SYNTHESIS OF BUTYL ACETATE BY LIPASE-CATALYSED ESTERIFICATION

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LIST OF SYMBOLS

Symbol	Description	Unit
V_{max}	Maximum rate of reaction	mol/min.g
$K_{m(A)}$	Michealis Menten constant of acetic acid	mol/dm^3
$K_{m(B)}$	Michealis Menten constant of isoamyl alcohol	$mol\ dm^{-3}$
K_I	Inhibitory constant of acetic acid	$mol\ dm^{-3}$
[HA]	Concentration of weak acid	M
$[A^-]$	Concentration of the conjugate	M
[A]	Concentration of acetic acid	$mol\ dm^{-3}$
[B]	Concentration of isoamyl alcohol	$mol\ dm^{-3}$

LIST OF ABBREVIATION

CAGR	compound annual growth rate
HPMC	hydroxypropyl methylcellulose
IU	International unit
OFAT	one-factor-at-a-time
PVA	polyvinyl alcohol
SDGs	Sustainable Development Goals

SINTESIS BUTIL ASETAT MELALUI PROSES ESTERIFIKASI DIMANGKINKAN OLEH LIPASE

ABSTRAK

Ester adalah bahan aroma yang dijumpai secara semula jadi dalam tumbuh-tumbuhan dan buah-buahan. Ester merupakan bahan tambahan penting untuk makanan, minuman, industri farmasi dan kosmetik kerana memberikan rasa dan wangian. Ester dibekalkan oleh sumber semula jadi atau melalui sintesis kimia. Lipase sebagai biomangkin menunjukkan potensi tinggi dalam sintesis ester aroma kerana produk daripada tindak balas yang dimangkinkan oleh lipase disahkan sebagai produk semula jadi. Butil asetat disintesis daripada asid asetik dan esterifikasi n-butanol yang dimangkinkan oleh lipase *Candida rugosa* yang disekat gerak. Parameter untuk memaksimakan sekat gerak lipase termasuk masa tindak balas, jumlah lipase, suhu, dan nisbah molar asid asetik/etanol dikaji dengan menggunakan kaedah satu-faktor-pada-satu-masa (OFAT). Penghasilan optimum untuk butil asetat iaitu pada 81.33% diperoleh dalam masa inkubasi 40 minit dengan 50 U/ml jumlah lipase dan nisbah molar substrat 1: 1 (0.1 M) pada suhu 40 ℃ dan 150 rpm. Kajian ini juga mengira parameter kinetik menggunakan data sekunder yang diambil dari kerja kumpulan penyelidik lain. Data tersebut dimasukkan ke dalam mekanisme Random Bi Bi, Ordered Bi Bi dan Ping Pong Bi Bi untuk membandingkan parameter kinetik yang diperoleh. Analisis kinetik esterifikasi didapati mengikuti mekanisme Ping-Pong Bi Bi dengan perencatan oleh salah satu substrat, iaitu asid asetik. Parameter kinetik yang diperoleh adalah V_{max} = 249.05 mol/min.g, $K_{m(A)}$ = 46.25 mol/dm^3, $K_{m(B)}$ = 4.15 mol/dm^3, K_I = 15.09 mol/dm^3.

SYNTHESIS OF BUTYL ACETATE BY LIPASE-CATALYSED ESTERIFICATION

ABSTRACT

Ester is an aromatic compound naturally found in plants and fruits, which is an important additive for food, beverages, pharmaceutical and cosmetic industries, giving flavour and fragrance. The compound is either supplied by natural resources or chemical synthesis. Lipase as biocatalyst shows great potential in aroma ester synthesis since lipase-catalysed reaction product is verified to be natural. In this study, butyl acetate was synthesised from acetic acid and n-butanol esterification catalysed by immobilized *Candida rugosa* lipase. The process parameters which were optimized included reaction time, enzyme amount, temperature, and acetic acid/ethanol molar ratio were studied using one-factor-at-a-time (OFAT) method. Optimal molar conversion for butyl acetate of 81.33 % was obtained at 40 minutes incubation time with 50 U/ml of enzyme amount and substrate molar ratio of 1:1 (0.1 M) at 40 ℃ and 150 rpm. The kinetic parameters were also calculated using secondary data extracted from another researcher group. The data was fitted into Random Bi Bi, Ordered Bi Bi and Ping Pong Bi Bi mechanisms to compare the kinetic parameters obtained. The kinetic analysis of the esterification reactions was found to follow a Ping Pong Bi Bi mechanism with inhibition by one of the substrates, acetic acid. The fitted kinetic parameters obtained were V_{max} = 249.05 mol/min.g, $K_{m(A)}$ = 46.25 mol/dm^3, $K_{m(B)}$ = 4.15 mol/dm^3, K_I = 15.09 mol/dm^3.

CHAPTER 1
INTRODUCTION

This chapter describes the general introduction of transesterification of alcohol and ester and its current development with problem encountered. It gives an idea of the background of this research, the particular problem of interest and objectives to be achieved.

1.1 Market study of flavors and fragrances

Flavours and fragrances show wide application serving various sectors. Flavours and fragrances used in industries consist of several chemical categories such as esters, acids, alcohol, ketones, aldehydes and lactones.

The global market for flavours and fragrances was estimated at USD 13.7 Billion in the year 2020, growing at a CAGR of 3.3% to reach a revised size of USD 17.2 Billion by 2027. (*Global Food Flavors Industry*, 2020) Increasing demand of flavours and fragrances in the field of pharmaceuticals, medicines, cosmetic products, dietary supplements along with food & beverages is the main reason of growing market over the forecast period. Market size was categorized by region as shown in Figure 1.1 in which Asia Pacific is expected to grow into the largest flavours and fragrances market in global, taking over the place of North America (*Flavors & Fragrances Market Size By Ingredients, End-Use 2022*, 2018). Table 1.1 shows the compound annual growth rate (CAGR) of flavours and fragrances market at various region including Asia, America and Europe countries over a forecast period to 2027 (*Global Food Flavors Industry*, 2020).

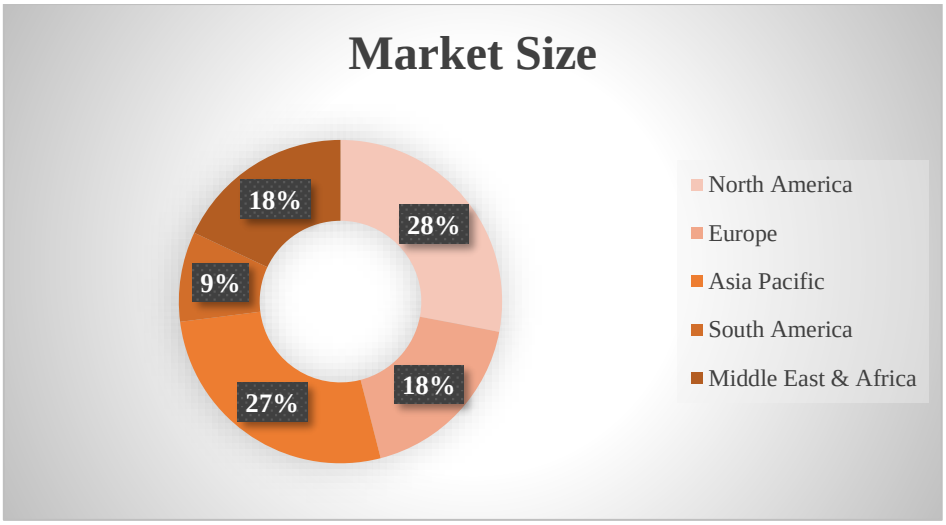


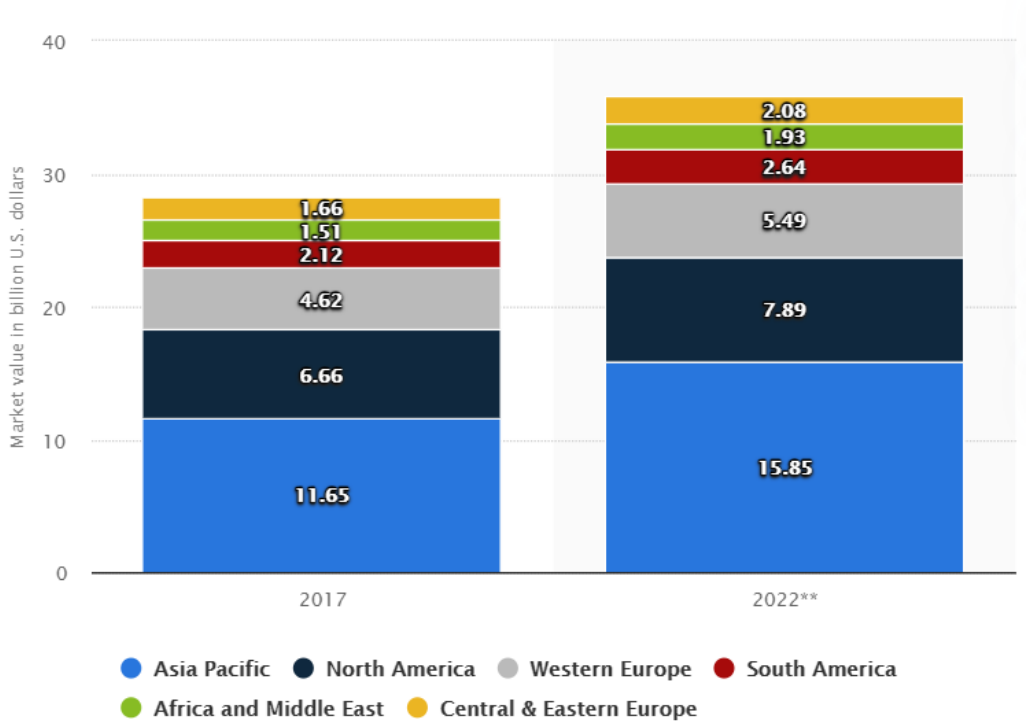
Figure 1.1 Market size of flavours and fragrances by region (*Flavors & Fragrances Market Size By Ingredients, End-Use 2022*, 2018)

Table 1.1 CAGR of flavours and fragrances market in 2020-2027 (*Global Food Flavors Industry*, 2020)

Region	CAGR (%)
Global	3.3
China	6.2
Japan	0.9
Canada	2.5
Germany	1.6

Market value of the flavours and fragrances shows a difference according to the region. Figure 1.2 shows the market value worldwide in 2017 and the estimated value in 2022 (*Flavor and fragrance market value region, 2017-2022*, 2020).

Figure 1.2 Market value of the flavours and fragrances in 2017 & 2022 (*Flavor and fragrance market value region, 2017-2022*, 2020)



The major limiting factor of the flavours & fragrances market is the strict regulations by several government institutions, such as the U.S. Food and Drug Administration (FDA) and International Fragrance Association (IFRA). Also, due to common application of essential oils or aroma in food and beverages, hygiene and personal care products, perfumes and household products, heath concern of using natural ingredients is gaining rising awareness which foster the market growth as well.

1.2 Research background

Ester is an aroma compound naturally found in plants and fruits, which gives flavour and fragrance. Owing to this property, esters are important additive for food, beverages, pharmaceutical and cosmetic industries, making their products more attractive and diversify. The compound is either supplied by natural sources or chemical synthesis. To obtain natural ester, it has to be collected and extracted from plants. However, limitations such as requirement

of specific condition for plant growth, influence of weather on yield and supply, crops diseases and difficulties in isolation drive industries to pick synthetic ester (Dubal *et al.*, 2008).

The conventional method of producing ester in chemical industries is well known as Fischer esterification (Gadewar, 2018). It is an easy and straightforward catalytic reaction between a carboxylic acid and an alcohol in the presence of strong acid to produce ester. Transesterification, another route to produce ester from an alcohol and an ester, uses acidic as well as alkali catalyst such as solid acid (Furuta, 2007), sodium hydroxide (Bonrath, Gaa and Stritt, 2013) and acidic ion-exchange resin NKC-9 (He *et al.*, 2010).

Despite of being implemented in large scale, it has several drawbacks which attention should be drawn. The most concerned problem is the use of strong acid as catalyst, which poses severe environmental issues in disposal. Due to the high acidity, special material with high resistance on corrosion is required for equipment which may lead to higher capital cost. As common chemical process does, metal catalyst also plays a role in esterification. Instead of using rare or precious metals such as yttrium and indium (Grasa, Singh and Nolan, 2004), alkali metals which are abundantly available can catalyse the synthesis of aryl ester with 94% yield using potassium carbonate (Chen *et al.*, 2018). However, involvement of metal in synthesis route is not favourable in food industries.

In order to produce ester in a greener way, researches using environmentally friendly catalyst were carried out. Heterogeneous borated zeolite catalyst was used to produce butyl acetate from bio-butanol and bio-ethyl acetate with 96% yield, making it a potential catalyst (Nandiwale, Galande and Bokade, 2015). In the meanwhile, biocatalyst was also a possible catalyst, but was yet to be the first-line option in large-scale production. Lipase as biocatalyst is used in esterification and transesterification due to its high selectivity, short reaction time, ability to produce chiral esters and being environmental benign. Therefore, researches on

optimization of lipase-catalysed transesterification parameters attracted an enormous attention in the past decade (Pedrine *et al.*, 2017).

1.3 Problem statement

Synthetic aromatic compound ester which can provide flavours and fragrance is widely used in industries, creating large market value. Industries applying conventional route, which implements sulphuric acid as catalyst, are restricted by legislation and society responsibility on environment issues. Hence, producing ester using lipase from substrates alcohol and acid, become a plausible and potential solution. General study shows that lipase is highly selective, effective and non-hazardous to be used as catalyst. The modern approach lipase-catalysed esterification in large-scale ester production shows great potential to be developed. Optimization of process parameters was done by researchers on esterification between various alcohol and acid but there are only a few reports of butyl acetate from butanol and ethyl acetate. Butyl acetate as aromatic compound gives a scent and flavour of banana which is one of the common varieties in food. Moreover, butyl acetate was recently found to be a safer biodiesel additive compared to ethyl acetate owing to its lower freezing point and higher flash point (Ali *et al.*, 2011). Therefore, this study will focus on optimizing the operation parameters of lipase-catalysed esterification of butanol and acetic acid. The effects of operation parameters and the parameter set which gives the best conversion will be determined.

1.4 Objectives

i. To produce butyl acetate from n-butanol and acetic acid mediated by lipase catalysed esterification
ii. To optimize the operating parameters on acetic acid conversion using OFAT method
iii. To determine kinetics constants from secondary data extracted from external source and validate reaction rate equation

CHAPTER 2
LITERATURE REVIEW

2.1 Catalytic esterification using lipase

Enzymes are naturally occurring biocatalyst which provide an alternative pathway in chemicals synthesis. They bind with the substrates, converting them into product and in the meanwhile play a role of catalyst to speed up the reaction. Among the various groups of enzymes, lipase, esterase and protease are capable of hydrolysing and forming ester bonds. Protease catalyses the transesterification producing carbohydrates. Lipase can hydrolyse longer chain of carboxylic ester bond in compare with esterase, which promotes the wide range of ester production using lipase (Hari Krishna and Karanth, 2002). Besides possessing no hazard impact on environment, lipase fulfils the requirements of chemical manufacturing in terms of selectivity and efficiency. Lipase is highly selective in producing the desired product without side reaction, which reduces the waste generated and downstream processing step. The reaction time required for reaction is shorter compared to that of conventional method, which is feasible for production scheme. Mild temperature and pressure offer less energy consumption in agreement with the green chemistry principals. However, the cost of lipase is always a major drawback. The extracellular lipase obtained from fermentation, extraction and purification result in higher cost which has to be covered from other aspects. The most used lipases are from *Mucor miehei, Candida rugosa, Candida antarctica and Pseudomonas cepacia*. (Gog *et al.*, 2012; Sen and Puskas, 2015)

2.2 Solvent

Water is thermally unstable for transesterification process; hence the non-aqueous medium is adapted. One of the advantages using lipase as biocatalyst is its thermal stability in

organic solvent as well as biphasic medium. Among the common solvents used are toluene, hexane, cyclohexane, heptane and so on. Although these solvents might raise an environmental issue, they favour the process in great extent. Solubility between hydrophobic acyl donor and hydrophilic short chain alcohol (methanol to propanol) can be improved. It also helps to stabilise the transition state of enzyme and reduce the binding of alcohol with enzyme to form dead-end complex which inhibit the binding of ester (Vidya and Chadha, 2010). The strength of organic solvent can be evaluated by log P value, where a higher value indicates a higher catalytic power of lipase and hence a higher conversion (Yadav and Devendran, 2012). Several researches showed that ionic liquid could be used as solvent for transesterification. Ionic solvent is undeniably more environmental benign, but lower conversion was reported (Nara, Harjani and Salunkhe, 2002) where more studies are needed and the separation from product mixture is also an issue. Going on to a greener approach in chemical synthesis, recent research started to work on a solvent-free system. This is very advantageous due to less chemical used, safer for food industries, reduce in downstream process and lower cost of production. A study showed immobilized *Candida cylindracea* lipase on HPMC/PVA films gave 99% yield which means solvent-free system might be future trend of ester synthesis route (Jawale and Bhanage, 2021).

2.3 Lipase immobilization

Full utilization of lipase to pay off the high cost can be done by immobilization technique. Free lipase will lose their activity and reuse is not possible. This can be overcome by immobilizing the lipase on carrier to enhance its reusability, stability, resistance to pH and temperature. There are five main immobilization techniques, which are adsorption, covalent binding, membrane confinement, entrapment and cross-linking. Physical adsorption and covalent binding immobilized the lipase on the carrier material by weak van der Waals force

and strong covalent bond, respectively. Membrane confinement (encapsulation) and entrapment methods trap the lipase in semipermeable membrane and polymer matrix, respectively. The substrates have to penetrate through to bind with the lipase. Cross-linking is the flocculation of lipase to create a three-dimensional structure. Leaking of lipase might be a problem in adsorption and entrapment. For covalent binding, the lipase is reversibly chemically modified and for cross-linking, the active site might be altered significantly. Mass transfer limitation in membrane confinement might be due to small pore size. (Thangaraj and Solomon, 2019)

2.4 Parameters of ester synthesis via transesterification

Several literatures which are similar to this project, regarding transesterification between alcohol and ester with parameters optimization were reviewed. Table 2.1 shows the synthesis of various flavour esters under optimum conditions with respective yield. The effect of some parameters on the reaction conversion were reviewed in section followed.

2.4.1 Enzyme loading

Enzyme loading indicates the amount of immobilized enzyme used to catalyse the reaction, in which it is well known that enzyme loading will give positive impact to reactant conversion. When the higher values of conversion and product were achieved, the cost of enzyme raises as a concern in the meanwhile. Hence, an optimum value of enzyme loading should be taken to balance both high conversion and enzyme efficiency.

A previous study has reported the reaction conversion increased when the amount of lipase increases from 30 to 60 IU in the synthesis of 1-butyl oleate from equimolar 0.1 M butanol and oleic acid (Ghamgui *et al.*, 2004). The lipase used is Crude *Rhizopus oryzae* immobilized on carbonate of calcium ($CaCO_3$). Similar trend has been observed in production of butyl acetate where maximum conversion yield is obtained at 500 IU of immobilized lipase

(Salah *et al.*, 2007). The reaction is carried out by using immobilized *Rhizopus oryzae* lipase with equimolar substrates at 0.2 M. Both studies show that upon the optimal point of enzyme amount, the differences in conversion were levelled off indicating that the optimal enzyme amount is the rate-limiting factor of conversion in given amount of substrates. Enzymatic esterification of citronellyl laurate also give an optimal value at 80 mg/mL of *Candida rugosa* lipase immobilized on Amberlite MB-1 which has an activity of 910 U/mg (Serri, Kamaruddin and Long, 2006) . Decrease in esterification yield will also occur when the lipase is added in excessive. Effect of lipase concentration is also studied by Kumar and Kanwar (2011) by using Steapsin lipase immobilized on Celite 545 to catalyse the synthesis of ethyl ferulate. It shows that excessive lipase will give negative impact on production yield. Beyond 20 mg/mL of immobilized lipase, the active sites of lipase are unexposed to the substrates. The lipase will agglomerate in solvent-free system, leading to reducing conversion. The lower optimal enzyme amount value of 20 mg/mL compared to 80 mg/mL might be due to the difference in lipase activity, where activity of immobilized Steapsin lipase (5.61 U/mg) is lower than that of immobilized *Candida rugosa* lipase (910 U/mg).

Table 2.1 Review of ester synthesis via esterification

Substrates		Product	Lipase	Operating conditions	Reaction media	Conversion (%)	Reference
Alcohol	Acid						
Methanol and ethanol	Butyric acid	Methyl butyrate and ethyl butyrate	*Candida antarctica* Lipase B (CALB) immobilized on iron magnetic nanoparticles	Alcohol/Acid ratio: 1 T = 25 °C Stirring = 150 rpm Time = 8 h 10 mg of immobilised lipase	heptane	> 90	(Souza *et al.*, 2017)
Isoamyl alcohol	Acetic acid	Isoamyl acetate	*Bacillus aerius* lipase immobilized on silica gel matrix	Alcohol/Acid ratio: 1 T = 55 °C Time = 10 h 1% of immobilized lipase by acid weight	Solvent-free	6800%	(Narwal *et al.*, 2016)
Butanol	Oleic acid	1-butyl oleate	*Rhizopus oryzae* lipase	Alcohol/Acid ratio: 1 T = 37 °C Stirring = 200 rpm Time = 120 h 60 IU of immobilised lipase	Solvent-free	73	(Ghamgui *et al.*, 2004)
Butanol	Acetic acid	Butyl acetate	*Rhizopus oryzae* lipase immobilized on Celite 545	Alcohol/Acid ratio: 1 T = 37 °C Stirring = 200 rpm Time = 24 h 500 IU of immobilised lipase	Solvent-free	60	(Salah *et al.*, 2007)

Isobutyl alcohol	Propionic acid	Isobutyl propionate	*Candida antarctica* Lipase B (CALB) immobilized on macroporous polyacrylic resin beads	Alcohol/Acid ratio: 3 T = 40 °C Stirring = 300 rpm Time = 10 h 5 % (w/w) of immobilised lipase	Solvent-free	92.52	(Kuperkar *et al.*, 2014)
Citronellol	Lauric acid.	Citronellyl laurate	*Candida rugosa* lipase immobilized on Amberlite MB-1	Alcohol/Acid ratio: 1.5 T = 37 °C Stirring = 200 rpm Time = 24 h 60 mg/mL of immobilised lipase	Iso-octane	89	(Serri, Kamaruddin and Long, 2006)
Ethanol	Ferulic acid	Ethyl ferulate	Steapsin lipase immobilized on Celite 545	Alcohol/Acid ratio: 1 T = 45 °C Stirring = 120 rpm Time = 6 h 10 mg/mL of immobilised lipase	Dimethylsulfoxide (DMSO)	68	(Kumar and Kanwar, 2011)
Isoamyl alcohol	Acetic acid	Asoamyl acetate	Novozym 435 from *Candida antarctica* supported on acrylic resin	Alcohol/Acid ratio: 1 T = 40 °C Stirring = 200 rpm Time = 2 h 6.3 g of immobilized lipase/mol alcohol	n-hexane	20	(Romero *et al.*, 2005)

2.4.2 Temperature

Temperature plays a significant role in enzymatic esterification. Suitable temperature has to be considered due to volatility of compounds such as alcohol, preventing evaporation which will reduce the participation of substrate in reaction. High temperature can increase the reaction conversion by reducing the mixture viscosity, increasing mixture solubility and enhancing molecular collision between enzymes and substrates (Yadav and Devendran, 2012). However, taking into account the thermal stability of biocatalyst used, high temperature could lead to denaturation of enzymes and hence drastic drop of conversion might occur.

According to the literature, ethyl butyrate synthesis from esterification of ethanol and butyric acid is temperature dependent (Souza *et al.*, 2017). The conversion is stable from 20 up to 40 °C, but drops drastically at 45 °C due to volatility of methanol which has a boiling point at 65 °C. It reduced the molar concentration of methanol in reaction media, leading to reduction in conversion. Esterification of butanol and acetic acid to produce butyl acetate was carried out using immobilized *Rhizopus oryzae* lipase immobilized on Celite 545 in solvent-free system (Salah *et al.*, 2007). By increasing the temperature from 30 up to 37 °C, the conversion is tripled. The maximum conversion yield (60 %) was observed at 37 °C and further increase in temperature resulted in lower conversion because the lipase might be thermal inactivated at temperature higher than 37 °C. Other authors studied the synthesis of banana flavour ester which is isoamyl acetate. The reaction is catalysed by Novozym 435 lipase from *Candida antarctica* immobilized on acrylic resin in n-hexane solvent system. The study on effect of temperature showed increment of initial reaction rate along with temperature within the range of 30 to 65 °C which is below the boiling point of solvent. Reduction in conversion due to thermal inactivation is prevented since Novozym 435 lipase can tolerate and work at temperature as high as 100 °C (Romero *et al.*, 2005).

2.4.3 Molar ratio of reactants

Stoichiometric molar ratio suggests equimolar ratio of acyl donor and alcohol in producing ester, but higher ratio is practiced pushing the equilibrium to the side of desired product. In the meanwhile, the optimum ratio should be investigated considering both criteria, which are inhibitory effect of substrates on enzymes and utilization of acetylating agent per unit of conversion. When increasing the ratio, either acyl donor or nucleophile concentration is being chosen as excess reagent depending on possible inhibition effects of substrate on enzymes.

Earlier study done by (Narwal *et al.*, 2016) have found that acetic acid and isoamyl alcohol at ratio of 1:1 at 1 M was optimal and achieved highest yield at 68.38% to produce isoamyl acetate. The reaction mixture was incubated at 55 °C for 10 hours with 1% immobilized lipase from *Bacillus aerius* on silica gel matrix by acid weight. The higher molar ratio of nucleophile (alcohol) decreased the yield when the ratio increased from 1:1 to 1:4.

Another study in synthesis of 1-butyl oleate is carried out where the esterification was catalysed by immobilized *Rhizopus oryzae* lipase in a solvent-free medium with 1 g of acid and alcohol taken at various molar ratio of oleic acid: butanol (Ghamgui *et al.*, 2004). Similarly, the excess molar quantities of alcohol caused a reduction in reaction conversion when the ratio increased from 1:1 to 1:3. When the amount of acid is increased by 3 and 5 times, the conversion decreased indicating that 1:1 molar ratio of oleic acid: butanol is optimal for the esterification. Noticeably, the conversion percentage is relatively higher than the one observed when butanol is the major component in reaction medium, which means that butanol exists as more significant inhibitor to the lipase as compared to oleic acid.

Immobilized *Rhizopus oryzae* lipase has been used to catalyse an esterification but instead of oleic acid and butanol, 2 g of acetic acid and butanol were used (Salah *et al.*, 2007) . The results obtained also showed that ester yield was maximal at 1:1 molar ratio of acid:

alcohol which is in line with previous findings (Ghamgui *et al.*, 2004). Both showed that increasing molar ratio of butanol reduced the conversion due to inhibition. However, opposite result was shown when conversion dropped greater in higher acetic acid concentration than in higher butanol concentration. This indicated that acetic acid expresses a higher inhibitory effect on *Rhizopus oryzae* lipase as compared to oleic acid.

In other studies, such as synthesis of isobutyl propionate and citronellyl laurate, a distinct trend was observed where increasing alcohol concentration can improve the conversion percentage. Isobutyl propionate is produced by esterification of 0.1 M propionic acid with various concentration of isobutyl alcohol catalysed by immobilized lipase Novozyme 435. The conversion increased with acid: alcohol ratio from 1:1 up to 1:3 of but marginal increase was obtained in further increase to 1:4. Citronellyl laurate is produced by esterification of 0.02 M lauric acid with various concentration of citronellol catalysed by *Candida rugosa* lipase immobilized on Amberlite MB-1. Among the various ratio of 1:1, 1:1.5, 1:2, 1:2.5 and 1:3, 1:1.5 showed the highest conversion at 89.7%. It decreased slightly to 80% at higher ratio while ratio of 1:1 gave the lowest conversion at 40% which means increasing the alcohol concentration by 50% can double the acid conversion. It could be explained when alcohol is not inhibitor to lipase, higher nucleophile concentration will push the equilibrium of reaction in a forward direction.

2.5 Kinetics and mechanisms of catalysed reaction

The rate of reaction plotted against substrate concentration falls into two categories, i.e., early and late regimes. At low substrate concentration, the initial rate is substrate dependent (early regime) in which the rate increases with substrate addition. At high substrate concentration, the initial rate is substrate independent (late regime) in which addition of

substrate does not increase the reaction rate, reaching a maximum rate. It is referred as saturation point of enzyme.

The kinetic mechanisms are analysed based on velocity-substrate curve. The curve is linearized by plotting the curve using reciprocal values of velocity and substrate. This double reciprocal curve is also known as Lineweaver-Burke plot (Lineweaver and Burk, 1934). This curve has been used to differentiate between different types of inhibition: competitive, uncompetitive, and non-competitive (Waldrop, 2020). For competitive inhibition, increase in amounts of inhibitor will show a steeper slope, while the x-intercept decreases in the meanwhile. The y-intercept remained constant, indicating that the maximum rate is not affected by the inhibitor amount. For uncompetitive inhibition, the slope will not change but y-intercept and y-intercept decreases when amounts of inhibitor increases. The plot shows parallel lines without any interception between them. Lineweaver-Burke plot of non-competitive inhibition is similar to competitive inhibition, but the interception point does not fall in y axis.

Besides the double reciprocal plot, a few other methods are presented. The direct linear plot can estimate kinetic constants with elimination of bias and the constants can be read from the plot directly without calculations (Eisenthal and Cornish Bowden, 1974). A method of combination plot is introduced, presenting the kinetics data as a single linear plot (W-c Chan, 1995). Dissociation constant and nature of inhibition are revealed directly from the slopes and intercepts.

Enzyme-catalysed reactions follow a sequence of steps, especially for the complex reactions or reactions involving multiple substrates (Ulusu, 2015). Kinetic mechanism focuses on the order of substrates bind with the enzyme and the transformation of enzymes and substrates can be either ordered or random. Focusing on bisubstrate reactions, the mechanism can be either sequential or non-sequential. In sequential mechanism, substrate and enzyme form an enzyme–substrate complex as the first step when a reaction takes place, followed by

the formation and release of products. Sequential mechanism can be extended further to separate into random and ordered mechanisms. In random mechanisms, the order of substrate to bind with enzyme does not in specific order. Substrate that bind to the enzyme, and product that produced first are arbitrary. In contrast, the order of substrates binding, and product release are specific in ordered mechanisms.

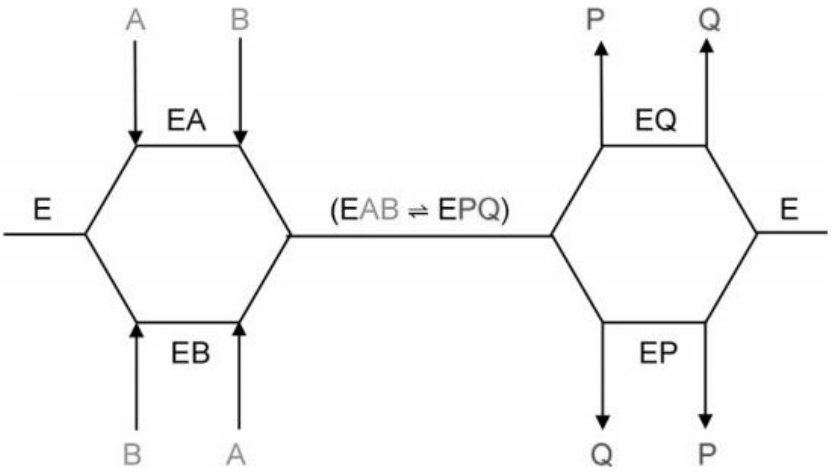


Figure 2.1 Schematic representation of the Random Bi-Bi mechanism. E represents enzyme, A and B represents substrates and P and Q represents products.

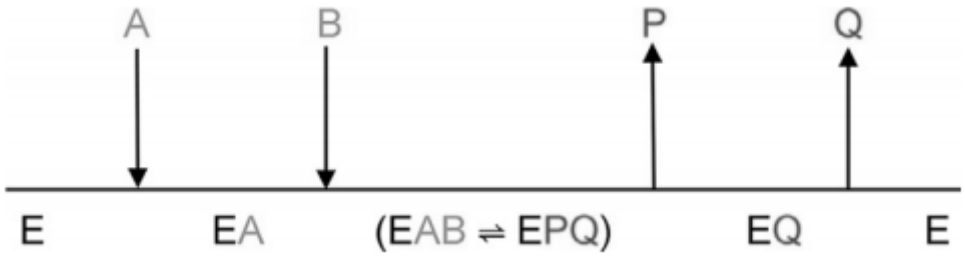


Figure 2.2 Schematic representation of the Ordered Bi-Bi mechanism. E, A, B, P and Q represents enzyme, first substrate, first product, second substrate and second product respectively.

Non-sequential mechanism is so called as the Ping Pong Bi Bi mechanism. The characteristic of this mechanism is shown in the transition of enzyme into an intermediate form. The product of one or more will be released between the binding of two substrates. This

mechanism is also known as double placement reaction and always involved in group transfer reaction. The scheme of Ping-Pong Bi-Bi mechanism is shown in Figure 2.3 (Roskoski, 2015).

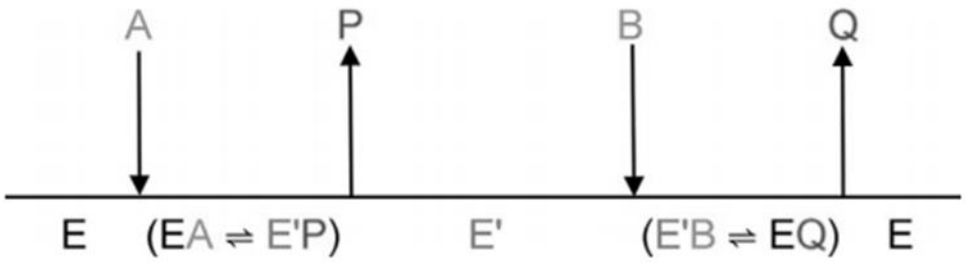


Figure 2.3 Schematic representation of the Ping-Pong Bi-Bi mechanism. E, A, P, B and Q represents enzyme, first substrate, first product, second substrate and second product respectively.

One of the most crucial aspects when looking into reaction kinetics is the effect of presence of inhibitors on enzymes. Inhibitors are substances that can suppress the catalytic reaction. Reaction kinetics and mechanism are greatly affected by presence of inhibitors, which will reduce the reaction rate. Products of reaction which is enzyme-catalysed, typically water, usually will inhibit the reaction when the product concentration starts to increase in the reaction media. Synthesis of isoamyl acetate from esterification of acetic anhydride and isoamyl alcohol catalysed by Novozym 435 was not inhibited by the alcohol and produced acetate. However, the studies showed that inhibition was exerted by the acetic anhydride (Romero *et al.*, 2005, 2007). For reaction between different combinations of substrate, the inhibition effect is distinct. Synthesis of isobutyl propionate from propionic acid and isobutyl alcohol catalysed by immobilized lipase Novozym 435 fitted Ping-Pong Bi–Bi mechanism in which both acid and alcohol substrates show inhibitory effect (Kuperkar *et al.*, 2014).

CHAPTER 3
METHODOLOGY

This chapter shows the materials, equipment with instruments and research methodology.

3.1 Materials

Free lipase from *C.rugosa* was purchased from Sigma-Aldrich with 1176 U/mg. Celite 545 (Fluka), acetic acid (R&M Chemicals) and sodium hydroxide (Merck KGaA) were purchased. Hexane, n-butanol, disodium hydrogen orthophosphate (Na2HPO4) and sodium dihydrogen orthophosphate dihydrate (NaH2PO4.2H2O) and phenolphthalein were purchased from Fisher Scientific. Olive oil used was the brand of Naturel (extra virgin). All chemical reagents were of analytical grade.

3.2 Equipment and instruments

Table 3.1 List of equipment and instruments used

Equipment / Instrument	Brand / Model	Manufacturer
Benchtop pH Meter	Delta 320	Mettler Toledo
Micropipette	Fisherbrand 0.5-5mL	Thermo Fisher Scientific
	Joanlab 0-1000μL	Joanlab
0.22 μm Nylon syringe filter	LabServ	Thermo Fisher Scientific
Incubation shaker	Incu-Shaker 10 L	Benchmark Scientific

3.3 Experimental Procedures

The overall procedures were illustrated in flow chart below.

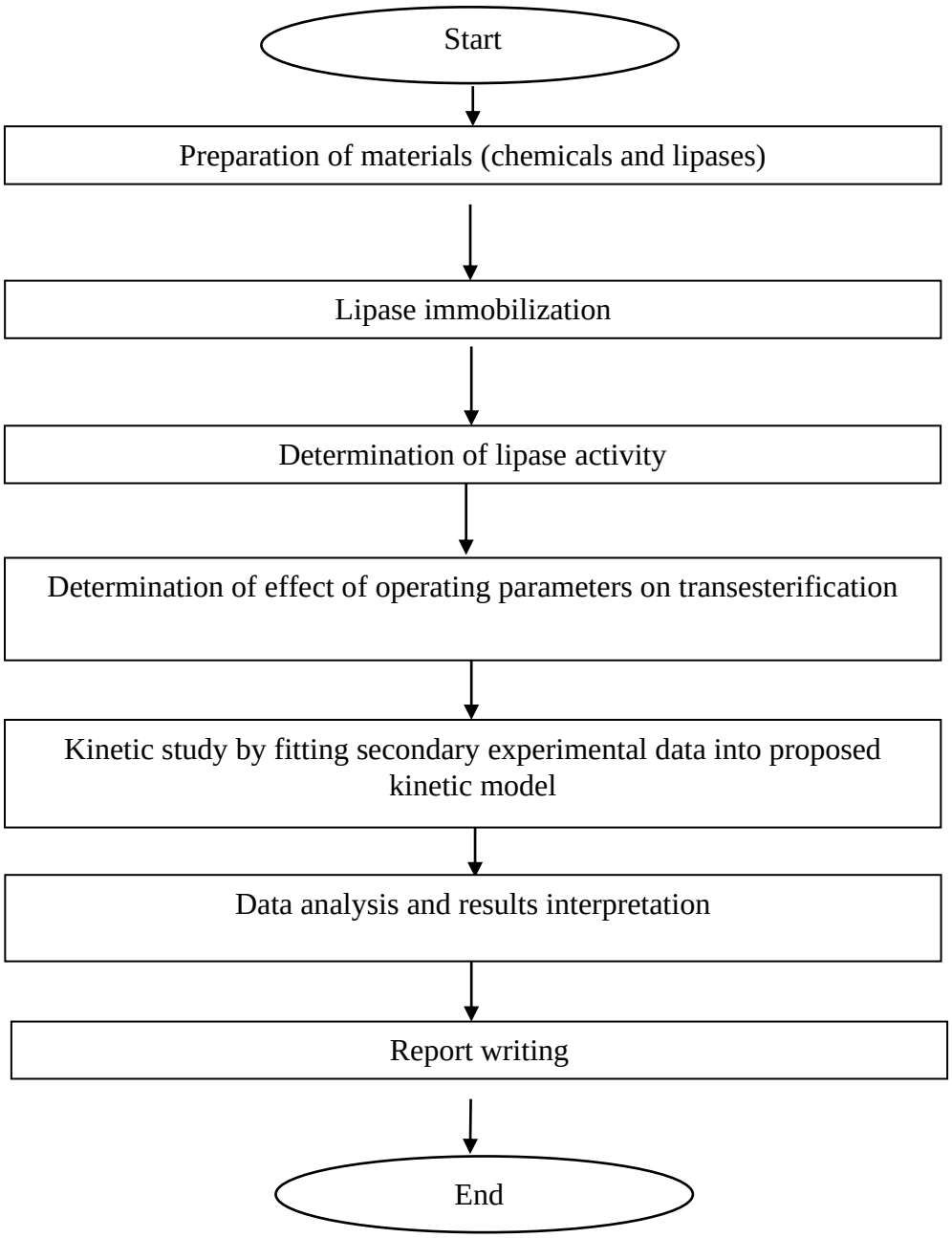


Figure 3.1 Flow chart of project methodology

3.3.1 Buffer solution at pH 7.0

Buffer solution used was prepared using disodium hydrogen orthophosphate (Na_2HPO_4) and sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$). The buffering capacity is determined using the Henderson-Hasselbalch Equation. Each component was transferred to volumetric flask and bring the solution to volume with distilled water.

$$pH = pKa + \log([A^-] / [HA])$$

where [HA] = concentration of weak acid and [A-] = concentration of the conjugate base.

3.3.2 Lipase immobilization

Free *C.rugosa* lipase (0.5 g) was dissolved in 3 ml of buffer solution (pH 7.0) and mixed with 1 g of Celite 545. Mixture was added with 15 ml of cold acetone (cooled in fridge) and stirred for 60 min at 20 °C. The immobilized lipase was filtered and washed twice with acetone to remove the unadsorbed lipase. The immobilized lipase was dried at room temperature and stored at 4 °C until use. The optimal immobilization conditions were referred to Chang *et al.* (2007) .

3.3.3 Lipase assay

The lipase activity was determined by titration method using 0.05 M sodium hydroxide based on monitoring the synthesised fatty acids. The reaction medium composed of 2.5 ml of olive oil, 2.5 ml of 7 % (w/v) Arabic gum solution and 2 ml of pH 7.0 buffer solution. The mixture was incubated at 35 °C. The reaction was started by adding 5 mg of immobilized enzyme into the mixture. A blank solution was prepared without immobilized lipase. After 30 min, the reaction was quenched with 10 ml of acetone-ethanol mixture. Enzyme activity was determined titrimetrically in triplicates. One unit of lipase activity is defined as the amount of

enzyme required to release 1 nmol (10^{-9} mol) of fatty acids per minute at pH 7.0 and 35 °C using olive oil as substrate.

3.3.4 Butyl acetate synthesis

Butyl acetate synthesis was carried out by filling a total volume of 5 mL with 0.1 M of acetic acid, 0.1 M of n-butanol and solvent hexane. The container was placed in incubation shaker. The mixtures of acetic acid, n-butanol, and immobilized lipase (30 mg) were stirred in an incubator shaker at various reaction temperatures. A sample was run without the enzyme under the same conditions to act as control. The sample taken should be covered appropriately to prevent vaporization. For all assays, three replicates were done, and average values were taken.

3.3.5 Optimization of butyl acetate synthesis by OFAT approach

The influential parameters were subjected to one-factor-at-a-time (OFAT) method to identify the influence of each factor and optimize the conversion yield of butyl acetate. This approach altered a single factor while other were held constant, and the optimal value of altered factor was fixed in subsequent experiments. OFAT method focuses on impact of single factor without considering the effect of interaction between factors on the ester yield. It was to determine the optimum range of the esterification variables, including the reaction time, enzyme loading, temperature and substrate molar ratio. The effect of reaction time on acetic acid conversion was studied by titrating the samples every 20 min up to 140 min. The enzyme loading was varied from 0 and 90 mg at 10 mg interval and the impact of reaction temperature ranging between 25 ^{o}C and 45 ^{o}C was evaluated. The effect of substrate molar ratio was determined by varying acetic acid concentration from 0.1 M to 0.5 M while n-butanol remained at 0.1 M and vice versa. For all experiment runs, the conversion of acetic acid was measured by mean of 0.05 M sodium hydroxide titration.

3.3.6 Conversion

The conversion of substrates into butyl acetate was calculated using equation 3.1:

$$X(\%) = \frac{C_A - C_{A0}}{C_A}.100 \quad \text{… Eq. (3.1)}$$

where X is the conversion of acetic acid, C_{A0} is initial concentration of acetic acid and C_A is concentration of acetic acid after reaction.

3.3.7 Reaction kinetic modelling

The kinetic study on lipase catalysed esterification of acetic acid by butanol was not conducted due to time constraint. Alternatively, reaction kinetic on esterification between acetic acid and alcohol was studied from research work "Kinetics and mechanism of esterification of isoamyl alcohol with acetic acid by immobilized lipase" (Gogoi and Dutta, 2009). Secondary data of initial reaction rate with various substrate concentration were extracted using online tool "PlotDigitizer". Lineweaver-Burk double reciprocal graph was plotted using the extracted data. The secondary data was fitted into the kinetic mechanism equations to calculate the kinetic parameters by non-regression analysis using Polymath software.

The initial reaction rate equation for Ping-Pong Bi Bi mechanism with acid inhibition can be expressed as:

$$v = \frac{V_{max}[A][B]}{[A][B] + K_{m(A)}[B] + K_{m(B)}[A]\left(1 + \frac{[A]}{K_{I(A)}}\right)} \quad \text{… Eq. (3.2)}$$

where v is initial reaction rate (mol/min.g), V_{max} is maximum reaction rate (mol/min.g), *[A]* is concentration of acetic acid (mol dm^{-3}), *[B]* is concentration of isoamyl alcohol (mol dm^{-3}), $K_{m(A)}$ is Michealis Menten constant of acetic acid (mol dm^{-3}), $K_{m(B)}$ is Michealis Menten constant of isoamyl alcohol (mol dm^{-3}), K_I is inhibitory constant of acetic acid (mol dm^{-3}).

The initial reaction rate equation for Random Bi Bi mechanism with acid inhibition can be expressed as:

$$v = \frac{V_{max}[A][B]}{[A][B] + K_{m(B)}[A] + K_{m(A)}[B]\, K_I K_{m(B)}} \quad \text{… Eq. (3.3)}$$

The initial reaction rate equation for Ordered Bi Bi mechanism with acid inhibition can be expressed as:

$$v = \frac{V_{max}[A][B]}{[A][B] + K_{m(B)}[A] + K_{m(A)}[B]\left(1+\frac{[B]}{K_{I(A)}}\right) + K_{m(A)}K_{m(B)}\left(1+\frac{[B]}{K_{I(B)}}\right)} \quad \text{… Eq. (3.4)}$$

CHAPTER 4
RESULT AND DISCUSSION

4.1 Lipase activity

Method of lipase immobilization on solid support includes adsorption, covalent binding, membrane confinement etc. Simple adsorption was applied in this study due to insoluble property of lipase in organic media. Celite 545, which has high porosity and high specific surface area, is suitable to be used as catalyst support for adsorption (Datta, Christena and Rajaram, 2012). Sagiroglu and Telefoncu (2004) reported that immobilization of lipase on Celite 545 has higher operational stabilities compared to that of Amberlite IRA-938. The optimum immobilization parameters were referred and modified from the work of Chang *et al.* (2007). Immobilization of lipase *Candida rugosa* was done at temperature 20 °C, agitation speed 150 rpm, enzyme/support ratio 1:2 for 90 min. Hydrolytic lipase activity was measured in triplicate and the results were shown in Table 4.1. Average hydrolytic activity of immobilized lipase was 0.375 U/mg under the assay conditions of 100 mg of immobilized lipase in 100 mM pH 7.0 sodium buffer, at 35 °C within 30 min reaction time. The value, however, was much lower than the value (15.8 U/mg) reported by Chang *et al.* (2007). It could be explained by measuring hydrolytic lipase activity of free lipase. The result showed that the free lipase had an activity at 0.43 U/mg, much lower than the value labelled at the bottle. Therefore, a slight decrease of lipase activity from 0.43 to 0.375 due to immobilization was reasonable.

Table 4.1 Hydrolytic activity of immobilized lipase

Sample	Hydrolytic activity (U/mg)
1	0.355
2	0.398
3	0.372
Average	0.375