KINETIC MODELLING OF LIPASE-CATALYSED FLAVOUR ESTERS SYNTHESIS FOCUSING ON FACTOR AFFECTING THE REACTION CURVES

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by

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LIST OF SYMBOLS

C_s	Substrate concentration	mol/L
C_{so}	Initial substrate concentration	mol/L
D	Dilution rate	min^{-1}
F_j	Volumetric flow rate	L/min
F_{jo}	Initial volumetric flow rate	L/min
$\frac{dN}{dt}$	Rate of accumulation	mol/min
K_{MA}	Michaelis-Menten constant for substrate A	mol/L
K_{MB}	Michaelis-Menten constant for substrate B	mol/L
K_{MAEs}	Michaelis-Menten constant of acid in esterification reaction	mol/L
K_{MBEs}	Michaelis-Menten constant of alcohol in esterification reaction	mol/L
K_{MATEs}	Michaelis-Menten constant of ester in transesterification reaction	mol/L
K_{MBTEs}	Michaelis-Menten constant of alcohol in transesterification reaction	mol/L
K_{IA}	Inhibition constant of substrate A	mol/L
K_{IB}	Inhibition constant of substrate B	mol/L
K_{IAEs}	Inhibition constant of acid in esterification reaction	mol/L
K_{IBEs}	Inhibition constant of alcohol in esterification reaction	mol/L
K_{IATEs}	Inhibition constant of ester in transesterification reaction	mol/L
K_{IBTEs}	Inhibition constant of alcohol in transesterification reaction	mol/L
V	Rate of reaction	mol/L.min
V_{max}	Maximum reaction rate	mol/L.min

LIST OF ABBREVIATIONS

A	Substrate A
B	Substrate B
CAL-B	Candida Antartica Lipase B
CRL	Candida Rugosa Lipase
CSTR	Continuous Stirrer Tank Reactor
FAME	Fatty acid methyl ester
FAEE	Fatty acid ethyl ester
ODE	Ordinary Differential Equation
P	First product from the reaction
Q	Second product from the reaction
$-r_A$	Rate of disappearance of substrate A
r_p	Rate of formation of product, P
$ScCO_2$	Supercritical carbon dioxide

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PEMODELAN KINETIK REAKSI ESTERS PERISA PEMANGKIN LIPASE MEMBERI TUMPUAN KEPADA FAKTOR YANG MEMPENGARUHI LENGKUNG TINDAK BALAS

ABSTRAK

Esters perisa adalah kompaun bernilai tinggi kerana permintaan yang lebih tinggi dalam pelbagai industri. Ia boleh dihasilkan melalui esterifikasi dan transesterifikasi. Kinetik lipase-pemangkin sintesis esters perisa telah disiasat.Objektif kerja ini adalah untuk mengkaji kesan kepekatan substrat atas kadar tindak balas awal dan menentukan konfigurasi reaktor terbaik untuk kedua-dua model Ping-Pong Bi-Bi dan Ordered Bi-Bi. Kepekatan substrat adalah salah satu faktor utama dalam tindak balas enzim. Kepekatan optimum setiap substrat perlu dinilai untuk meningkatkan kadar tindak balas esters perisa. Selain itu, pemilihan penderma asl terbaik untuk pengeluaran asetat isoamil dikaji berdasarkan kadar permulaan yang diperolehi; anhidrid asetik sesuai untuk pembentukan ester ini. Selain itu, asid yang berbeza dengan alkohol yang serupa dan sebaliknya juga dikaji untuk menentukan substrat dengan kesan perencatan dan substrat yang disukai untuk meningkatkan kadar tindak balas.Tambahan pula, data kinetik secara konklusif menunjukkan bahawa CAL-B adalah lebih cekap daripada CRL untuk sintesis butil butirat menggunakan asid butirik dan butanol.Konfigurasi reaktor juga mengkaji untuk model bi-substrat dalam sintesis esters perisa pemangkin lipase, menyebabkan reaktor kelompok sesuai untuk Ordered Bi-Bi manakala CSTR untuk model Ping-Pong Bi-Bi.

KINETIC MODELLING OF LIPASE-CATALYZED FLAVOUR ESTERS SYNTHESIS REACTION FOCUSING ON FACTOR AFFECTING THE REACTION CURVES

ABSTRACT

Flavour esters are high value compound due to higher demand in various industries. They can be produced via esterification and transesterification. The kinetics of lipase-catalyzed of flavour esters synthesis were investigated. The objectives of this work were to study the effect of substrate concentration on initial reaction rate and determine best reactor configuration for both Ping-Pong Bi-Bi and Ordered Bi-Bi models. Substrate concentration is one of the main factors in enzymatic reaction. Optimum concentration of each substrate needs to be evaluated in order to increase the reaction rate of flavour esters. Besides, selection of best acyl donor for isoamyl acetate production was studied based on the obtained initial rate; acetic anhydride was suitable for this ester formation. Apart from this, different acids with similar alcohol and vice versa also investigated in order to determine the substrate with inhibition effect and substrate which favored to increase the reaction rate. Furthermore, the kinetic data conclusively showed that CAL-B was catalytically more efficient than CRL for synthesis of butyl butyrate using butyric acid and butanol. Reactor configuration also studied for the bi-substrate models in lipase-catalyzed flavour esters synthesis, resulting batch reactor was suitable for Ordered Bi-Bi model whereas CSTR for Ping-Pong Bi-Bi model.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Esters are natural chemical compounds with the functional group carboxyl. Alcohol and carboxylic acid are widely used to make esters, with water being removed as a by-product. However, ketone, acid chlorides, acid anhydrides, alcohol, and ester exchange can all be used to prepare esters instead of carboxylic acid, which serves as the acyl donor for ester formation. Esters are widely employed in the chemical industry for a variety of applications. For example, isoamyl acetate, furfuryl acetate, hexyl acetate, isoamyl butyrate, and butyl butyrate are utilized as flavouring agents, ascorbyl ester is utilized as an antioxidant and surfactant (Karmee, 2011) and isopropyl and methyl acetate are utilized as scent additives.

The demand for natural products in various applications have paved a way towards to increasing production of ester, either from esterification or transesterification reaction. Therefore, the production of ester becomes a concern and have keen widely explored for the past few decades with the aim to meet the global demand. Before the development of technologies for ester synthesis, flavour esters such as isoamyl acetate and isoamyl butyrate can be obtained naturally from plants and flowers, because they contain abundant of esters. Thus, extraction method was used to obtain the ester from the natural resources. However, there are a few limitations from using the process ,such as the expensive method and only small quantity of ester can be extracted, which is not feasible to meet the world demand as well as higher cost for commercial exploitation (Krishna *et al.*, 2001).

Researchers developed the ester production through chemical synthesis since there are limitations of extracting esters from the natural compounds. The chemical method can be used to extract almost all types of esters. Esters can be produced through esterification and transesterification reactions, as previously stated. Various esters can be obtained via esterification using different acids and alcohols and biodiesel production is the common production from transesterification of oil or fat with alcohol. There are also other esters obtained via transesterification such as furfuryl acetate (Mathpati *et al*., 2016), butyl butyrate (Madras *et al.*, 2008) and butyl-4-methyl-3-oxopentanoate (Yadav and Shinde, 2012). In terms of ester yield, chemical synthesis can achieve higher yield compared to the traditional method. However, it has major disadvantage, where the product formed is restricted to labelled as green product (natural). Especially, in food industry, ester production through chemical synthesis is not advisable and need an additional method to separate the chemical catalyst from the product which increase the operating cost. Therefore, there is a green alternative method to overcome this problem, which enzyme is commonly used as the catalyst that has the similar function as chemical catalyst. Lipase as the biocatalyst is opted with many advantages such as allowing for better reaction control, has mild condition, able to produce high quality of ester as well as environmentally compatible This research focused on the kinetics of lipase-catalyzed flavour ester synthesis via esterification and transesterification processes and to investigate the relationship between the substrate concentration and product formation rate in batch and continuous reactors.

1.2 Problem Statement

At first, the esters used for flavoring were extracted using the traditional approach, which utilized esters derived from natural resources such as flowers and plants (Bansode *et al.,* 2017). However, the shortage of supply was the main problem because the amount extracted traditionally is very small. Due to the higher cost and lower yield, large scale production could not be carried out. Though, the demand for esters keeps increasing but the supply is inefficient to meet the demand. As a result, researchers are looking at new alternative processes that can be commercialised. As a result, chemical synthesis is used to replace the old way of producing ester. The chemical synthesis can be used for all ester production but extraction of ester from plant material is only applicable for flavour esters However, there are some disadvantages to this process, including the fact that the product is not 100% natural and that the acid catalyst employed is toxic to humans. People are becoming interested in using lipase as a catalyst alternative for the ester synthesis reaction because of the increased demand for esters in many applications such as cosmetics, pharmaceuticals, and food, and it is a biotechnological approach for long-term sustainability. Therefore, the use of lipase must be increased to meet the demand of current market. The operating conditions such as temperature, pH and enzyme concentration in the process must be adjusted to enhance the lipase to catalyze the reaction and maintain quality and quantity of desired end-product, ester.

Previously, there is less research been conducted on the mathematical modelling using kinetic model of lipase-catalysed flavour ester synthesis. Most of the previous researchers conducted experiment-based approach, which is time and energy consuming to be performed compared to simulation work. The kinetic parameters were

evaluated using non-linear regression method in MATLAB® software at optimum condition of pH, temperature and enzyme loading which have obtained from the experiment. Both the kinetic model and the evaluated kinetic parameters for the respected ester synthesis were used to perform the simulation. Simulation is a tool to evaluate the performance of a system. Therefore, the goal of this project is to use MATLAB® software to perform simulation for various types of lipases and substrates used in the lipase-catalysed flavour ester synthesis by analyzing the effect of substrate concentrations used on the initial reaction rate of product formed in batch and continuous reactors and determining the reactor configuration that is best suited the lipase-catalysed flavour ester synthesis.

1.3 Research objectives

To complete the research, there are some objectives that must be followed:

1) To study the reaction curves of ester synthesis reaction using various esters with the given kinetic parameters.
2) To analyze the effect of substrates concentrations on the initial rates of product formed from simulated reaction curves.
3) To compare the reaction curves between batch and continuous reactor which best for the lipase-catalysed flavour ester synthesis.

1.4 Scope of study

In this study, the effect of substrate concentrations on the product rate in lipase-catalysed ester synthesis from esterification and transesterification reactions for batch and continuous reactors using kinetic model were studied. The kinetic model for each ester is depends on the inhibition of substrates and product. The kinetic parameters were obtained at optimum conditions of temperature, pH and enzyme concentration from the experimental data, and they are used to perform the simulation. The changes of substrate concentration over the initial reaction rate of product were investigated for various types of esters for both esterification and transesterification reactions. The kinetic models for batch and continuous reactors were used for specific ester product to analyze the effect of reactor configuration on the rate of product formed and compared to determine which reactor has best suited the lipase-catalysed flavour ester reaction.

1.5 Organization of Thesis

This thesis is divided into five chapters as follows:

Chapter 1: This chapter includes the overview and background study of this research. It gives a brief introduction about the enzyme lipase, ester synthesis methods and lipase-catalysed flavour ester synthesis. This chapter covers problem statement, scope of study and objectives of this study.

Chapter 2: This chapter describes the previous works and literature reviews on production of lipase, types of lipases used for ester products. This explains the reaction mechanism of esterification and transesterification. Besides that, discussion

on the operating conditions on lipase-catalyzed ester synthesis reaction. Moreover, this chapter also covers the reaction kinetic models used for various esters which will be used in this study.

Chapter 3: This chapter covers the methodology used to conduct this study. It also describes how to develop the rate equations for substrates and products using the kinetic model taken from the literature for batch reactor and for the continuous reactor was developed using the rate equation taken from Elements of Chemical Reaction Engineering (Fogler, 2006). Numerical software being used in this study is being introduced here, such as MATLAB ODE function.

Chapter 4: This chapter covers the results and discussion part. The simulated results are analyzed and discussed. The effect of substrate concentrations over the initial reaction rates of the ester formed and best reactor configuration for this reaction were studied.

Chapter 5: This chapter covers conclusion which is the summary of the results obtained in present study and also recommendations for future works on lipase-catalysed flavour ester synthesis were discussed.

CHAPTER 2

LITERATURE REVIEW

In this chapter, the background research of the recent research was discussed. The development of flavouring industry is reviewed with the usage of suitable lipase for the flavour ester production. Besides that, operating conditions and the possible kinetic models for bi-substrate reactions were explained in detail.

2.1 Flavouring Industry

The demand for flavourings, particularly in the food and cosmetics industries, has steadily increased over the years as people's attention to food taste has grown. The flavouring industry has undergone significant changes over time in order to efficiently produce a variety of flavours. Natural resources, particularly plant sources, were used to obtain flavour compounds ranging from single to complex substances back then. However, because of the elucidation of the structure and the lower yield of the desired product, it is more difficult to extract ester from natural compounds, and the cost of obtaining the optimum quantity of ester is higher (Schrader *et al.*, 2004). As a result, researchers looked for another way to solve the problem, which was to employ a chemical method to synthesize the flavours. However, because this method produced undesirable side products that are harmful to the environment and contain colour impurities, an additional separation technique is required, which is costly, even though chemically synthesized flavours appear to yield more products in shorter time. Furthermore, chemically synthesized flavours were not certified as natural compounds, which consumers not preferred (Shaaban *et al.*, 2016). The drawbacks of both methods paved the way for biotechnological flavour production using enzyme as a catalyst

instead of a chemical catalyst. This enzyme functions as a biocatalyst, allowing flavours to be produced from reactants in an alternative way that requires less activation energy. This biotechnological method has the benefit of being more environmentally friendly and economically profitable.

Table 2.1 Flavours and fragrances of flavour esters

Ester	Flavour	Reference
Butyl butyrate	Pineapple	(Madras *et al*.,2008)
Isoamyl butyrate	Banana	(Bansode and Rathod, 2014)
Cinnamyl butyrate	Winey	(Waghmare *et al.*, 2017)
Tetrahydrofurfuryl butyrate	Fruity	(Yadav and Devi, 2004)
Ethyl butyrate	Banana/pineapple	(Devi et al., 2017)
Geranyl acetate	Lavender/rose/fruity	(Murcia *et al.*, 2018)
Octyl acetate	Orange	(Garlapati *et al.*,2013)
Citronellol acetate	Fruity	(Yadav and Borkar, 2009)
Isoamyl acetate	Banana	(Kumari *et al.*, 2009)
Ethyl hexanoate	Apple	(Thomas, 2017)
Ethyl isovalerate	Apple/fruity	(Chowdary and Prapulla, 2005)

2.2 Enzyme

Enzymes are biological polymer, which act as biocatalyst to accelerate biochemical reactions. The high molecular weight protein, enzymes react with molecules known as substrate molecules whereby they bind to the active sites of an enzyme to produce into a new molecule called products. The binding of a substrate to

an enzyme structure is known as lock and key model which illustrated in Figure 2.1. The enzyme used in the reaction only requires minimal amount to catalyze (Bhatia, 2018) and it does not influenced the chemical reaction equilibrium (Charles *et al.*,2019). Enzymes are highly specific with respect of substrate, so certain substrate, which is suitable to the function of enzyme is allowed to bind to the active sites of the enzyme. The enzymes are only produced by living organisms. They consist in linear chain of amino acid, which provides 3D structure. The amino acid sequence then determines the enzyme's catalytic activity. Besides that, the enzymes are highly sensitive to temperature, whereby it can be denatured beyond the optimum temperature due to loss of its biological structure of the enzyme, eventually it cannot convert the substrates into products.

Enzymes tend to increase the reaction rate by lowering the activation energy for the substrates to be converted into products, by providing the alternative pathway. However, some enzymes require cofactors for the conversion of substrates into products as it cannot be functioned alone (Charles *et al.*, 2019). Cofactors can be metal ions which help to carry out the enzymatic activity in an effective way. Cofactors, on the other hand, are organic molecules known as coenzymes that help the catalyst return to its original condition to complete the catalytic process. The presence of cofactor is required for enzymes that require it to improve their catalytic activity and function properly (Voet, 2016).

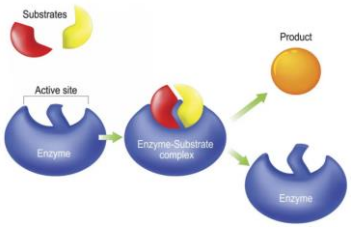

Figure 2.1 Lock and key model of an enzyme (Newman, 2018)

Furthermore, the main factor which affects the enzymatic reaction is the presence of inhibition. Inhibitor is the species which binds with on the active site of a free enzyme and eventually hinder the enzyme to catalyze the reaction. Therefore, the rate of reaction is lower with the presence of inhibitor compared to reaction rate without inhibitor. There are two categories of inhibitions: reversible and irreversible inhibition. In reversible inhibition, comprises of three types of inhibition which are competitive, non-competitive and uncompetitive (Fromm and Hargrove, 2011).

When an inhibitor competes with substrates and binds to an enzyme's active site, it is known as competitive inhibition. As a result, the product cannot be produced since the substrate is unable to bind to the enzyme's active site. It can be overcome, however, by increasing the substrate concentration so that the substrate binds to the enzyme instead of the inhibitor. Non-competitive inhibition occurs when inhibitor binds to both enzyme and the enzyme–substrate complex. Unlike competitive inhibition, this situation cannot be overcome using the high substrate concentration. The inhibitor binds to the enzyme-substrate complex but not to the free enzyme in uncompetitive inhibition, rendering the enzyme-substrate complex inactive. Therefore, the substrate cannot be converted into the products.

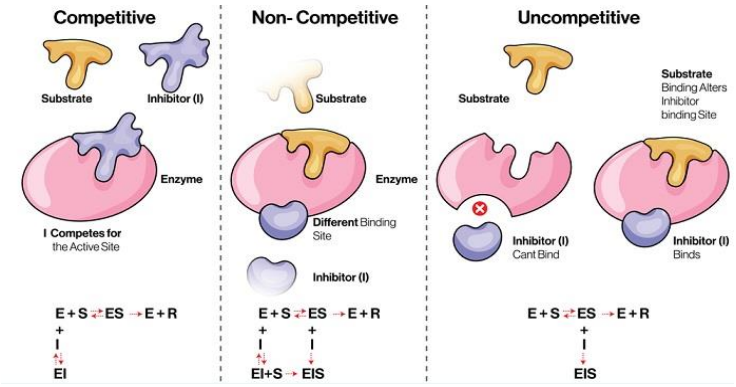

Figure 2.2 Types of reversible inhibition (Ranga, 2021)

2.2.1 Lipase

In rapid development of ester synthesis industries due to the demand of multiple flavour ester products by the customers, the enzyme as the biocatalyst is widely used due to the drawbacks of using chemical catalyst in the synthesis such due to the drawbacks of using chemical catalyst in the process such as low yield, cause pollution, presence of undesirable by-products and gives poor results and the product cannot be labelled as natural product. Therefore, the compatible enzyme is used which can increase the process efficiency in order to obtain higher yield of product and reduces downstream costs. There are many enzymes used in ester production industry to produce flavours, fragrances, plasticizer and pharmaceutical products. Among them, the most promising enzyme is lipase. Lipase (triacylglycerol acylhydrolases; EC 3.1.1.3) were a family of hydrolytic enzymes that functioned as catalysts for both the hydrolysis and synthesis of esters. The characteristics and substrate specificity of microbial lipases were diverse, making them attractive tools for both research laboratories and in industries (Annapurna, 2018). Lipase as biocatalyst is widely used because it has advantages over the chemical catalyst as it can function under mild condition whereby only lower temperature is required for the reaction to occur and prevent the formation of by-product (García *et al.*, 2000; Zhang *et al.*, 2020), improve the quality of the product (Kraai *et al.*, 2008), highly specific which allow to synthesize the required product by lipase (Paiva et.al, 2000; Mathpati *et al.*, 2016). Besides that, the use of lipase allows manufacturers to classify end product as green which is known as natural. Many microbes produce lipase, which can be used to catalyze a variety of reactions, as shown in Table 2.2.

Table 2.2 Lipase production from different substrates

Substrate	Microorganism	Product	References
Corn steep liquor and soybean oil	*Geotrichum candidum NRRLY-552*	Lipase	(Maldonado, *et al.*, 2014)
	Geotrichum sp	Lipase	
Soybean meal	*Penicillium P58* and *P74*	Lipase	(Rigo *et al.*, 2010)
Triturated nut and barley bran	*Lipolytica CECT 1240*	Lipase	(Domínguez *et al.*, 2003)
Soybean oil cake	*Bacillus sp*	Lipase	(Bhosale and Kadam, 2015)
Wheat bran and olive oil	*Aspergillus flavus*	Lipase	(Toscano *et al.*, 2013)

Lipase is commonly employed in flavour ester synthesis, although it has a number of disadvantages, including high cost, instability, and sensitivity. To address this issue, the industry has shown interest in immobilized lipase, which can be achieved in a variety of techniques, including entrapment, adsorption, encapsulation, cross-linking, and covalent bonding (Nisha, *et al.*, 2012; Sirisha *et al.*, 2016; Ismail and Baek, 2020). Immobilization methods can be classified into two, which include chemical and physical methods. The chemical method requires creating a covalent bond between the lipase and the support material or agglomeration of lipase molecules by cross-linking, whereas the physical method just includes the binding of lipase to the support material (Ismail and Baek, 2020).The immobilized lipase is easily handled, can be reused to minimize the operating costs and can be separated from the reaction for further use compared to free lipase.

2.3 Esterification

Esterification is reaction of producing esters using carboxylic acid reacts with an alcohol with elimination of water. A catalyst is required in most of the esterification process which used to speed accelerate the reaction rate of esterification process so that the ester can be produced in shorter time. The reaction occurs in a way that –OH from carboxylic acid and +H from alcohol is removed to form water which is a by-product of this process. The esterification mechanism between alcohol and acid was illustrated in Figure 2.3**.** Esters can also be formed through other reactions such as acid anhydrides, acid chlorides, amides, nitriles, unsaturated hydrocarbon, ethers, aldehydes, ketones, alcohols and esters (through ester interchange). The esterification reaction is not a complete reaction because it involves a reversible reaction. Therefore, to achieve 100% conversion, a separation process is required to separate the desired product (ester) and undesired product (water). Esters can be produced through both batch and continuous processes.

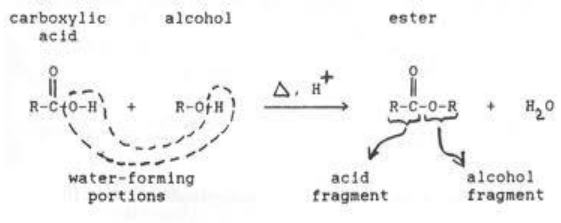

Figure 2.3 Molecular equation for esterification process

2.4 Transesterification

Transesterification reaction also another reaction for producing ester by using alcohol and ester as shown in Figure 2.4. Apart from esterification reaction, this also required a catalyst to enhance the reaction rate of product, flavour ester. Acid or base

catalyst is commonly used in transesterification process. However, there is a drawback when chemical catalyst is used, which contaminated the end-product, flavour ester especially when used in food industry. Therefore, enzyme was introduced into the transesterification process as a biocatalyst which has similar role as that of chemical catalyst. Lipase is commonly used because it overcome the problems of using chemical catalyst and better alternative in term of purity of the ester. Though, biodiesel production is one of the application from transesterification reaction whereby the triglycerides (oil and fat) react with any alcohol, typically methanol or ethanol is used as alcohol to produce fatty acid methyl ester (FAME) or fatty acid ethyl ester (FAEE) and glycerol as by-product (Ezzati et al., 2021), apart from biodiesel, flavour esters also can be obtained through this reaction.

Figure 2.4 Molecular equation for transesterification process

There are various types of substrates used in flavour esters production using these two reactions as listed in Table 2.3. In esterification reaction, the substrates involved are acid and alcohol whereas in transesterification reaction, ester and alcohol are used as substrates.

Table 2.3 Types of reaction and substrates involved in flavour ester synthesis

Flavour ester	Reaction	Substrates		References
		Acid/ester	Alcohol	
Butyl butyrate	Esterification	Butyric acid	Butanol	(Madras *et al.*, 2008)
Cinnamyl butyrate	Esterification	Butyric acid	Cinnamyl alcohol	(Waghmare *et al.*, 2017)
Citronell**o**l acetate	Transesterification	Vinyl acetate	Citronellol	(Yadav and Borkar, 2009)
Ethyl hexanoate	Esterification	Hexanoic acid	Ethanol	(Annapurna *et al.*, 2018)
Ethyl isovalerate	Esterification	Isovaleric acid	Ethanol	(Chowdary and Prapulla, 2005)
Geranyl acetate	Transesterification	Vinyl acetate	Geraniol	(Murcia *et al.*, 2018)
Isoamyl butyrate	Esterification	Butyric acid	Isoamyl alcohol	(Bansode *et al.*, 2017)
Isoamyl acetate	Acetylation	Acetic anhydride	Isoamyl alcohol	(Romero *et al.*, 2007)
	Transesterification	Ethyl acetate	Isoamyl alcohol	(Rizzi *et al.*, 1992)
	Esterification	Acetic acid	Isoamyl alcohol	(Gogoi and Dutta, 2009)
Octyl acetate	Transesterification	Vinyl acetate	Octanol	(Yadav and Trivedi, 2003)
Tetrahydrofurfuryl butyrate	Esterification	Butyric acid	Tetrahydrofurfuryl alcohol	(Yadav and Devi, 2004)

2.5 Operating Parameters

There are many factors which influences the yield of the ester produced in the bioreactor such as temperature, catalyst concentration, substrate concentration, pH and addition of organic solvent. Optimum condition of each factors needed to obtain to increase the production of ester using various lipases.

2.5.1 Enzyme concentration

Enzyme concentration is an important factor in lipase-catalyzed ester reaction. The reaction rate increases as the enzyme concentration increases because increasing number of active sites of enzyme binds with the substrates (Mathpati *et al.*,2016). The enzyme will reduce the activation energy by providing the alternative pathway which reduce the reaction time to produce the ester. García et al.(2000) reported that the reaction rate is directly proportional to the enzyme concentration which indicates the first-order relationship between the concentration of enzyme which acts as catalyst and the reaction rate. This showed that the reaction rate was favored when the higher amount of enzyme was used (Bansode *et al.*, 2017; Costa *et al.*, 2021).

2.5.2 Substrate concentration

There were several factors that had a significant impact on the rate of product formation. The concentration of the substrate is one of the factors. By varying the substrate concentration and determining which substrate has an inhibitory effect in the reaction, the inhibition effect on the final product can be reduced by using a lower concentration of the inhibitory substrate. Furthermore, the optimal concentration of substrate is required to maximize the amount of ester to be synthesized. By varying one substrate concentration with a fixed concentration of another substrate and vice versa,

the optimum concentration of each substrate can be achieved. Chowdhury and Mitra (2015) reported that increasing both substrate concentrations linearly increased the reaction rate, indicating that there was no inhibition involved. However, Yadav & Shinde (2012) reported that increasing the ester concentration in the transesterification process reduces the reaction rate, indicating that the ester has a strong inhibitory effect. Similar trend observed by Mathpati et al. (2016) in transesterification reaction between furfuryl alcohol and vinyl acetate, indicating that furfuryl alcohol has an inhibitory effect, implying that increasing the concentration slowed down the reaction rate. However, increasing the isovaleric acid and ethanol concentrations from 0.2 M to 1.0 M, reduced the rate indicating both are inhibitors (Chowdary and Prapulla, 2005).

2.5.3 Reaction pH

Because enzymes are pH sensitive, pH is one of the factors in lipase-catalyzed reactions. It has the ideal pH to efficiently perform catalytic activity. This is because a change in pH either higher or lower caused the enzyme to lose its catalytic activity, resulting in deactivation. The pH of different lipases varies. The best pH for immobilized *Candida rugosa* in n-amyl isobutyrate ester synthesis using n-hexane as organic solvent was 8 (Milainovi *et al.*, 2014), whereas Novozym 435 which is known as immobilized CAL-B in isoamyl acetate synthesis was 7.7 (Romero *et al.*, 2007).

2.5.4 Solvent

Higher yields are obtained when various lipases are used in an organic solvent to synthesize ester. According to Kraai et al. (2008) the properties of organic solvent have a significant impact on enzymatic activity. The effect of solvent characteristics on the selection of organic solvents is that hydrophobic solvents are preferred over

hydrophilic solvents because the reverse reaction can be avoided (Yadav and Shinde, 2012; Zhang *et al.*, 2020). However, the organic solvent's toxicity is a major disadvantage that has limited its use in the food, pharmaceutical, and cosmetics industries. As a result, supercritical carbon dioxide ($ScCO_2$) is used to replace organic solvents because it is non-toxic, has a lower cost, and is easier to separate. Although several studies have been published on the synthesis of flavour esters via esterification and transesterification in various solvents, there has been little research on the synthesis of flavour esters in $ScCO_2$. Table 2.4 showed various types of solvents used by previous researchers for flavour ester production.

Table 2.4 Solvents used for flavour esters

Ester	Solvent	Reference
Isoamyl acetate	n-hexane	(Romero *et al.*, 2007)
Citronellyl butyrate	n-hexane	(Dahlan *et al.*, 2012)
Furfuryl acetate	Toulene	(Mathpati *et al*., 2016)
Octyl acetate	n-heptane	(Yadav and Trivedi, 2003)
Terpinyl acetate	$ScCO_2$	(Liaw and Liu, 2010)
Butyl butyrate	$ScCO_2$	(Madras *et al.*,2008)

2.6 Kinetics of Enzyme Reaction

The study of the rates of chemical reactions involving enzymes is known as enzyme kinetics. In enzyme kinetics, the velocity or rate is the primary concern, and the factors that influence the rate are discussed. The study of independent variables such as temperature, substrate concentration, pH, and reaction rate on the dependent variable, enzyme kinetics, is known as kinetic analysis. The kinetic mechanism for a reaction can be determined through kinetic analysis. The rate of reaction at different substrate and enzyme concentrations is used to investigate the mechanism of an enzyme-catalysed reaction. When the enzyme is mixed with the substrate in the bioreactor, the concentration of substrate and product, as well as the rate of reaction, will change as the reaction proceeds.

2.6.1 Enzymatic reaction with two substrates

In the enzymatic reaction, the common kinetic equation used is Michaelis-Menten equation. This is the fundamental equation which used to study the enzyme kinetics by analyzing the time taken for the reaction take place based on the concentration of reacted substrate. This equation defines the relationship between the substrate concentration and the rate of enzyme-catalyzed reaction. However, it is valid for reaction involving one substrate. Most of the enzyme able to catalyze the reaction which has more than one substrate. Therefore, Bi-Bi mechanism are used for two substrates which are sequential and non-sequential reaction. Sequential reaction is single displacement reaction which classified into two:(1) Ordered Sequential Bi-Bi mechanism, (2) Random Sequential Bi-Bi mechanism whereas Ping-Pong Bi-Bi mechanism is the non-sequential double displacement reaction. The example of Ordered Sequential Bi-Bi reaction, Random Sequential Bi-Bi reaction and Ping-Pong

Bi-Bi mechanism are as follows (Voet, 2016) which showed in Figure 2.5,2.6 and 2.7 respectively.

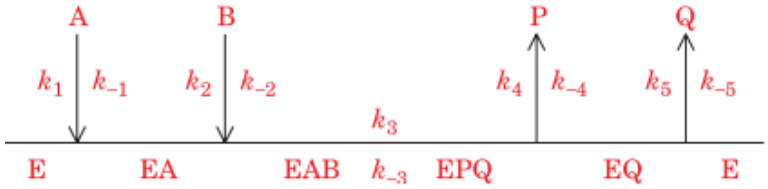

Figure 2.5 Ordered Sequential Bi-Bi mechanism

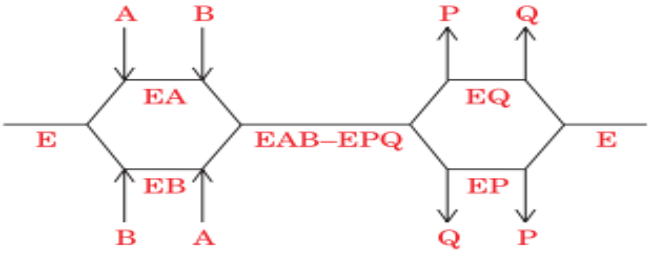

Figure 2.6 Random Sequential Bi-Bi mechanism

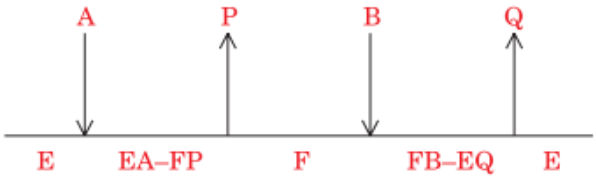

Figure 2.7 Ping-Pong Bi-Bi mechanism

2.6.2 Ordered Sequential Bi-Bi mechanism

The mechanism shows that the reaction will not take place until both substrates are bind at the active site of enzyme. The enzyme will bind with A in orderly manner followed by addition of B which results in a ternary complex, EAB. The rate equation of ordered reaction for two substrates is as follows:

$$v = \frac{V_{max}[A][B]K_s^B}{K_s{}^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B]} \quad (2.1)$$

2.6.3 Random Sequential Bi-Bi mechanism

This reaction mechanism does not have a particular order to bind with active site of enzyme for formation of ternary complex, EAB. There are two possible ways for the enzyme to bind with either A or B, these two paths depend on the actual substrate concentration. The rate equation of Random Sequential Bi-Bi mechanism scheme is expressed as follows:

$$v = \frac{V_{max}[A][B]K_S^B}{K_S^A K_m^B[B] + K_S^B K_m^B[A] + K_S^A K_S^B K_m^B + [A][B]} \quad (2.2)$$

2.6.4 Ping-Pong Bi-Bi mechanism

This mechanism, the enzyme bind with substrate A first followed by the release of first product P and the new enzyme, E* is formed. Then, E* is bind with second substrate B and E*B complex is formed, followed by breaking down of complex to free enzyme and second product Q. This mechanism does not have ternary complex. The rate equation of Random Sequential Bi-Bi mechanism scheme is expressed as follows:

$$v = \frac{V_{max}[A][B]}{K_m^B[A] + A[B] + [A][B]} \quad (2.3)$$

2.6.5 Kinetic Mechanism of Lipase-catalyzed Ester Synthesis Reaction

The kinetics of lipase-catalyzed ester synthesis reactions are the description of the reaction for the particular ester synthesis. The kinetic mechanism is developed based on the design of experiment (DoE) whereby the independent assessment of changes in substrates concentration. It is useful in not only quantifying a reaction rate but also in revealing the details inclusion of substrate or product inhibition. In lipase-catalyzed ester synthesis reaction, most of the reaction mechanisms are reported to follow Ping–Pong Bi–Bi mechanism. Numerous kinetic studies by lipase have been reported with a variety of substrates and including inhibition employed in the reaction for various

flavour ester production. In flavour esters production which induced bi-substrate model usually followed Ping-Pong Bi-Bi or Ordered Bi-Bi model with inclusion of substrate/substrates inhibition and type of lipase used as showed in Table 2.5.

Table 2.5 Kinetic models of various flavour esters for both reactions

Flavour ester	Reaction	Lipase	Model	Inhibition	References
Butyl butyrate	Esterification	CAL-B	Ping Pong Bi-Bi	Both acid and alcohol	(Madras *et al.*, 2008)
Cinnamyl butyrate	Esterification	CAL-B	Ping Pong Bi-Bi	Alcohol	(Waghmare *et al.*, 2017)
Citronell**o**l acetate	Transesterification	CAL-B	Ping Pong Bi-Bi	Both ester and alcohol	(Yadav and Borkar, 2009)
Ethyl hexanoate	Esterification	CRL	Ping Pong Bi-Bi	Both acid and alcohol	(Annapurna *et al.*, 2018)
Ethyl isovalerate	Esterification	Immobilised Rhizomucor miehei	Ping Pong Bi-Bi	Both acid and alcohol	(Chowdary and Prapulla, 2005)
Geranyl acetate	Transesterification	CAL-B	Ping Pong Bi-Bi	None	(Murcia *et al.*, 2018)
Isoamyl butyrate	Esterification	CAL-B	Ping Pong Bi-Bi	Alcohol	(Bansode *et al.*, 2017)

Isoamyl acetate	Acetylation	CAL-B	Ping Pong Bi-Bi	Acetic anhydride	(Romero *et al.*, 2007)
	Transesterification	Immobilised Mucor miehei	Ping Pong Bi-Bi	Both ester and alcohol	(Rizzi *et al.*, 1992)
	Esterification	Immobilised Mucor miehei	Ping Pong Bi-Bi	Acid	(Gogoi and Dutta, 2009)
Octyl acetate	Transesterification	CAL-B	Ordered Bi-Bi	Alcohol	(Yadav and Trivedi, 2003)
Tetrahydrofurfuryl butyrate	Esterification	CAL-B	Ping Pong Bi -Bi	Alcohol	(Yadav and Devi, 2004)