ESTERIFICATION OF ETHANOL AND ACETIC ACID CATALYSED BY IMMOBILIZED *Candida rugosa* LIPASE

NURUL ADILA BINTI MOHD RADZI

UNIVERSITI SAINS MALAYSIA

2020

ACKNOWLEDGEMENT

Throughout the completion of my final year project, I have received a lot of help and encouragement from a lot of people. Hereby, I would like to express my gratitude and gratefulness to those who have directly or indirectly involve in this project.

First and foremost, I would like to express my deepest gratitude to my supervisor, Prof Datin Dr. Azlina binti Harun@Kamaruddin, for the endless guidance and support throughout the duration of this project. Your informative remarks have encouraged me to improve my thoughts and raise the quality of my work.

My gratitude extends my sincere thanks to the postgraduate student, Mr. Jackson Robinson, for his helpful advice and guidance in my lab work. His invaluable assistance have helped me in structuring my experiment procedures and methods. Special thanks also to all the technicians and staffs in School of Chemical Engineering who have kindly assisted me throughout the completion of the lab work.

Apart from that, many thanks to my fellow laboratory-mates, Sim Wan Ting and Muhammad Firdaus, for the stimulating discussion regarding our lab work. Also, I am also grateful for my friends and classmates for the precious time we have spent together in my degree studies.

In addition, I would like to thank the management of School of Chemical Engineering for allowing me to carry out my lab work despite the ongoing COVID-19 outbreak. Thanks should also go to Prof Dr. Mohd Roslee Othman, the coordinator for the final year project of School of Chemical Engineering, for the incredible arrangement that aid us to finish this project.

Last but not least, I would like to thank my family, especially my parents that are always there for me. Their excellent advice and compassionate ear have helped me to go through my

hard times. It would have been difficult for me to finish my studies without their wonderful understanding and encouragement throughout the last few years.

Nurul Adila Binti Mohd Radzi

June 2021

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

$[A]$	Concentration of A	M
$[B]$	Concentration of B	M
$K_{i,A}$	Inhibition constant of substrate A	M
$K_{i,B}$	Inhibition constant of substrate B	M
$K_{m,A}$	binding constant of substrate A	M
$K_{m,B}$	binding constant of substrate B	M
pK_a	negative log of acid dissociation constant	
R^2	Coefficient of determination	
v	Initial rate of reaction	µmol/min/mg
V_{max}	maximum reaction rate	µmol/min/mg

LIST OF ABBREVIATIONS

CRL	Candida rugosa lipase
E	Enzyme
EAB	Enzyme-substrate complex
EPQ	Enzyme-product complex
F	Enzyme intermediate
P	First product
Q	Second product
RSM	Response surface methodology
SDG	Sustainable development goals

PENGESTERAN ETANOL DAN ASID ASETIK DIMANGKINKAN OLEH LIPASE TERSEKATGERAK

ABSTRAK

Etil asetat adalah ester serbaguna yang digunakan sebagai pelarut dan pencair. Tindak balas pengesteran etanol dan asid asetik secara konvensional dimangkinkan oleh asid sulfurik, H_2SO_4. Enzim adalah pendekatan lestari untuk tindak balas pengesteran untuk menghasilkan etil asetat. Dalam kajian ini, pengesteran etanol dan asid asetik yang dimangkinkan oleh lipase *Candida rugosa* (CRL) tersekatgerak dalam medium n-heksana telah dioptimumkan. CRL bebas telah disekatgerak dengan kaedah penjerapan fizikal pada sokongan, Amberlite XAD7 yang menghasilkan aktiviti spesifik sebanyak 0.13 U / mg. Pengaruh masa tindak balas, suhu, nisbah molar substrat dan pemuatan enzim telah dikaji. Penukaran maksima sebanyak 88% dicapai pada masa 2 jam masa tindak balas, suhu pada 50°C, nisbah asid asetik kepada etanol sebanyak 0.5 dan pemuatan enzim sebanyak 80 U. Seterusnya, pemodelan kinetik untuk transesterifikasi berenzim dwi-substrat, etil butirat telah dikaji dengan menggunakan data sekunder dari kertas penyelidikan. Data diselaraskan pada persamaan kadar untuk model kinetik menggunakan regresi tak lelurus untuk mendapatkan parameter kinetik. Berdasarkan plot salingan kembar, Lineweaver-Burk, tindak balas transesterifikasi mengikuti mekanisme ping-pong bi-bi dengan perencatan bersaingan oleh etil kaprat. Parameter kinetik yang diperoleh adalah, V_{max} = 1.1918 M, $K_{m,\,A}$ = 0.0117 M, $K_{m,\,B}$ = 0.1674, $K_{i,\,A}$ = 0.1091 M, dan $K_{i,\,B}$ = 0.0031.

ESTERIFICATION OF ETHANOL AND ACETIC ACID CATALYSED BY IMMOBILIZED *CANDIDA RUGOSA* LIPASE

ABSTRACT

Ethyl acetate is a versatile ester used as solvent and diluents. The conventional esterification reaction of ethanol and acetic acid was catalysed by sulphuric acid, H_2SO_4. Enzyme is a sustainable approach for the esterification reaction to produce ethyl acetate. In this study, the esterification reaction of ethanol and acetic acid catalysed by immobilized *candida rugosa* lipase (CRL) in n-hexane was optimized. The free CRL was immobilized by physical adsorption on support, Amberlite XAD7 which resulted in specific activity of 0.13 U/mg. The effect of reaction time, temperature, substrate molar ratio and enzyme loading were studied. Maximum conversion of 88% was attained at 2 hours of reaction time, temperature of 50°C, acetic acid to ethanol ratio of 0.5 and enzyme loading of 80 U. Next, the kinetic modelling of bi-substrate enzymatic transesterification of ethyl butyrate was studied using secondary data from research paper. The data was fitted to the rate equation of the kinetic model using non-linear regression to obtain the kinetic parameter. Based on the Lineweaver-Burk double reciprocal plot, the transesterification reaction follows ping-pong bi-bi mechanism with competitive inhibition by ethyl caprate. The kinetic parameters obtained was, V_{max} = 1.1918 M, $K_{m,A}$ = 0.0117 M, $K_{m,B}$ = 0.1674, $K_{i,A}$ = 0.1091 M, and $K_{i,B}$ = 0.0031.

CHAPTER 1

INTRODUCTION

Enzymatic esterification is a green alternative to the current chemical synthesis. This reaction utilizes mild condition and reduces side product. Thus, the purification and separation cost will be reduced. Immobilized *Candida rugosa* lipase (CRL) is employed as the biocatalyst in esterification of ethanol and acetic acid to produce ethyl acetate. Parameters such as temperature, enzyme loading, reaction time and substrate concentration are manipulated in order to determine the optimum operating condition for ethyl acetate synthesis. Then, kinetic of bi-substrate enzymatic reaction are studied using secondary experimental data from findings of one research study.

1.1 Research Background

Ethyl acetate, $C_4H_8O_2$ also known as ethyl ethanoate is an aromatic compound that are broadly used as solvent and diluent for various industries, such as, pharmaceutical, paint, coating, and adhesive. It is also used in foods and pharmaceuticals industry as flavour enhancer since it has the fruity taste when it is diluted. Ethyl acetate is also used to manufacture artificial leather, perfumes, paint, and even nails polish remover. Due to its polarity, non-toxicity and biodegradability, ethyl acetate is an eco-friendly solvent replacing any other toxic aromatic compound (Kruis et al., 2017; Wu et al., 2021). Recently, ethyl acetate has been recognized as one of an interesting acyl acceptor for transesterification of vegetable oil catalysed by lipase to produce biodiesel.

Ethyl acetate is a natural compound that is rich in fruits, such as apples, pears, oranges and grapefruits. However, the extraction of this compound is cost extensive, while the extraction yield is too small since the composition in the natural resources is quite low (de

Oliveira et al., 2019). Moreover, the extraction process will produce large amount of waste (Csanádi et al., 2012).

Currently, the synthesis of ethyl acetate is from fossil fuels by chemical reactions. All the reactants of the processes to produce ethyl acetate are either ethylene-based or originated from ethylene, which is known to be produced from steam cracking from natural-gas or crude oil constituents (Löser et al., 2014). This clearly shows that this reaction is not sustainable due to the limitations of the resources. Furthermore, production from chemical reaction involves in high temperature and strong acid.

1.2 Ethyl Acetate Market

As mention before, ethyl acetate has various applications in industry, but it is most known as solvent and diluents. The paint and coating industry has shown a healthy growth in demand for ethyl acetate. The main usage of ethyl acetate in paints is as activator and hardener. It is also used in coatings formulation such as epoxies, acrylic, vinyl and many more. These paint and coatings are consumed in different industries such as construction, wood furniture, automotive, marine and others. Since there are increasing in populations, income-levels and construction in countries like China, India and Brazil, the demand for ethyl acetate is growing well (*Ethyl Acetate Market | Growth, Trends, and Forecasts (2020 - 2025)*, 2019).

Although ethyl acetate is widely used in paint and coatings industries, but it is only taking 57% of the whole global demand in 2019. There has been an increased in demand in printing ink and food and beverage industries. Emerging countries such as China, India and Indonesia are thriving in printing ink industry. The printing industry in Asia Pacific are expected to be expanding which expected to give positive impact on the ethyl acetate's demand. In food industry, ethyl acetate has been adopted as the solvent and also as the flavor enhancer (*Methodology - Ethyl Acetate Market | Fortune Business Insights*, 2020). In emerging countries

where cost of the food is more pronounced, the consumption of artificial flavor is on the rise as the cost is reduced. Hence, the global demand for ethyl acetate is forecasted to be growing steadily until 2026 (*Ethyl Acetate Market | Growth, Trends, and Forecasts (2020 - 2025)*, 2019).

The market size of ethyl acetate in 2019 in Asia Pacific reported to be USD 2,312.3 million. The global market size of ethyl acetate was USD 3,323.2 million and expected to increase to USD 4,368.9 million by 2027. During the forecasted period, Asia Pacific is projected to dominate the demand for the ethyl acetate market in the wake of urbanization and rising in household incomes (*Methodology - Ethyl Acetate Market | Fortune Business Insights*, 2020).

Enzyme is a great alternative to replace the current unsustainable process. Enzyme is a biocatalyst that has a remarkable performance as catalyst. This is due to the selectivity and specificity for their substrates (Y. Yu & Lutz, 2010). Lipase is a versatile enzyme, under hydrolase group which involves in hydrolysis as catalyst. There are many types of lipases that can be used in the industry, for example, *Candida antarctica* lipase A (CALA), *Candida antarctica* lipase B (CALB) and also *Candida Rugosa* lipase (CRL). Lipase can be obtained from plants, animals and microorganism since these organisms producing lipases. However, the one produced by microorganism is the most relevance due to their potential in biotechnology (Y. Yu & Lutz, 2010). For ease in the enzyme recovery, lipase is usually immobilized. By this, enzyme can be reused and the cost for enzyme usage will be lower.

Organic solvent is frequently used in the industrial application. Organic solvents offers many advantages including, increase the substrate solubility and enzyme stability and also easy recovery of enzyme since it is insoluble in organic solvent (Patel et al., 2015). N-hexane, n-heptane, cyclohexane and cyclo-octane are among the organic solvent that is used in the enzymatic biocatalysis.

There are many factors affecting the enzymatic transesterification such as the temperature, amount of enzyme, the concentration of substrate and the reaction time. Temperature can influence the reaction rate and the stability of the enzyme (de Oliveira et al., 2019). This will alter some properties of the substrate and products. Theoretically, increase in substrate concentration will result in the shifting of the reaction towards the product and effecting the reaction rate. However, there are possibility of the inhibition occur (Patel et al., 2015). The enzyme loading and the reaction rate also will affect the yield of the ethyl acetate.

1.3 Problem Statement

The production of ethyl acetate conventionally uses fossil fuels as one of the resources by chemical reaction. The reaction needed a high temperature and a strong acid as the catalyst. Although chemical synthesis is considered as economical, but the process involves a strong acid and employ high operating conditions, such as, temperature and pressure. On top of that, there will be formation of side product which will requires additional separation and purification sections. Enzymatic esterification is a good alternative to replace the conventional production. Using enzymes as a catalyst is somewhat more environmentally friendly and more sustainable in the way that it requires low temperature and pressure, and also offers high selectivity to the substrate. This will reduce the formation of undesired product and will ease the purification section. Moreover, the use of immobilized enzyme will enable the enzyme to be extracted and reused after one reaction cycle. Therefore, in this study, immobilized enzymes will be used as the catalyst for esterification of ethanol and acetic acid to produce ethyl acetate. This process will be optimized in order to obtain the optimum operating condition that will yield the highest amount of ethyl acetate.

1.4 Objectives

i. To study the performance of the esterification of ethanol and acetic acid catalyzed by immobilized lipase in organic solvent.
ii. To establish the optimum operating parameters such as temperature, reaction time, enzyme loading, and acid and alkali concentration.
iii. To study the kinetic model of the bi-substrate enzymatic synthesis based on the kinetic data using secondary data from a research.

1.5 Scope of Research

In this study, the main focus is the synthesis of ethyl acetate from esterification of ethanol and acetic acid over *Candida rugosa* lipase (CRL) as the catalyst in organic solvent, which is n-hexane. The parameters that are studied are the temperature (20, 30, 37, 40, 50 and 60°C), enzyme loading (30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 U), substrate molar ratio (0.05, 1, 1.5, 2, 2.5 and 3) and the reaction time (1, 2, 3, 4, 5, 6 and 7 hour). From this study, the optimum operating condition for the ethyl acetate synthesis will be determine. The kinetic of the esterification reaction will be proposed accordingly.

CHAPTER 2

LITERATURE REVIEW

Green technology for producing ethyl acetate can be done in two ways which is fermentation and enzymatic esterification. There are a lot of research that have been done on the fermentation of sugar to produce ethyl acetate in the past 10 years. Yeast is the commonly used microorganisms to produce ethyl acetate. Fermentation will convert sugar to ethyl acetate (Kruis et al., 2017; Löser et al., 2013). This process involves cultivation of yeast, which is somewhat tedious.

2.1 Enzymatic Esterification

Enzymatic esterification is a technology which utilizes enzymes to catalyse the reaction of alcohol and carboxylic acid to produce esters. Enzymes is a non-toxic biocatalyst which has a very high selectivity to the substrates under mild conditions. This particular circumstance will prevent the formation of by-products which in the end will turn into waste. Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are usually involved in the hydrolysis of lipids to fatty acids and glycerol, and also acts as catalyst in other reactions such as, transesterification, interesterification, esterification and many more.

As being said previously, enzymatic esterification is one of the green technologies in synthesizing chemicals, such as ethyl acetate. The enzymatic esterification provides a sustainable approach to the chemical industries since it is an environmentally friendly method of synthesizing chemicals thanks to using less energy, producing less waste while utilizing less raw materials. This approach corresponds to the Sustainable Development Goals (SDGs), “Goal 9: Industry, Innovation and Infrastructure”, which specify about developing sustainable industrialization by 2030. The sustainable industrialization can be achieved by using enzyme as the catalyst as it requires low reaction temperature compared to the chemical esterification.

This way, the amount of energy used in the reaction can be reduced. Apart from that, enzymatic esterification also in-line with the SDGs, "Goal 12: Responsible consumption and production", since the production of esters and other chemicals using enzyme have little to no by-products. In addition to that, since the selectivity of the enzymes towards substrates is high, less amount of substrates need to be used compared to the conventional chemical esterification (*#Envision2030: 17 Goals to Transform the World for Persons with Disabilities | United Nations Enable*, n.d.).

Esterification can be carried out in solvent-free medium or in the medium with the presence of organic solvent. Organic solvents possess advantages of increasing the solubility of the substrate and the product while shifting the reaction equilibrium in the forward direction. However, the organic solvents can somehow inhibit the reaction by directly interacting with the enzyme, or the substrates, or the product, or even water layer at the vicinity of the enzymes. Hence, solvent with the value of log *P* (P is the partitioning coefficient of solvent between 1-octanol and water) of higher than 2 should be used. Hydrophilic solvent can strip off the hydration layer at the surface of the enzyme and causing denaturation. Organic solvent, such as n-hexane, n-heptane, isooctane, can maintain the activity of the enzyme due to the immiscibility nature of the solvent with water. In various study shows that, a higher yield of esters are obtained in the presence of organic solvent in the reaction medium (Devi et al., 2017; Ozyilmaz & Gezer, 2010; Patel et al., 2015). Patel et al. (2015) suggest that cyclic alkanes, e.g., cycloalkane, is better than the straight chain alkane, e.g., n-hexane. However, Devi et al. (2017) uses n-hexane as the organic solvent, which is more suitable for food applications.

2.2 Immobilization of Enzyme

The drawbacks of utilizing the enzymatic biocatalysis is that enzyme is expensive. The immobilization of enzyme is necessary to maintain the reusability of the enzyme and reduce

the cost to repurchase the enzyme. Enzymes can be immobilized on different shapes of support, such as, membranes or beads, by either covalent binding, entrapment, ionic bonding, encapsulation or adsorption. The support of the enzyme should possess characteristics of large surface area, able to offer rapid mass transfer between substrates and products, and can facilitate great chemical and mechanical stability (Bayramoğlu et al., 2011; Patel et al., 2015). It is worth noting that immobilization of enzyme helps in the catalytic activity of enzyme by providing extra protection against inhibitory effect of organic solvents (Patel et al., 2015) and have a higher thermostability compared to free enzymes (Bayramoğlu et al., 2011).

In the study of de Oliveira et al. (2019), *Rhizomucor miehei* lipase is immobilized onto chitosan to reduce the cost for the enzyme in the synthesis of ethyl butyrate. Chitosan is a natural biopolymer that shows great suitability properties to be used as enzyme support for immobilization. The properties including low cost, nontoxicity, high affinity for proteins, hydrophilicity and physiological inertness. The immobilized enzyme can be used up to seven consecutives cycle without loss of the enzyme activity. The reduction of esterification percentage may be due to the denaturation caused by either one of the substrates or both.

Immobilization of enzyme can also be done on membrane and nano-materials. Bayramoğlu et al. (2011) employing hydrophobic polypropylene chloride membrane (PPC). Same activity values were obtained within the first five cycles, and start declining afterwards. Patel et al. (2015) employing graphene oxide as the enzyme support. The enzyme was able to retain its activity up to 92% after 10 continuous cycles. Possible cause of the declining enzyme activity is due to the washing of enzyme by solvent after every cycle that limit the mass transfer and the reduction of accessibility of enzyme to the substrate (Patel et al., 2015). Table 2.1 shows different types of enzyme immobilization techniques on different types of enzymes.

Table 2.1: Type of enzyme immobilization

Lipase	Support	Method of Immobilization	Reusability	Product	Reference
Candida rugosa	Aminated polypropylene (APP) membrane	Covalent binding	5 cycles	Ethyl valerate	(Bayramoğlu et al., 2011)
Rhizumucor miehei	Chitosan	Adsorption	7 cycles	Methyl butyrate and ethyl butyrate	(de Oliveira et al., 2019)
Candida rugosa	Exfoliated graphene oxide	Direct reaction	10 cycles	Ethyl caprylate	(Patel et al., 2015)
Candida rugosa* and *porcine pancreatic	Calcium alginate (Ca-Alg)	Entrapment	-	Isoamyl acetate, ethyl valerate and butyl acetate	(Ozyilmaz & Gezer, 2010)

2.3 Enzymatic Esterification Reaction Condition

Ethanol and acetic acid are the two substrates used for the synthesis of ethyl acetate via enzymatic esterification. The biocatalysis commonly carried out under two conditions which is solvent-free and in the presence of organic solvent. The optimization of the reaction is vital in order to establish the optimum condition to obtain a maximum yield of ethyl acetate. The parameters that affected the production of ethyl acetate is temperature, enzyme loading, acid and alkali concentration and also reaction time. Table 2.2 shows the optimization of enzymatic esterification and transesterification done by other researchers.

Table 2.2: Summary of enzymatic esterification and transesterification of immobilized enzyme

No	Enzyme used	Substrate used		Parameter manipulated	Optimum operating condition	Reaction media	Analysis	Reference
		Alcohol	Acid					
1	Immobilized *Candida rugosa* lipase	Ethanol	Valeric acid	Temperature (℃)	40	n-Hexane	FTIR	(Bayramoğlu et al., 2011)
				Reusability	5 cycles			
				Acid to alcohol molar ratio	2			
2	Immobilized *rhizomucor* lipase	Ethanol	Butyric acid	Temperature (℃)	25	n-Hexane	FTIR	(de Oliveira et al., 2019)
				Substrate concentration	0.02 mol/L			
				Alcohol to acid molar ratio	1:1			
				Protein load	1.61 mg protein/g support			
				Enzyme load	200 mg			
				Stirring speed	150 rpm			
				Time	6 hours			
				Reusability	7 cycles			
3	Immobilized *Candida rugosa* lipase	Ethanol	Caprylic acid	Enzyme load	40 mg/ml	Cyclic alkanes	FTIR	(Patel et al., 2015)
				Temperature (℃)	40			
				Substrate molar ratio	0.1:0.15			
				Time	48 h			
				Reusability	10 times			
4	Immobilized *Candida rugosa* lipase	Ethyl caprate	Butyric acid	Substrate concentration	0.5 M	n-Hexane	-	(Devi et al., 2017)
				Enzyme concentration	15 g/l			
				Temperature (℃)	50			
				Molar ratio	equimolar			
5	Immobilized *Candida rugosa* lipase	Ethanol	Valeic acid	Temperature (℃)	50	n-Hexane	-	(Ozyilmaz & Gezer, 2010)
				Enzyme concentration	3 mg/ml			
				Substrate concentration	acid = 100mM			
				Time	10 hours			

2.3.1 Effect of Reaction Time

The reaction time for the enzymatic transesterification is one of the important parameters in optimizing the synthesis of aromatic ester. In a large-scale production, the short reaction time with a high conversion is needed. These parameters can give a positive impact on the economic performance and the efficiency of the process (de Oliveira et al., 2019).

Previous studies by Ozyilmaz & Gezer (2010) have determined the reaction time for a high synthesis of aromatic esters is within 10 hours. After 10 hours of reaction time, the ester production slightly reduced. In synthesis of geranyl acetate, the increase in reaction time above 8 hours does not increase the production of the particular ester (Rosa et al., 2017). The same situation is observed for the synthesis of ethyl butyrate, which showed the conversion rate is increasing up until six hours of operation only. The conversion percentage starts declining after six hours of operation.

This situation may be due to the water produced during the catalysis that disrupt the mass transfer between the substrate and enzyme. Although the hydration layer may acts as the protective layer from denaturation by organic solvents, the large amount of water possibly will lead to hydrolysis of ester product (Ozyilmaz & Gezer, 2010).

2.3.2 Effect of Reaction Temperature

In most cases, the increase in temperature will improves the conversion rate, however the enzyme stability will drop. Temperature has a remarkably impact on the properties of the substrate, such as solubility and ionization. Temperature improves the mass transfer limitation by decreasing the viscosity of the mixture and promotes the molecular collision interface (Almeida SA et al., 2017). This condition will reduce the energy barrier substrates and the enzyme-substrate complex. However, at some extent, enzyme will lose its activity and experience thermal inactivation. Hence, the optimum temperature for the enzymes needs to be determined to avoid thermal inactivation of enzyme.

The temperature for the synthesis of ethyl acetate in gas phase is said to be at optimum value at 50℃ as the enzyme activity will drop after 60℃ (Csanádi et al., 2012). Studies from Bayramoğlu et al. (2011) and Patel et al. (2015) agrees that the optimum temperature for the immobilized *Candida rugosa* lipase to synthesize ethyl ester is 40℃. At 25℃, the conversion for the esters is too low while the temperature beyond 40℃ caused in a lower product formation. This is due to the loss of enzyme's activity at high temperature, while, at low temperature, the reaction rate is low due to the limitations in mass transfer.

However, the study by Devi et al. (2017) and Ozyilmaz & Gezer (2010), says that the optimum temperature is 50℃ for immobilized *Candida rugosa* lipase since the product yield is higher. The increased in temperature beyond 50℃ shows a rapid reduction in the percent of esterification. According to (Ozyilmaz & Gezer, 2010), optimal temperature depends on the ester systems as well as the type of enzyme used.

On the other hand, study by de Oliveira et al. (2019) adopted 25℃ as the optimum temperature for *Rhizomucor miehei* lipase. The increase in temperature up to 37℃ did not resulted in significant change to the conversion rates. The reduction in immobilized enzyme activity at high temperature may be due to the restriction on the enzyme movements caused by the covalent bonds form between enzyme and support, or low restriction for substrate diffusion.

2.3.3 Effect of Amount of Biocatalyst

The amount of enzyme added into the reaction is another factor affecting enzymatic esterification reaction. The conversion rates of esters are positively affected by the increase in the amount of catalyst within a certain limit. However, increase in the amount of enzyme will impact the economic feasibility of the entire process (Almeida SA et al., 2017). According to Patel et al. (2015), the highest conversion of the product is obtained with the amount of enzyme of 40 mg/ml. However, the conversion is declining with the further increase of the amount of

enzyme. The same situation is observed in other researches as well (de Oliveira et al., 2019; Devi et al., 2017; Ozyilmaz & Gezer, 2010).

At higher enzyme loading, the enzyme will be dispersed randomly in the reaction media and reduce the contact between the enzyme and the substrate (de Oliveira et al., 2019). The enzyme also said to be agglomerated together and insufficient protein unfolding, hence, substrate cannot access to the active sites of the enzyme (Ozyilmaz & Gezer, 2010; Patel et al., 2015).

2.3.4 Effect of Substrates Molar Ratio

The enzymatic esterification involves acid and alcohol as the substrate. High concentration of acid may act as an inhibitor and cause a decreased in enzymatic catalytic activity. High concentration of alcohol can counter the inhibitory effects. However, the conversion may reduce as the reactant becomes more polar.

Low concentration of acid and alkali are preferable in enzymatic esterification with organic solvent. de Oliveira et al. (2019) has reported that the substrate concentration of 0.02 mol/L of both substrates yield maximum esterification percent for synthesis of ethyl butyrate. The increase in substrate concentration resulted in significant decrease of product formation. The similar pattern observed in the study by Devi et al. (2017), which reported that the maximum transesterification percent of ethyl butyrate using *Candida rugosa* lipase is achieved at the substrates concentration of 0.05 M.

The increase in acid or alkali concentration will lead the reactants to be more polar, which will exhibit hydrophilic characteristics. The hydrophilic structure will interact with water layer on the enzyme surface and will lead to changes in the protein structure (Almeida SA et al., 2017). This may result in the inhibition which occurs in the reaction media (de Oliveira et al., 2019; Devi et al., 2017).

The molar ratio of both alcohol and carboxylic acids is one of the key parameters that affects the esterification percent. As the reaction is reversible, the increased in the amount of substrates may shift the reaction equilibrium forward. However, the possible inhibitory effects caused by the substrates and the product need to be determined.

Studies by de Oliveira et al. (2019) and Devi et al. (2017) reported that the acid to alcohol molar ratio of 1:1 is ideal to obtain the highest ester conversion. A higher acid concentration may result to a potent inhibition on the enzyme activity due to acid dissociation. The pH of the microaqueous environment of the enzyme maybe drops. Moreover, another potential inhibition of enzyme activities for short chain carboxylic acids such as acetic, propionic and butyric acid which can bind to the serine residue in the catalytic site of enzyme.

On the other hand, Bayramoğlu et al. (2011) and Patel et al. (2015) suggest a higher acid to alcohol ratio of 2 and 1.5, respectively. They reported that higher concentrations of alcohol will lead to inhibitory effects on the reaction which can slow the reaction down. During the first reaction step in lipase-catalysed esterification, acid molecules will preferentially bind to the enzyme. The large amount of alcohol will compete with the acid to bind onto the enzyme, which will then reduce the amount of acid on the enzyme. The reaction rates will be limited by the amount of acid in the vicinity of the enzyme (Bayramoğlu et al., 2011; de Oliveira et al., 2019). Furthermore, low molecular weight alcohol, such as ethanol and methanol, can accumulate in the microaqueous environment of the enzyme to the point that can lead to protein denaturation that blocks the nucleophilic structure of its active site. Another possible caused of enzyme denaturation is by the high polarity of the substrates that can remove the hydration layer essential for maintaining the structure of the enzyme (de Oliveira et al., 2019).

2.4 Kinetics Study of Enzyme

Kinetic modelling and study are vital in order to understand the overall process and for scaling up purposes. The lipase-catalysed reactions is usually described as ping-pong bi-bi mechanism or ordered bi-bi model (Almeida SA et al., 2017). Gas phase ethyl acetate synthesis catalysed by *Lipozyme* IM can be explain using ordered bi-bi kinetic model (Perez et al., 2007). On the other hand, the enzymatic transesterification of ethyl butyrate is described as ping-pong bi-bi mechanism and exhibits competitive inhibition by both substrates (Devi et al., 2017).

Kinetics mechanism for two substrates reaction consists of sequential mechanism and double-displacement mechanism. The sequential mechanism can be further divided into ordered and random mechanism.

2.4.1 Sequential Mechanism

In this mechanism, both substrates must bind to the enzyme before the release of any product. This binding leads to the formation of ternary complex of the enzyme and both substrates. There is a single chemical step, then follows by the formation of ternary enzyme-substrate complex (EAB). The system is in equilibrium if the chemical step is slower than the substrate binding processes (L. Stein, 2011). The type of binding of substrate defines further classification of the reaction mechanism into ordered or random mechanism.

2.4.1(a) Ordered Bi-Bi Mechanism

Ordered bi-bi mechanism is where the binding of either one of the substrates on the enzyme becomes obligatory before the binding of the other substrate. The details of mechanism are demonstrated in Figure 2.1.

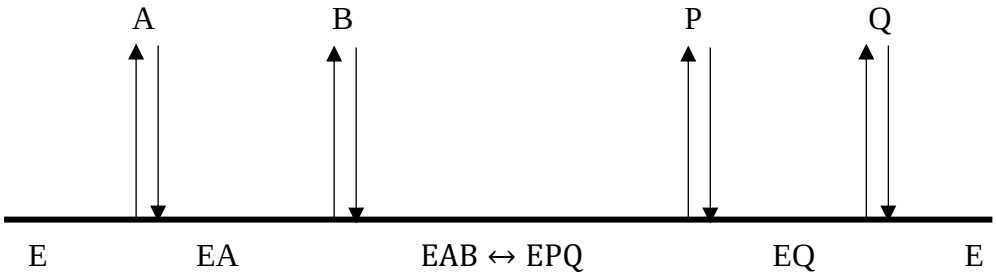

Figure 2.1: Mechanism of ordered bi-bi model

This sequential mechanism can be further classified in terms of the location of rate limiting step which is rapid equilibrium mechanism and steady state mechanism. The rate limiting step for rapid equilibrium is the chemistry of the reaction while for steady-state mechanism, step except chemistry of the reaction controls the reaction rate. In some cases, ordered mechanism exhibits exceptional case of Theorell-Chance mechanism, which demonstrated in Figure 2.2 where central complex decomposes so rapidly that its stationary concentration can be neglected and disregarded in rate equation (Bisswanger, 2017; Yadav & Magadum, 2017).

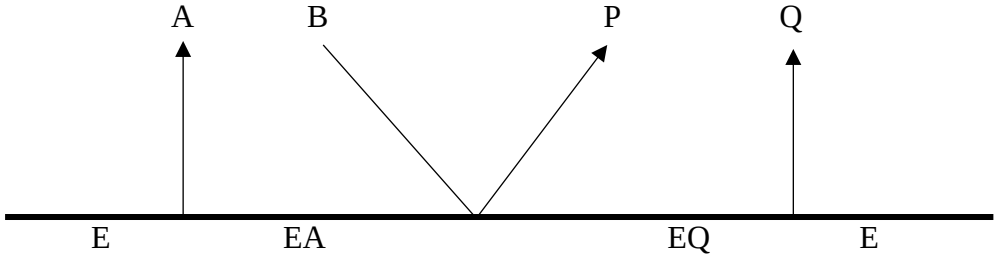

Figure 2.2: Mechanism of Theorell-Chance model

2.4.1(b) Random Bi-Bi Mechanism

Random bi-bi mechanism is where the order of the binding of the substrate and the release of the product is in random manner (Berg et al., 2002) which is demonstrated in Figure 2.3. Equation derived for the rapid equilibrium random bi-bi mechanism is similar to the ordered bi-bi mechanism, which only differs in their inhibition and Michaelis constant (Bisswanger, 2017).

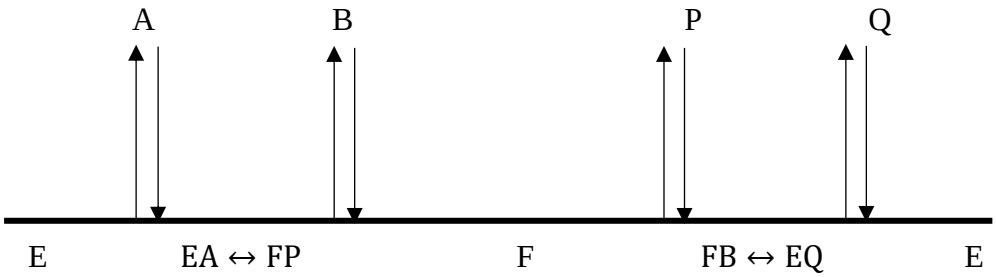

Figure 2.3: Mechanism of random bi-bi model

2.4.2 Ping-Pong Bi-Bi Mechanism

Ping-pong bi-bi mechanism or double displacement mechanism is when the reaction proceed with binding of only one of the substrates on the enzyme. The first product is released while leaving some of its portion inside the active sites upon the completion of the first catalytic cycle. The stringent feature of this mechanism is the formation of stable substituted enzyme intermediate (F), where the enzyme is temporarily modified after the release of the first product. Product Q is then form and released after the binding of the second substrate (Yadav & Magadum, 2017). The ping-pong bi-bi scheme is shown in Figure 2.4.

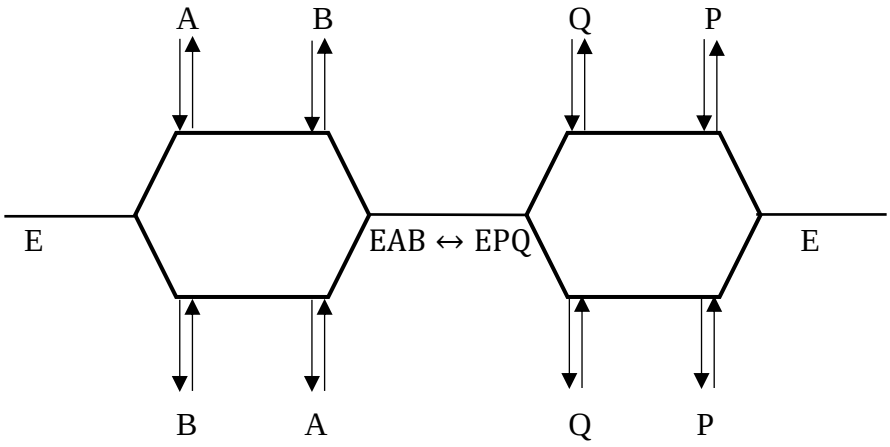

Figure 2.4: Mechanism of ping-pong bi-bi model

Plot of initial velocity and substrate concentration cannot demonstrate the changes in different type of mechanism. Hence, double reciprocal plot, or Lineweaver-Burk plot is employed to present the difference between sequential and double displacement mechanism.

The rate equations and type of double reciprocal plot for different type of mechanism are tabulated in Table 2.3.

Table 2.3: Different rate equation and double reciprocal plots for different type of mechanisms (Yadav & Magadum, 2017)

Mechanism	Rate equation	Double reciprocal plots
Rapid equilibrium random bi-bi mechanism	$v = \frac{V_{max}[A][B]}{K_{ia} + K_{m,b}[A] + K_{m,a}[B] + [A][B]}$	Intersecting lines
Steady-state ordered bi-bi mechanism	$v = \frac{V_{max}[A][B]}{K_{i,a}K_{m,b} + K_{m,b}[A] + K_{m,a}[B] + [A][B]}$	Intersecting lines
Theorell-Chance mechanism	$v = \frac{V_{max}[A][B]}{K_{ia}K_{m,b} + K_{m,b}[A] + K_{m,a}[B] + [A][B]}$	Intersecting lines
Rapid equilibrium ordered bi-bi	$v = \frac{V_{max}[A][B]}{K_{ia}K_{m,b} + K_{m,b}[A] + [A][B]}$	Intersecting lines
Ping-pong bi-bi mechanism	$v = \frac{V_{max}[A][B]}{K_{m,b}[A] + K_{m,a}[B] + [A][B]}$	Parallel lines

2.5 Inhibition of Bi-substrate Enzymatic Esterification

The inhibition of enzyme is a natural occurring phenomenon. The inhibitors are commonly classified into two groups which is reversible and irreversible. Reversible inhibitor is in the case where the inhibitors bind with the enzyme with a non-covalent bond that can be reversed anytime by dilution or dialysis. Reversible inhibition is further divided into competitive, uncompetitive and non-competitive inhibition. On the other hand, irreversible inhibitors, as the name implies, cannot be revert back as the inhibitors bind covalently or tightly that causing permanent damage to the active sites. Lineweaver-Burk graph is suitable to determine the inhibition occurs from substrate (Yadav & Magadum, 2017).

2.5.1 Ordered Bi-Bi Inhibition

As mentioned previously, the ordered bi-bi mechanism, as the name suggest, will prioritize the sequence of binding of the substrate to the enzyme. The binding of substrate (A)

will be interfered by combination of both substrates or either one. Hence, the inhibition can be eliminated or reduced by increasing the concentration of substrate, B, as it increases the competitiveness of A to bind to the enzyme (M. Yu et al., 2006). The steady-state ordered bi-bi mechanism can be described as (Jaiswal & Rathod, 2018):

$$v = \frac{V_{max}[A][B]}{[A][B]+K_{m,B}[A]+(K_{m,A}[B])\left(1+\frac{[B]}{K_{i,A}}\right)+K_{i,A}K_{m,B}\left(1+\frac{[B]}{K_{i,B}}\right)+} \quad (2.1)$$

From equation 2.1, V_{max} is the maximum rate of reaction, A and B are the substrates, K_m is the binding constant and K_i is the inhibition constant.

2.5.2 Random Bi-Bi Inhibition

The random bi-bi mechanism is similar to the equilibrium ordered bi-bi but can be distinguish as the varying any substrate concentration in random bi-bi mechanism can reduce the inhibition. In this mechanism, the inhibitor may compete with both of the substrates (M. Yu et al., 2006). The rate equation for this mechanism is as follows (Jaiswal & Rathod, 2018):

$$v = \frac{V_{max}[A][B]}{K_{i,A}K_{m,B}[B]K_{m,A}+K_{m,B}[A]+K_{m,A}[B]+[A][B]} \quad (2.2)$$

Which, V_{max} is the maximum rate of reaction, A and B are the substrates, K_m is the binding constant and K_i is the inhibition constant.

2.5.3 Ping-Pong Bi-Bi Inhibition

In this mechanism, the inhibition occurs when enzyme react with substrate (A) to form dead-end EA complex or react with other substrate (B), forming EB complex. EB complex will further converted to intermediate, enzyme-acyl (EAc). Eac complex will react with acid and produce the wanted product, P (Devi et al., 2017; Romero et al., 2007). The general rate equation for ping-pong bi-bi with inhibition can be expressed as (Zaidi et al., 2002):

$$v = \frac{V_{max}[A][B]}{K_{m,B}[A]\left(1+\frac{[A]}{K_{i,A}}\right)+K_{m,A}[B]\left(1+\frac{[B]}{K_{i,B}}\right)+[A][B]} \quad (2.3)$$

Which, V_{max} is the maximum rate of reaction, A and B are the substrates, K_m is the binding constant and K_i is the inhibition constant.

2.5.4 Reversible Inhibition

The reversible inhibition is divided into competitive, uncompetitive and non-competitive inhibition based on their binding with the enzyme, enzyme-substrate complex or both. Similar to that of one substrate enzymatic reaction, multiple substrate reaction also applies similar reversible type of inhibition to explain the inhibition occurs in the reaction.

2.5.4(a) Competitive inhibition

This type of inhibition occurs when the substrate or inhibition bind to the active sites. Both of the substrates and the inhibitor compete for the same active site resulted to the reduce in velocity of the reaction. This can also cause allosteric inhibition where after the inhibitors bind with the enzyme, no other molecules can bind to the enzyme. The Cleland notation for the competitive inhibition can be described as below.

$$\begin{array}{c} E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \xrightarrow{k2} E + P \\ + \\ I \\ \uparrow\downarrow \\ EI \end{array}$$

The Lineweaver-Burk plot of competitive inhibition is demonstrated in Figure 2.5. The value of K_m changes while the V_{max} unchanged. Hence, the amount of inhibitor is crucial in this study. In double displacement model, the high concentration of second substrate may act as the competitive inhibitor (Yadav & Magadum, 2017).

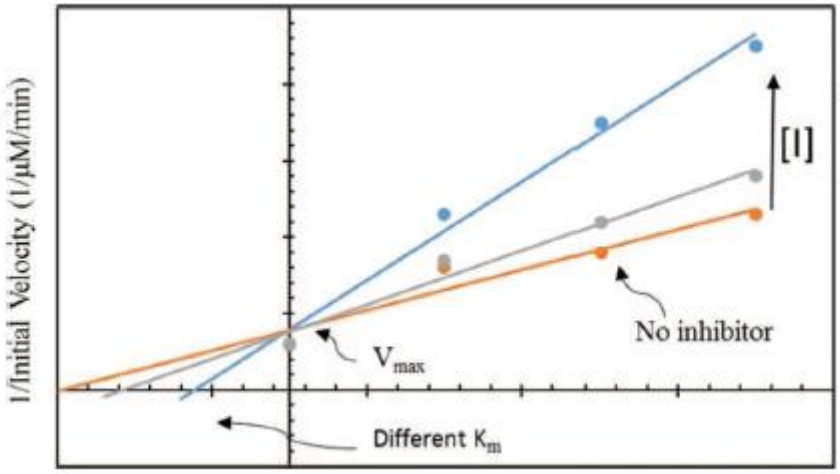

Figure 2.5: Lineweaver-Burk plot of competitive inhibition (Yadav & Magadum, 2017)

2.5.4(b) Uncompetitive Inhibition

Uncompetitive inhibition happens when inhibition binds with the enzyme-substrate complex instead of enzyme itself in the reaction. When the substrate binds with the enzyme, the binding site of the inhibitor forms. This type of inhibition is a rare case. The Cleland notation is written as:

$$E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \xrightarrow{k2} E + P$$
$$+$$
$$I$$
$$\uparrow\downarrow$$
$$EAI$$

The Lineweaver-Burk of uncompetitive inhibition is presented in Figure 2.6. The parallel line is caused by the binding with the enzyme-substrate complex where slope and intercept are difference (Yadav & Magadum, 2017).

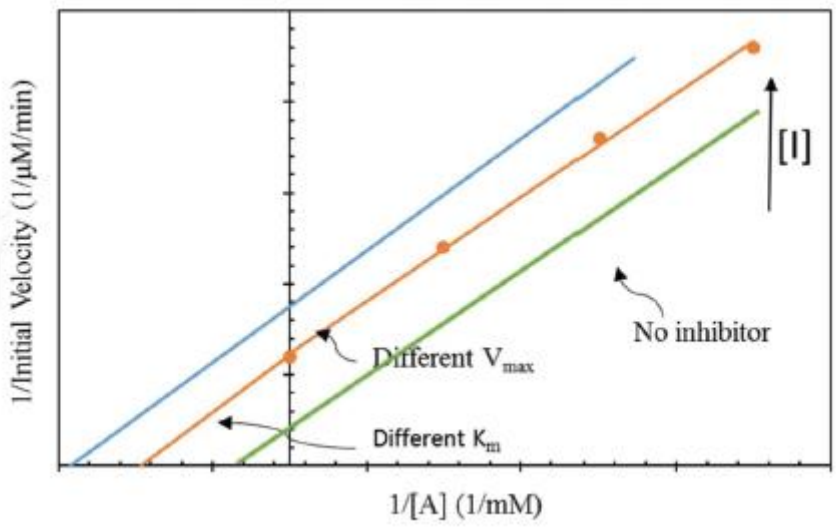

Figure 2.6: Lineweaver-Burk plot of uncompetitive inhibition (Yadav & Magadum, 2017)

2.5.4(c) Non-competitive

Non-competitive inhibition occurs when the inhibitor binds with the enzymes and the enzyme-substrate complex. The inhibitor binds to different active site than the substrate and hold no resemblance to the substrate. There is no competition between the substrate and the inhibitor as the inhibitor binds at other place than the substrate. The Cleland notation is as below:

$$E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \overset{k2}{\rightarrow} E + P + I \rightleftarrows EI + I \rightleftarrows EAI$$

The Lineweaver-Burk plot of non-competitive inhibition is presented in Figure 2.7. This type of inhibition cannot be counter by addition of more substrate.

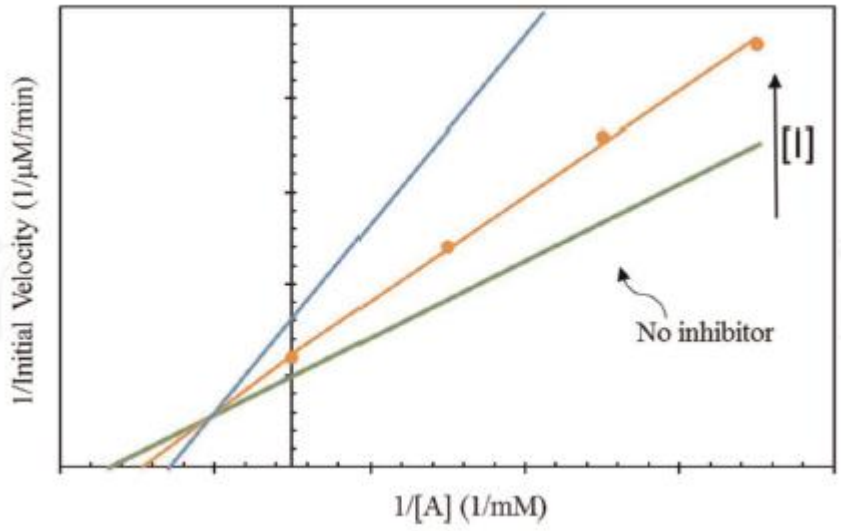

Figure 2.7: Lineweaver-Burk plot of non-competitive inhibition (Yadav & Magadum, 2017)

2.5.5 Irreversible Inhibition

This type of inhibition cannot be reverted back as the inhibitor bind so strongly that it does not dissociate with the enzyme. The enzyme activity will decline exponentially as the inhibitor binds with the enzyme. Rate of reaction reduces linearly at lower concentration. Covalent modification and tight binding are the example of irreversible inhibitions. Any physical separations are ineffective in removing the inhibitor from the enzyme.

CHAPTER 3

METHODOLOGY

In this chapter, the details on chemicals used and the experimental work of the esterification of ethanol and acetic acid to formed ethyl acetate is disclosed. The experimental work includes the enzymatic assay of lipase, optimization of ethyl acetate and kinetic study of the enzymatic esterification.

3.1 Chemicals and Equipment

Table 3.1 shows the chemicals and equipment needed for the synthesis of ethyl acetate via enzymatic esterification.

Table 3.1: Chemicals and equipment needed

Chemicals	Acetic acid (CH_3COOH), 99.0%
	Ethanol (C_2H_5OH), 99.4%
	n-Hexane (C_6H_{14}), 99.0%
	Candida rugosa lipase
	Sodium hydroxide (NaOH)
	Phenolphthalein
	Amberlite XAD7
	Gum acacia
	Olive oil
	Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.H_2O$)
	di-Sodium hydrogen orthophosphate anhydrous (Na_2HPO_4)
	Methanol (CH_3OH), 99.0%
Equipment	Incubation shaker
	pH meter
	Micropipette
	Magnetic stirrer

3.2 Experimental Procedures

The overview of the overall experimental procedures for this final year project is presented in Figure 3.1.