase (Prabha et al., 2012). Hence, lipase is commonly used as an enzyme for esterification reactions (Jiang, 2019; Moo-Young, 2019; Plou, 1999; Baek et al. 2020).

Biocatalysts are widely applied in industrial applications such as animal feed, textile, food and beverage and pharmaceutical industries. For instance, the use of biocatalyst in order to obtain a large-scale chiral chemical is one of the main focuses of pharmaceutical industries. Chirality is one of the most important factors in determining whether a chemical is active or harmful. Stereoselectivity is possible because enzymes are selective by nature.

Lipases and transaminases are among the most often employed biocatalysts in the pharmaceutical sector to produce chiral chemicals. Chirality is one of the most important factors in determining whether a chemical is active or harmful. Stereoselectivity is possible because enzymes are selective by nature. Lipases and transaminases are among the most often employed biocatalysts in the pharmaceutical sector to produce chiral chemicals (Wu et al., 2020; Sheldon et al., 2020).

2.2 Immobilization of Enzyme

Enzymatic esterification using lipase appears appealing and promising as it eases product separation, low wastewater treatment requirements, and a lack of adverse reactions (Ravindra, 2006). In addition, the lipase enzyme is used as a catalyst because it has the ability to transform into a product with low product inhibition, high activity, yield in non-aqueous media, and reusability of the immobilized enzyme (Bajaj et al., 2010). Furthermore, lipase is a friendly environment and has low toxicity. However, the most significant drawbacks of commercial enzyme synthesis include high cost, availability of optimum enzymes, and safety concerns.

To address this issue, the enzyme is commonly utilized in an immobilized form, allowing it to be reused several times, lowering costs and improving product quality. The localization or confinement of an enzyme on a solid support or a carrier matrix is referred to as immobilization. Mechanical strength, microbiological resistance, thermal stability, chemical durability, chemical functionality, hydrophobic/hydrophilic character, ease of regeneration, loading capacity, and cost are all considerations to consider when selecting a carrier for industrial processing applications (Karube et al., 1977). For instance, Chiou & Wu (2004) justify the low efficiency and operational stability exhibited by immobilizing lipase of *Noosa* on dry chitosan beads due to chitosan-swelling behaviour in aqueous solutions compared to wet chitosan beads as the carrier.

The immobilization of enzymes is experiencing massive development. In the past, biotechnological methods were used to immobilize enzymes; however, due to the rapid development of nanotechnology and the synergistic interaction of the two technologies, traditional methods such as noncovalent adsorption, covalent binding, entrapment, and encapsulation are now being used to develop immobilized enzymes on various nanomaterials (Jegannathan et al., 2008; Khan et al., 2010). Various techniques and carriers have been explored to immobilize lipase for biodiesel production. In Modi (2007)'s studies, the adsorption technique was used to immobilize *Candida Antartica* on macroporous acrylic resin as a support carrier. The result states that ethyl acetate is a feasible acyl acceptor to produce biodiesel as it improves the operational stability of immobilized lipase through multiple cycles, potentially saving money on the enzyme. Furthermore, the studies of Razack & Duraiarasan (2016) also justify the immobilization of *B. Cepacian* and *B. Subtilis* on sodium alginate by using the encapsulation technique to mitigate the cost of the process in terms of high enzyme reusability in the reaction. In addition, the immobilization enzyme also improves and enhances fatty acid methyl ester production by 93.61% of conversion. Table 2.1 below depicts an overview of the various techniques and carriers utilized to immobilize lipase.

Table 2.1 Overview of different technique and carrier for lipase immobilization.

Technique	Carrier	Enzyme	Conversion (%)	Reusability	Reference
Adsorption	Macro porous acrylic resin	*Candida antartica*	91.3	12	(Modi et al., 2007)
Adsorption	NA	*Candida antartica*	92	NA	(Xu et al., 2005)
Encapsulation	Silica aerogel	*B. cepacia*	64	NA	(Orçaire et al., 2006)
Encapsulation	Sodium alginate	*B. cepacian B. subtilis*	93.61	20	(Razack & Duraiarasan, 2016)
Entrapment	Silicate sol–gel glasses	*Candida qntarctica*	96	NA	(DiCosimo et al., 2013)
Covalent binding	magnetic core–shell nanocomposites	*Candida rugosa*	NA	4	(Xie & Zang, 2016)

2.3 Enzymatic Esterification Reaction Operating Condition

Methyl formate is produced from carboxylic acid and alcohol with biocatalyst via the esterification process. The optimization of the reaction is vital to achieve the highest yield of the product. The factor that affects the production is the molar ratio of substrates and oil, enzyme concentration, and temperature. Table 2.2 illustrates the summary of optimum operating conditions for enzymatic esterification reaction.

Table 2.2 Summary of optimum operating condition for each parameter; (A) Substrates molar ratio (carboxylic acid: alcohol), (B)enzyme concentration (%), (C) temperature (°C), (D) reaction rate (hr)and (E) yield percentage (%) on enzymatic esterification reaction

Enzyme	**Substrates**		**Parameter**	**Optimum operating condition**	**Analysis**	**Reference**
	Carboxylic acid	Alcohol				
Novozym 435	Formic acid	Phenethyl alcohol	A	1:5	Gas chromatograph	(Shin et al., 2020)
			B	15		
			C	40		
			D	4		
			E	95.92		
Candida rugosa	Oleic acid	Stigmasterol	A	1:3	Gas chromatography–mass spectrometry	(Chang et al., 2020)
			B	8		
			C	45		
			D	16		
			E	97.33		
Novozym 435	Formic acid	Hexanol	A	1:5	A gas chromatograph using an HP-INNOWax capillary column	(Seo et al., 2021)
			B	15		
			C	40		
			D	1.5		
			E	95		
Candida antartica	Octanoic acid	Hexanol	A	1:1	HPLC using the UltiMate 3000 Dionex (Luna C18 column from Phenomenex, ID 4.6 mm, L 250 mm	(Lopresto et al., 2014)
			B	NA		
			C	35		
			D	1		
			E	90		
Candida antartica	Propionic acid	Geraniol	A	1:6	Gas chromatography using a capillary column of	(Paroul et al., 2010)
			B	5		
			C	40		
			D	0.5		

			E	100	fused silica INOWAX	
Candida antarctica lipase B	Levulinic acid	Methanol	A	1:3	Gas chromatography	(Song et al., 2021).
			B	4		
			C	30		
			D	9		
			E	80		

2.3.1 Effect of Reaction Time

Reaction time is essential for enzyme performance and reaction progress. Optimizing the enzymatic reaction time is important for economic feasibility as the enzyme activity tends to decrease over time. In addition, it is possible to achieve maximum yield in the shortest amount of time while lowering the operating cost. For instance, Chang (2020) stated the yield of enzymatic esterification between oleic acid and stigmasterol was sharply increased to 80.85% yield at 8 hours, and then the reaction started to slow down by reaching 90.66% yield at 16 hours. Over time, most of the enzymes in the system bind to the substrate, and the remaining substrate molecules will have to wait until the enzyme becomes available once the reaction is finished. This indicates that the reaction rate will slow down when the enzyme concentration drops (Bingly, 2019). Moreover, Saha & Goud (2014) reported the optimum conversion for reaction time on the esterification process is at around 60 minutes. Increasing the reaction time tend to reduce the rate of conversion. Furthermore, the reaction tends to proceed in the reverse direction when the excess time occurs, resulting in a decrease in reaction conversion and an increase in the formation of the by-product, water.

2.3.2 Effect of Enzyme Loading

The amount of enzyme loading is important as it decides the reaction rate and the time for the reaction to achieve equilibrium (Song et al., 2021). In addition, enzyme loading is to increase the reaction and manages the enzyme cost by avoiding excess enzymes in the reaction. The effect of enzyme loading studied by Song et al (2021) report, which involves enzymatic esterification of levulinic acid with methanol and the conversion is directly proportional to enzyme loading. For instance, in the first 5 hours, the conversion increases from 10% to 68% when using 2 g/L to 8 g/L of enzyme loading. Meanwhile, when 10 g/L was used, the conversion slightly increased to 72%. As enzyme loading increases, the binding of enzyme and substrates becomes saturated, resulting in low conversion. Thus, 8 g/L is the optimum choice due to the economical usage of an enzyme. Findings by Song et al (2021) was similar to Chang et al (2020) as when the enzyme loading was at 2% to 10%, and the conversion increased from 25.22% to 96.58%. Therefore, there is no significant difference when the lipase loading range is from 8% to 10% due to aggregation of lipase at the reaction surface tends to reduce the effective concentration of lipase and the contact area of lipase and substrate. The elaboration is illustrated in Figure 2.1 below.

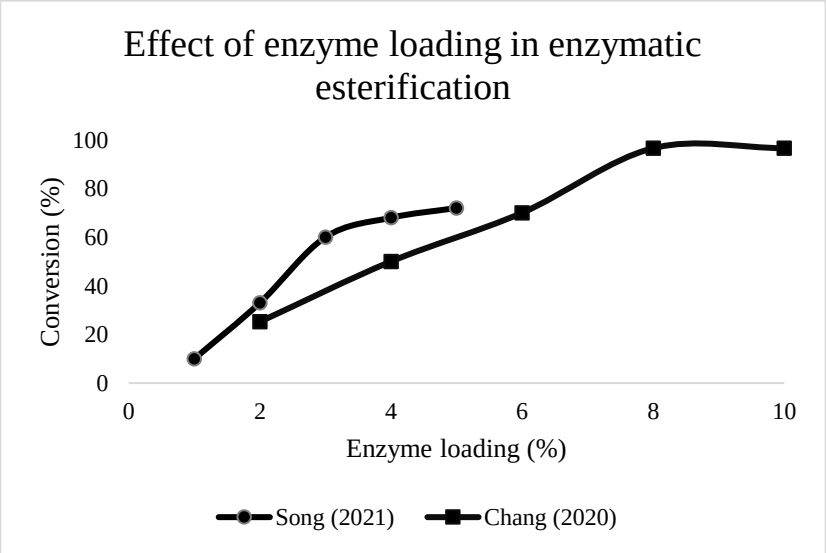

Figure 2.1 A trend of enzyme loading affecting towards conversion of product (Song et al, 2021; Chang et al, 2020)

2.3.3 Effect of Molar Ratio on Methanol and Formic Acid

The molar ratio of carboxylic acid and alcohol is vital as it is to be determinant in the equilibrium composition, which affects the product conversion. Generally, when equimolar quantities of both substrates react, the reaction proceeds until two-thirds of the acid and then comes to a stop. Since the esterification reaction is reversible in nature, varying the molar ratio of carboxylic acid and alcohol promotes a forward reaction up to 66% yield of desired product due to the equilibrium change, which obeys Le Chatelier's principle. According to Pandian (2020), alcohol is necessary in high quantities in order to force the reaction forward towards the product formation. Excessively utilizing alcohol is able to decrease the viscosity of the reaction medium and increase the area of contact between the reactant (Gan et al., 2012).

However, Shin (2010) stated there is a limit to the use of alcohol. Increasing the alcohol concentration beyond the optimized value, decreases the conversion of the desired product. For instance, conversion towards the effect of molar ratio on esterification reaction of formic acid to phenethyl alcohol from Shin et al (2010), increases from 1:1 to 1:5 (optimum point). Yet the conversion is reduced when the concentration of alcohol exceeds the optimum point because a structural analogue of a substrate joins at the active site of an enzyme but does not undergo reaction or known as dead-end inhibition. Finding from Pandian et al (2020) and Shin et al (2010), stated that where an excess of alcohol causes the alcohol molecules to deactivate the catalyst by binding to the active sites. This produces non-reacted alcohol, which leads to material wastage.

2.4 Kinetic Studies of Enzyme

The study of enzyme kinetics is crucial as it reveals information on an enzyme's behaviour that includes the sequence of substrates binding and products released from an enzyme's active site, also known as kinetic mechanism (Bisswanger, 2008; Cook & Cleland, 2007).

Furthermore, Hari Krishna & Karanth, (2002) has stated that the kinetic study of enzyme provides information on designing a reactor as it influences the rate of product formation and changes the system condition.

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{2.1}$$

Where,

v = Velocity of reaction

V_{max} = Maximum rate achieved by the system

K_m = Michaelis constant

S = Substrate concentration

The common equation to study the enzyme kinetic is Michaelis-Menten, shown in Equation 2.1 . This equation, however, may only be used on a single substrate system. Hence, the mechanisms for multiple substrate systems are classified into two types: sequential and nonsequential mechanisms. Sequential mechanisms have phase separation reactions where both substrates are attached to the enzyme, after which the reaction begins to produce a product that is subsequently released from the enzyme. Random and Ordered mechanisms are subgroups of sequential mechanisms. A Random mechanism is when substrates are binding and products are produced without a specific order, while an Ordered mechanism is vice versa. The Ping Pong mechanism, also known as the nonsequential mechanism, is defined by the transformation of the enzyme into an intermediate form. Between the additions of two substrates, the reaction occurs with the release of one or more products. This process is also known as the double placement response, and it is used often in group transfer (Ulusu, 2015).

For enzymatic esterification, the Ordered Bi-Bi mechanism is depicted in the report by Agustian (2016) and Parikh (2019). Yet, most of the enzymatic esterification reactions from previous studies have a great agreement with the Ping Pong Bi-Bi model (Bezbradica, Mijin, et al., 2006; Lopresto et al., 2014; Dave and Madamwar, 2008; Romero et al., 2007; Manan et

al., 2018). Thus, this report is to determine and compare the kinetics of esterification between oleic acid and methanol via three mechanisms.

2.4.1 Random Bi-Bi Mechanism

In a Random Bi-Bi mechanism, the order of binding of substrate and production of a product does not matter (Berg et al., 2002). In other words, the substrate can bind to the enzyme either before or after the other substrate. The same principle applied to the formation of the product. Hence, it develops a complex mechanism as one substrate on the enzyme can enhance, inhibit, or have no impact on the other substrates. Also, it is feasible for one substrate to be present with a product from the other substrate during the same period (Ulusu, 2015).

Figure 2.2 represents the mechanism of Random Bi-Bi. The mechanism starts when either substrate A or B binds an enzyme, forming either EA complex or EB complex, followed by the next binding of substrate producing EAB complex. Then, the EPQ complex is produced via chemical reaction, and products P and Q are released.

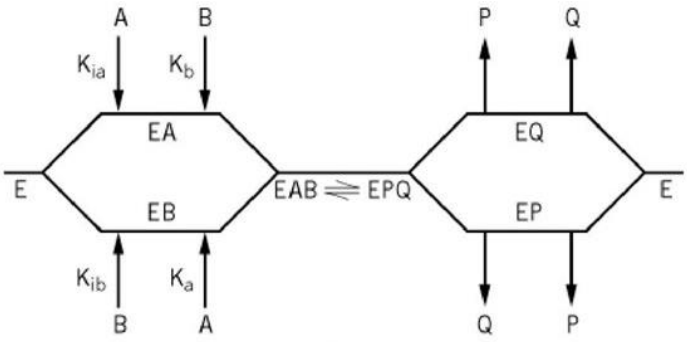

Figure 2.2 The kinetic study of Random Bi-Bi mechanism

The Lineweaver Burk Equation 2.2 for rate equation of Random Bi-Bi mechanism is shown as follows;

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_A}[B] + K_{m_B}[A] + K_i} \quad (2.2)$$

Where,

v = Initial reaction rate

A,B =Substrate A and B concentration, respectively

V_{max} = Maximum reaction rate

K_{mA},K_{mB} = Steady state Michaelis constant for substrate A and B, respectively

K_i = Inhibition constant

2.4.2 Ordered Bi-Bi Mechanism

The Ordered Bi-Bi mechanism is an obligatory order of adding substrates and releasing products. The mechanism is shown in Figure 2.3, where substrate A possesses a binding site on the enzyme, and EA complex is formed, which develops a binding site for substrate B. EAB complex is produced after EA complex has bind with substrates B, which EAB complex occur a chemical reaction which isomerized by a unimolecular reaction and form an EPQ complex. Lastly, products P and Q with free enzyme E are released.

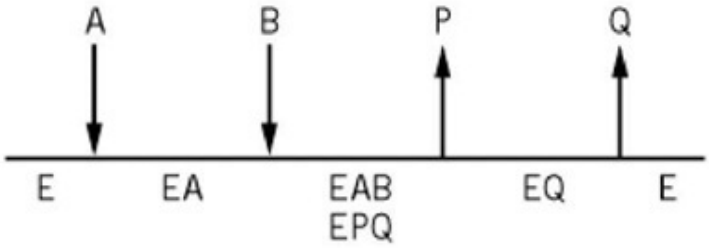

Figure 2.3 The kinetic study for Ordered Bi-Bi mechanism

The general rate equation for Ordered Bi-Bi mechanism of Lineweaver Burk is depicted as Equation(follow;

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_B}[A] + K_i K_{m_B}} \tag{2.3}$$

Where,

v = Initial reaction rate

A,B =Substrate A and B concentration, respectively

V_{max} = Maximum reaction rate

K_{mB} = Steady state Michaelis constant for substrate B

K_i = Inhibition constant

2.4.3 Ping Pong Bi-Bi Mechanism

The representative non-sequential mechanism is called Ping Pong Bi-Bi or double placement reaction, where the reaction releases one or more products between two substrates added. The most important aspect of this reaction is the existence of a substitute intermediate enzyme, which is a temporarily modified enzyme. The kinetic mechanism starts when a donor group of the first substrate, A binds to the enzyme, resulting in an enzyme-acyl complex, EA formed and transformed into an enzyme-acyl intermediate, EP by unimolecular isomerization. Then, a new stable form of enzyme F and product P is released. The mechanism continues with the second substrates, B binds at the site that P has vacated on the enzyme generated a tertiary complex enzyme acyl alcohol complex, FB. The breakdown of the tertiary complex forms the second product, Q and the free enzyme. The mechanism elaboration is depicted in Figure 2.4 below.

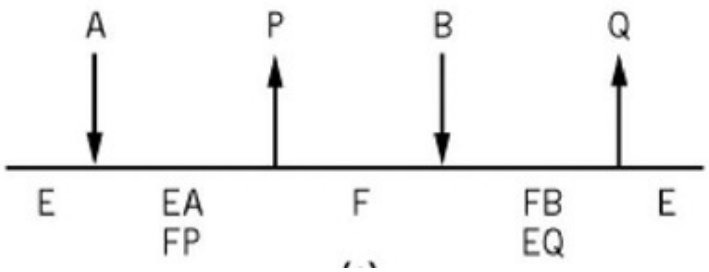

Figure 2.4 The kinetic study for Ping Pong Bi-Bi mechanism

The general equation for Ping Pong Bi-Bi mechanism of the Lineweaver Burk equation is shown in the Equation 2.4(below;

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_A}[B] + K_{m_B}[A]} \quad (2.4)$$

Where,

v = Initial reaction rate

A,B =Substrate A and B concentration, respectively

V_{max} = Maximum reaction rate

$K_{mA,}\, K_{mB}$ = Steady state Michaelis constant for substrate A and B, respectively

2.5 Inhibition in Bi-Substrate Reaction

Bi-substrate inhibitors contain two conjugated fragments, each directed to a distinct binding site on a bi-substrate enzyme. Bi-substrate inhibitor develops a ternary complex during the catalysed process. The benefit of bi-substrates development is the capacity to produce additional contacts with the target enzyme, which may lead to better affinity and selectivity of the conjugates (Lavogina et al., 2010). In order to study the effect of substrate inhibition in a bi-substrate reaction by varying a substrate concentration and another different fixed substrate concentration against the initial rate. The slope and intercept for a double reciprocal plot are observed. These phenomena are categorised as reversible inhibition, which includes competitive, uncompetitive, and non-competitive substrate inhibition, respectively (Leskovac, 2003).

2.5.1 Random Bi-Bi Inhibition Mechanism

As mentioned previously, the Random Bi-Bi mechanism randomly binds between substrates and enzymes. Increasing a varied substrate concentration (B) can reduce the inhibition in a reaction as the inhibitor may compete with both substrates to bind with an enzyme. It also

increases the competitiveness of fixed substrate concentration (A) by trapping the EA complex in the EAB complex from which the fixed substrate cannot exit from the enzyme (Yu et al., 2006). Equation 2.5 illustrates the rate equation for Random Bi-Bi with inhibition.

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_B}[A] + K_{m_A}[B]K_{i_A}K_{m_B}} \quad (2.5)$$

Where,

v = Initial reaction rate

V_{max} = Maximum reaction rate

A,B =Substrate A and B concentration, respectively

K_{mA}, K_{mB} = steady state Michaelis constant for substrate A and B, respectively

K_{iA} = inhibition constant for substrates A

2.5.2 Ordered Bi-Bi Inhibition Mechanism

Generally, the Ordered Bi-Bi mechanism is an evolving mechanism from a Random Bi-Bi mechanism. The Ordered Bi-Bi mechanism is synonymous with the equilibrium of Random Bi-Bi, yet a different result in the end. In the Ordered Bi-Bi mechanism, when a varied substrate concentration (B) increases, the competitiveness of fixed substrate concentration (A) increases, resulting in no chances of the inhibitor to bind with the enzyme as all enzyme is forced into central complexes. Hence, inhibition can be eradicated completely (Yu et al., 2006). The rate equation for Ordered Bi-Bi with inhibition is represented in Equation 2.6

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_B}[A] + K_{m_A}[B]\left(1 + \frac{[B]}{K_{i_A}}\right) + K_{i_A}K_{m_B}\left(1 + \frac{[B]}{K_{i_B}}\right)} \quad (2.6)$$

Where,

v =Initial reaction rate

V_{max} =Maximum reaction rate

A,B =Substrate A and B concentration, respectively

$K_{mA,}K_{mB}$ = Steady state Michaelis constant for substrate A and B, respectively

$K_{iA,}K_{iB}$ = inhibition constant for substrates A and B, respectively

2.5.3 Ping Pong Bi-Bi Inhibition Mechanism

According to Romero (2007) stated, the rate of reaction and product yield decreased due to either one or both substrates may form a dead-end inhibition with an enzyme. When there is no reaction occurs during a substrate that binds to an enzyme and produces an enzyme-acyl complex, it is known as a dead-end. Hence, there are three types of inhibition in the Ping-Pong Bi-Bi mechanism which are inhibition with the first substrate, inhibition with the second substrate and inhibition with both substrates. The general rate equation for Ping Pong Bi-Bi with inhibition is depicted in Equation 2.7

$$\frac{v}{V_{max}} = \frac{[A][B]}{[A][B] + K_{m_A}[B]\left(1 + \frac{[B]}{K_{i_B}}\right) + K_{m_B}[A]\left(1 + \frac{[A]}{K_{i_A}}\right)} \quad (2.7)$$

Where,

v =Initial reaction rate

V_{max} =Maximum reaction rate

A,B =Substrate A and B concentration, respectively

$K_{mA,}K_{mB}$ = Steady state Michaelis constant for substrate A and B, respectively

$K_{iA,}K_{iB}$ = inhibition constant for substrates A and B, respectively

2.6 Reversible Inhibition

Reversible inhibition is divided into three types of inhibition which are competitive, uncompetitive and non-competitive. The distinction between these three types of inhibition is their binding of inhibitor with enzyme, inhibitor with enzyme-substrate complex and inhibitor with both substrates.

2.6.1 Competitive Inhibition

Competitive inhibition takes place when either both substrates or inhibitors are binds towards the same active site on the enzyme resulting in a high competitiveness binding between them towards the active site. In addition, increased binding between inhibitor and enzyme leads to a reduction in reaction rate. The main principle behind research on competitive inhibition is that the inhibitor can only bind to the free enzyme's active site (Yadav & Magadum, 2017). The competitive inhibition can be described using the Cleland notation shown in Figure 2.5.

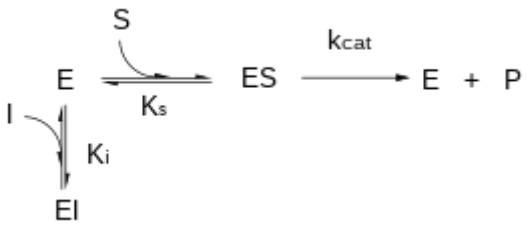

Figure 2.5 The mechanism of competitive inhibition via Cleland notation (Leskovac, 2003)

The Lineweaver Burk plot for competitive inhibition is shown in Figure 2.6, where the value for Michaelis constant, K_m changes yet maximum velocity, V_{max} unchanged. The value of Km is dependent on the concentration of the inhibitor. When the inhibitor concentration increases, the value of Km increases, resulting in low enzyme affinity for substrate (Ahern & Rajagopal, 2015).

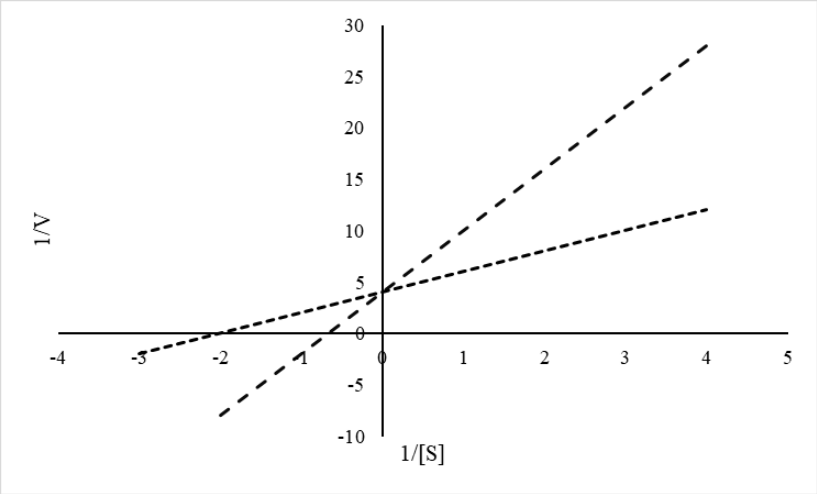

Figure 2.6 Lineweaver-Burk plot of competitive inhibition

2.6.2 Uncompetitive Inhibition

Uncompetitive inhibition happens after a substrate binds to the free enzyme, forming an enzyme-substrate complex with a binding site for the inhibitor. Hence, the inhibitor will bind to the enzyme-substrate complex. This elaboration can be visualized through Cleland notation in Figure 2.7.

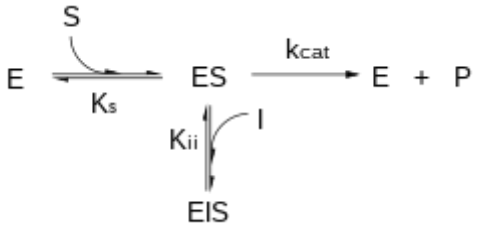

Figure 2.7 The mechanism of uncompetitive inhibition via Cleland notation (Leskovac, 2003)

Figure 2.8 depicts the Lineweaver-Burk plot for uncompetitive inhibition. Parallel lines were generated with the same slope yet different intercepts. As a result of the removal of the activated complex, uncompetitive inhibition reduces V_{max} and K_m due to improved binding efficiency in terms of Le Chatelier's principle and the effective elimination of the ES complex, decreasing K_m, which indicates a higher binding affinity (Wilkinson et al., 1984)