CONCEPTUAL STUDY OF ENZYMATIC SYNTHESIS OF PROPYL CAFFEATE IN PACKED BED REACTOR AT STEADY-STATE AND ISOTHERMAL CONDITIONS

by

AJAYAN RAJENDRAN

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LIST OF SYMBOLS

Symbol	Description	Unit
F_{AO}	Molar flowrate	mol/s
X	Conversion	-
W	Catalyst weight	kg
ΔP	Pressure drop	Pa
S	Substrate	-
E	Enzyme	-
ES	Enzyme-substrate complex	-
K_S	Equilibrium dissociation constant	mol/L
K_S^A	Equilibrium dissociation constant for the binary ES complexes of A	mol/L
K_S^B	Equilibrium dissociation constant for the binary ES complexes of B	mol/L
K^{AB}	Michaelis constants for the EAB ternary complexes	mM
K^{BA}	Michaelis constants for the EAB ternary complexes	mM
K’	Apparent Michaelis constant	mM
K_m	Steady-state Michaelis constant	mM
K_{mA}	Steady-state Michaelis constant of substrate A	mM
K_{mB}	Steady-state Michaelis constant of substrate B	mM
V_{max}	Maximum reaction rate	mol/Lh
C_A	Initial concentration of substrate A	mol/L
C_B	Initial concentration of substrate B	mol/L
K_{iB}	Inhibition constant for substrate B	mM
k_{cat}	Enzyme’s catalytic constant	1/s
k_{inact}	Thermal inactivation rate constant	-

t	Assay duration	s
k_B	Boltzman's constant	J/K
R	Gas constant	J/K.mol
T	Absolute temperature	K
h	Plank's constant	J.s
ΔG_{cat}	Activation energy of the catalyzed reaction	J/mol
ΔG_{inact}	Activation energy of the thermal inactivation process	J/mol
E_{act}	Active form of the enzyme	-
E_{inact}	Inactive form of the enzyme	-
Teq	Temperature at equilibrium	K
Topt	Temperature at optima	K

LIST OF ABBREVATIONS

PAE	Phenolic acid ester
DCC	Dicyclohexyl carbondiimide
CAL-B	Candida Antarctica lipase –B
PBR	Packed bed reactor
DPP4	Dipeptidyl peptide

SINTESIS ENZIMATIK PROPYL CAFFEATE DALAM REAKTOR (PBR) PADA KEADAAN STABIL DAN ISOTERMAL

ABSTRAK

Sintesis propil kafeat enzimatik dalam reaktor (PBR) pada keadaan stabil dan keadaan isotermal ditunjukkan dalam thesis ini. Simulasi dilakukan untuk mengkaji kesan suhu, nisbah mol substrat/alkohol, dan kepekatan substrat terhadap hasil propyl kafeat. Model matematik dikembangkan dalam perisian Polymath® untuk merangsang hasil untuk dibandingkan dengan data eksperimen. Hasil maksimum dicapai pada 60 ° C dengan 99.5% dalam data eksperimen sementara data simulasi yang dicadangkan memberikan 91.1% pada 60 ° C dengan peratusan ralat 8.44% yang dianggap peratusan ralat yang dapat diterima sejak kurang dari 10%. Kajian ini menunjukkan hasil maksimum dicapai dalam hasil simulasi dan eksperimen pada suhu optimum dengan julat 60 ° C hingga 70 ° C. Selain itu, kedua-dua data simulasi dan data eksperimen menunjukkan bahawa nisbah molar metil kafeat dan 1-propanol baik adalah antara 1:10 dan 1:40 yang menghasilkan hasil tinggi. Di samping itu, kajian mendapati bahawa kepekatan metil kafeat rendah sekitar 0.005 mol/L memberikan hasil maksimum dan meningkatkan reaksi enzimatik.

CONCEPTUAL STUDY OF ENZYMATIC SYNTHESIS OF PROPYL CAFFEATE IN PACKED BED REACTOR AT STEADY-STATE AND ISOTHERMAL CONDITIONS

ABSTRACT

The enzymatic propyl caffeate synthesis in packed bed reactor (PBR) at steady-state and isothermal conditions is presented in this thesis. The simulation carried out to study the effects of temperature, substrate/alcohol molar ratio, and substrate concentration on conversion. The mathematical model is developed in Polymath® software to simulate the results to compare with published experimental data. The highest conversion achieved at 60°C with 99.5% in the experimental data while the proposed simulated data gives 91.1% at 60°C with the error percentage of 8.44% which is considered acceptable error percentage since less than 10%. Based on this simulation results, both conversion and rate of reaction is decreases at reaction temperature above 80°C which shows the enzyme are start to deactivate at this temperature. This study shows the highest conversion is achieved in both simulation and experimental results at reaction temperature of 70°C and 60°C respectively. Moreover, both simulated and experimental results indicates that the perfect molar ratio of methyl caffeate to 1-propanol is with range of 1:10 and 1:40 which give higher conversion and producing high yield. In addition, the studies found that low concentration of methyl caffeate of 0.005 mol/L provide the highest conversion and enhances the enzymatic reaction.

CHAPTER 1

INTRODUCTION

The conceptual study of enzymatic propyl caffeate synthesis in packed-bed reactor at steady-state and isothermal conditions is studied in this project. Propyl caffeate is an ester of a hydroxycinnamic acid with molecular formula of $C_{12}H_{14}O_4$ and molecular weight of 222.24 g/mol. It also known as propyl 3-(3,4-dihydroxyphenyl) acrylate. It has the highest antioxidant activity among caffeic acid alkyl esters which has the great attraction by industries. Several chemical methods have been developed for the preparation of propyl caffeate. However, chemical methods have some drawbacks, such as poor reaction selectivity, leading to undesirable side reactions, pollution and high manufacturing cost. Thus, enzymatic synthesis of propyl caffeate catalyzed by lipase have been used. Researchers found that continuous-flow packed-bed enzyme reactor is an effective strategy to obtain greater overall yield in shorter amount of time. However, propyl caffeate is difficult to be synthesized with high number of carbon of aliphatic alcohols. Therefore, the feasibility and sustainability of packed-bed reactor for enzymatic propyl caffeate synthesis via transesterification reaction is to be evaluated in this paper. In order to evaluate the feasibility and sustainability of this enzyme reactor, the enzymatic kinetic model for packed-bed reactor is developed and studied in this project. The research background of the synthesis of propyl caffeate is discussed in detail and the problem statement and the objectives of this project also proposed in this paper.

1.1 Research Background

Phenolic acid ester (PAE) has various potential uses as functional food ingredients such as antioxidants and preservative agents (Stevenson et al., 2007). PAE is one major component that has biological properties including anti-inflammatory, anti-microbial, anti-oxidation, and anti-tumor activities. They usually act as reducing agents, hydrogen donators, and singlet oxygen quencher due to anti-oxidants capacity. Food processing, pharmaceutical and cosmetic industries widely used phenolic acid esters for their production (Xu et al., 2018).

Phenolic acid ester (PAE) could be extracted from plants. However, the isolation of this highly valuable PAE from natural product extracts inefficient, time consuming and uneconomical. Therefore, synthesis of PAE at reasonable price become attractive due to its economic benefits compared to extraction from natural sources (Wang et al., 2014). Many studies focused on chemical synthesis of PAE. In this case, usually caffeic acid and phenethyl alcohol used to synthesize ester with presence of dicyclohexyl carbodiimide (DCC) at room temperature. Besides that, phenolic acid ester also synthesized by using the enzymatic synthesis method. In this case, mostly lipase CAL-B as an enzyme from *Candida antarctica* is used for enzymatic esterification of phenolic acids and fatty alcohol (Chen et al., 2011).

The enzymatic synthesis of phenolic acid ester is more sustainable compared to chemical synthesis because the reagents used in chemical synthesis might cause environmental pollution and also harmful to humans (Chen et al., 2011). The enzymatic synthesis of PAE is the preferred route of synthesis because it can be achieved high yields compared to chemical synthesis (Stevenson et al., 2007). Moreover, enzymatic approaches to synthesis of PAE offers many advantages due to milder reactions and reagents (Chen et al., 2011).

Enzyme-catalysed reactions are generally labelled as natural flavors that make a great interest to industries. Enzyme-catalysed reaction has been attractive due to the catalytic selectivity and generated products are pure while chemical reaction not attractive due to high cost and impossibility of reuse. In addition, chemical synthesis is also low in thermal and chemical stability compared to enzymatic synthesis (Pätzold et al., 2019).

However, the drawbacks of enzyme-catalysed reaction are the enzymes are sensitive to temperature changes. At high temperature, the enzyme will be denatured, which affects the reaction. To overcome this issue, temperature changes should be carefully monitored and controlled. Apart from this, enzyme-catalysed reaction will be affected by lack of enzyme activity or stability in the chosen solvent. To overcome the drawbacks, enzymatic reaction should performed in non-conventional reaction media such as organic solvents, ionic liquids, and supercritical fluids (Pätzold et al., 2019).

In general, enzymatic-catalysed reaction is mostly preferred route of synthesis for phenolic acid ester because offers more advantages in terms of sustainability, environmental impact and energy consumption.

1.2 Problem Statement

Enzymatic propyl caffeate synthesis attracted by many industries due to the highest antioxidant activity among caffeic acid alkyl esters. However, the industrial production via enzymatic transesterification in batch reactors shows some drawbacks such as long reaction time and not efficient. In order to develop a rapid process for enzymatic propyl caffeate production, the packed-bed reactor is proposed for this enzymatic transesterification reaction. However, the enzyme is deactivated very fast due to the short half-life of the enzyme. Hence, the reaction is less productivity. Additionally, the inhibition of alcohol towards the enzyme also one of the factors that made the enzymatic synthesis not be sustainable.

Therefore, operating temperature of the enzymatic reaction could be manipulated to reduce the drawback of enzyme deactivation. Moreover, substrate to alcohol molar ratio need to be reduced to increase conversion and also reduced the inhibitory effect of alcohol towards the enzyme.

In this study, we develop the kinetic model for enzymatic packed-bed reactor to study the effect of reaction variables on its performance. The study shall emphasize on the effect of kinetic parameters on the operating parameters. The feasibility and sustainability of packed-bed reactor for enzymatic transesterification also will be discussed.

1.3 Objectives

i. To evaluate the sustainability and feasibility of enzymatic reaction by developing a kinetic model for enzymatic propyl caffeate synthesis via Polymath® software.

ii. To study the enzyme deactivation by manipulating operating temperature of enzymatic propyl caffeate synthesis in packed-bed reactor.

iii. To investigate the effects of substrate to alcohol molar ratio and substrate concentration on conversion in packed-bed reactor.

CHAPTER 2

LITERATURE REVIEW

2.1 Caffeic Acid

Caffeic acid is also known as 3,4-dihydroxycinnamic acid which is a simple phenolic compound consisting of only two hydroxyl groups on its aromatic ring and a C=C side chain which are pivotal for its antioxidant properties. The chemical structure of caffeic acid is shown in **Figure 2.1**. It is a naturally occurring phenolic acid, which widely found throughout a plant kingdom such as in fruits, grains, coffees, and vegetables. It is also used for many health benefits, including anti-flammatory, anti-cancer, and antiviral abilities and it may help to boost the performance of athletes. Caffeic acid (3,4-dihydroxycinnamic acid) has been shown to be a α-tocopherol protectant in low-density lipoprotein (LDL). Also, its conjugates such as chlorogenic and caftaric acids were demonstrated to be more powerful antioxidants in a number of different systems. The scientific community attracted to the anticarcinogenic properties of caffeic acid. The food contains caffeic acid will prevent the formation of nitro compounds which act as a protective effect against carcinogenesis that are the main inducers of this pathology (Monteiro Espíndola et al., 2019).

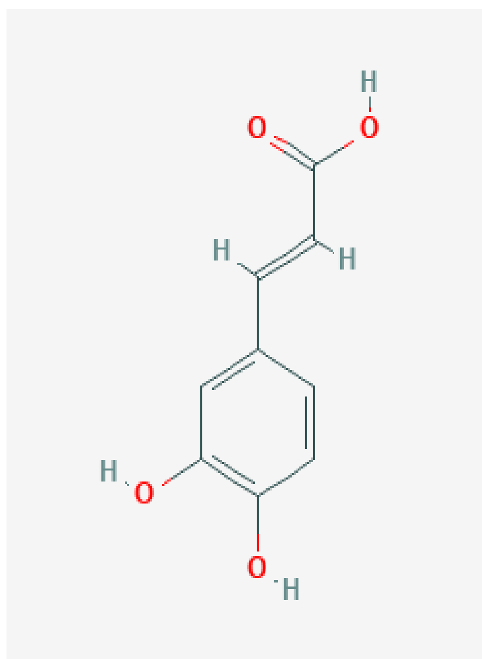


Figure 2.1: Chemical structures of caffeic acid (National Center for Biotechnology Information, 2020)

2.2 Type of Reactor for Enzyme-catalyzed Reaction

The lipase-catalyzed reactions mostly used slug-flow microchannel reactor. The reactor consists of a "T" slug-flow generator, a stainless-steel microchannel, two constant-flow pumps, and a temperature controller. This reactor is feasible because the contact area of the two immiscible liquids (or gas/liquids) in the slug-flow system is large, and the diffusion distance between the interface and slug core is short. Due to this, the interfacial mass and heat transfer intensities will be increased and speed up the chemical reaction. The substrate of lipase-catalyzed reactions is usually insoluble in water, but soluble in organic solvents, the reaction can be achieved by lipase in two-phase system in a slug-flow microchannel reactor which eventually preventing the usage of emulsifiers that creates difficulties in purification of product. For instance, astaxanthin esters in *H. pluvialis* oil were efficiently hydrolyses to free astaxanthin by lipase from *Aspergillus niger* by using microchannel reactor. This reactor have

characteristics of a high rate of conversion and lower chemical consumption compared to traditional reactors (Mei et al., 2020).

The packed-bed reactor (PBR) is one of the most feasible reactors that used for enzyme-catalyzed reaction. Researchers found that the packed-bed bioreactor in solvent-free system gives a 92% of conversion in 31 h at 50°C for esterification of fatty acids obtained from soybean soapstocks acid oil (Soares et al., 2013). Moreover, the conversion of over 84% of fatty acids to esters were maintained when the PBR was reused in successive 48 h esterification reactions for five cycles at 50°C and for six cycles at 45°C (Soares et al., 2013). Apart from that, the conversion of (5S)-N-(tert-butoxycarbonyl)-5-(methoxycarbonyl)-2-pyrroline to its corresponding amide is an important intermediate synthesis of dipeptidyl peptidase 4 (DPP4) inhibitor Saxagliptin in packed-bed reactor that packed with 40 g immobilized *Candida antarctica* lipase B *(Novozyme 435)* gives product yield greater than 98% and purity of 98.5% at steady-state in (Tirunagari et al., 2018).

2.3 The Effect of Operational Parameters

Enzymatic transesterification is affected by the reaction variables such as pressure, temperature, pH, reaction time, enzyme load, and substrate concentration or ratio. The effect of reaction temperature on the transesterification shows a significant impact on conversion of reactants. According to (Sun & Hu, 2017) , the conversion increased with increasing temperature from 50°C to 70°C. After that, from 70°C to 90°C the conversion maintained at higher level approximately 100%. However, the conversion sharply decreased at 100°C because at high temperature there is decrease of reaction system viscosity and fast mass transfer rate. This results in thermal enzyme deactivation and can lead to complete enzyme deactivation.

This is similar to pH, the enzymatic transesterification achieved higher conversion at optimum pH.

The substrate ratio also shows a significant effect on enzymatic transesterification because the increase of substrate ratio can enhance the transesterification rate and time taken to reach equilibrium become shorten (Sun & Hu, 2017). The increase of enzyme loading to 20% (relative to the weight of total substrates) will increase the conversion. However, at higher enzyme load approximately 25%, the immobile lipase (solid) agglomeration and diffusion problems of substrates has been found in (Sun & Hu, 2017).

2.4 Methods to Synthesize Propyl Caffeate

Propyl caffeate can be synthesized by chemical and enzyme-catalyzed reaction. Many researchers have studied the chemical synthesis of propyl caffeate from caffeic acid and phenethyl alcohol. The synthesis of propyl caffeate was performed with yield of 86% (Chen et al., 2011). However, the chemical synthesis still has some drawbacks due to the reagents used for the synthesis is very harmful to humans and may cause pollutions to the environment. Therefore, enzymatic synthesis of propyl caffeate has been developed through lipase-catalyzed reaction. The enzymatic synthesis offers many advantages compared to chemical synthesis because enzymatic reaction preferred milder reaction conditions and reagents. For instance, transesterification of methyl caffeate and 1-proponal catalyzed by Novozym 435 in [Bmim][CF_3SO_3] to synthesis propyl caffeate was performed with maximum yield of 99.5% (Wang et al., 2014).

2.5 Lipases for Propyl Caffeate Synthesis

Lipases are used as biocatalysts with excellent stability and activity in the presence of organic solvents, which manage to act on wide variety of substrates such as triacylglycerides, ester of fatty acids, lipids, synthetic and natural oils. They are originated from bacteria, yeasts and filamentous fungi. In general, lipases are used in a variety of application in both research laboratories and industrial scale. As example, they used in citric acid production, biodiesel synthesis, polymer synthesis, capsinoid production, pro-drug synthesis, fatty acid esters synthesis, ester synthesis, and glycerol succinic acid production. The maximum propyl caffeate yield of 98.5% obtained under optimum conditions with Novozym 435 as a biocatalyst, ionic liquid [Bmim][CF_3SO_3] as a medium, mass ratio of methyl caffeate to lipase of 1:20 and reaction temperature of 60 °C. The Novozym 435 type of lipase is the most active catalyst for synthesis of esters due to their thermostable compared to Lipozyme RM IM and Lipozyme TL IM (Pang et al., 2013). According to (Rønne et al., 2005), the Lipozyme TL IM provide fast reaction and also achieved equilibrium with shorter period of residence time compared to Lipozyme RM IM.

The Novozym 435 (N435) is one of the immobilized lipases which produced by Novozymes. It is based on immobilization through interfacial activation of lipase B from Candida antarctica on a resin, Lewatit VP OC 1600 (Ortiz et al., 2019). The characteristics for immobilized lipases is shown in **Table 2.1**. The transesterification reaction performed mostly by using this enzyme. Based on the literature by (Ortiz et al., 2019), it is the most efficient lipase that used for transesterification reaction. For instance, it used to catalyze the synthesis of Agave fructans mono- and diacylated with lauric acid.

Table 2.1: Characteristics for Immobilized Lipases (Rønne et al., 2005)

Brand	Species	Carrier	Specificity	Water content (wt%)	Porosity
Lipozyme TL IM	*Thermomyces lanuginosus*	silica granules	sn-1,3-specific	6.0	0.77
Lipozyme RM IM	*Rhizomucor miehei*	macroporous resin	sn-1,3-specific	3.2	0.45
Novozyme 435	*Candida antarctica* lipase B	macroporous	Non-specific	4.1	0.65

2.6 Design Equation for Packed Bed Reactor (PBR)

Packed-bed reactor (PBR) is a tubular reactor filled with catalyst particles. The PBR is modelled as having the fluid flowing in plug flow for PBR. The reactants are consumed as the reactants enter and flow axially down the reactor so that the conversion increases along the length of reactor. The generalized mole balance on species A over catalyst weight results in the equation as (Fogler, 2006):

$$In - Out + Generation = Accumulation$$

$$F_{A|W} - F_{A|(W+\Delta W)} + r'_A \Delta W = 0 \quad \ldots (2.1)$$

As first step, dividing equation (2.1) by ΔW and taking limit as catalyst weight, $\Delta W \rightarrow 0$. Then, the differential form of mole balance equation of PBR for species A in the reaction is expressed as:

$$\frac{dF_A}{dW} = r'_A \quad \ldots (2.2)$$

For a flow system, F_A has previously been given in terms of entering molar flowrate, F_{AO} and the conversion, X.

$$F_A = F_{AO} - F_{AO}X \quad \text{... (2.3)}$$

Then, differentiating the equation:

$$dF_A = -F_{AO}\, dX \quad \text{... (2.4)}$$

To give the differential form of design equation for PBR, substitute into equation (2.4) into equation (2.2):

$$F_{AO}\frac{dX}{dW} = -r'_A \quad \text{... (2.5)}$$

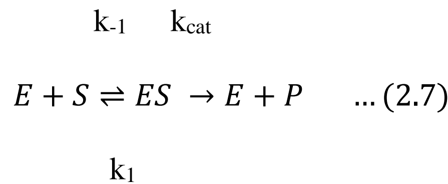
When analyzing reactor that have pressure drop along the length of reactor, the differential form of the design equation must be used. The equation (2.5) is integrated in the absence of pressure drop, ΔP= 0 with limits X= 0 at W= 0 to obtain equation (2.6):

$$W = F_{AO}\int_0^X \frac{dX}{-r'_A} \quad \text{... (2.6)}$$

This equation 2.6 can be used to find the weight of catalyst. When the total pressure remains same, it is necessary for W to achieve a conversion, X.

2.7 Enzyme Kinetic Model

An enzymatic reaction is usually modelled as two-step process. The formation of an enzyme-substrate (ES) complex appears when the substrate (S) binding by enzyme (E) and then the free enzyme and product (P) is formed by breaking down the enzyme-substrate complex. The following reaction series as (Marangoni, 2013):



$$\overset{k_{-1}\quad k_{cat}}{\underset{k_1}{E + S \rightleftharpoons ES \rightarrow E + P}} \quad ...(2.7)$$

The step of binding of substrate assumed to fast relative the rate of breakdown of the ES complex so that the substrate binding reaction is assumed to be at equilibrium. The equilibrium dissociation constant for the ES complex (K_S) is a measure of the enzyme-substrate affinity and corresponds to substrate concentration at 1/2 V_{max}:

$$K_S = \frac{[E][S]}{[ES]} \quad ...(2.8)$$

Therefore, the higher the affinity of the enzyme for substrate when value of K_S is lower. The rate of breakdown of the ES complex limits velocity of the enzyme-catalyzed reaction , which is expressed as:

$$v = k_{cat}[ES] \quad ...(2.9)$$

where the k_{cat} is defined as the effective first-order rate constant to form free product and free enzyme by splitting the ES complex. The total enzyme concentration is expressed as ($[E_T]$ = [E] + [ES]) and rate equation for enzyme is:

$$\frac{v}{[E_T]} = \frac{k_{cat}[ES]}{[E] + [ES]} \quad ...(2.10)$$

where [E] and [ES] is concentration of free enzyme and enzyme-substrate complex respectively. Substitute the equation (2.8) for [ES] into equation (2.10) to obtain equation (2.11) as:

$$\frac{v}{[E_T]} = \frac{k_{cat}([E][S]/K_s)}{[E] + [E][S]/K_s} \quad \dots (2.11)$$

The equation (2.11) divided by [E] and multiply by K_S for both numerator and denominator to obtain the expression for velocity of enzyme-catalyzed reaction as:

$$v = \frac{k_{cat}\,[E_T][S]}{K_S + [S]} \quad \dots (2.12)$$

The velocity of enzyme catalyzed reaction equation represented as Eq (2.13) when substituting $V_{max} = k_{cat}[ET]$,

$$v = \frac{V_{max}\,[S]}{K_S + [S]} \quad \dots (2.13)$$

There are some assumptions made for the Michaelis-Menten model such as substrate binding reaction is at equilibrium due to fast reaction, the concentration of substrate remains constant at time course of the reaction ([So]= [St]) and the conversion of product back to substrate is negligible.

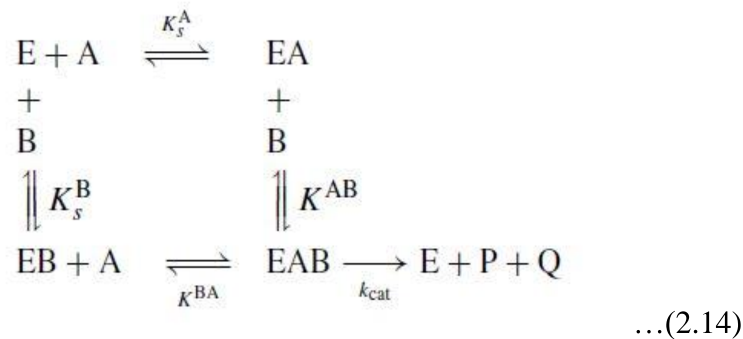
Two-substrate reactions:

Two-substrate reactions, also called as ping-pong or sequential reactions, are catalysed reactions that occur between two or more substrates. One or more products must be released before all substrates may react in ping-pong mechanisms. In a sequential mechanism, all substrates must bind with the enzyme before the reaction occur. Moreover, it can be classified

into ordered or random. In order sequential mechanism, it occur in a specific order for the substrates reaction with enzyme and product formation. In random sequential mechanism, substrate reaction with enzyme and product formation is not obligatory (Marangoni, 2013).

a) Random-sequential Bi Bi mechanism:

There is no particular order in the sequential substrates A or B reacts with enzyme to form the ternary complex EAB. The general scheme for random-sequential bi bi mechanism is expressed as:

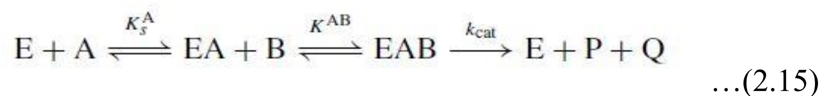


…(2.14)

where the equilibrium dissociation constant for the binary enzyme–substrate complexes EA and EB (K_S^A and K_S^B), the equilibrium dissociation (K_S) or steady-state Michaelis (K_M) constants for the formation of the ternary enzyme–substrate complexes EAB (K^{AB} and K^{BA}).

b) Ordered-sequential Bi Bi mechanism

There is a specific order for this mechanism, the enzyme combine with substrate A first, followed by binding with substrate B, to produce ternary complex EAB. The general scheme for ordered-sequential bi bi mechanism expressed as:

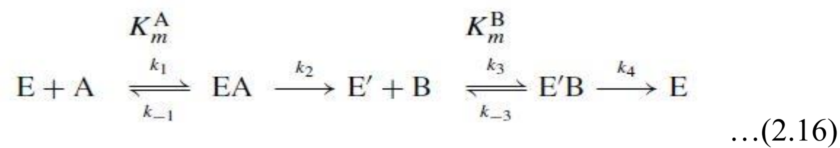


…(2.15)

where the equilibrium dissociation constant for the binary enzyme–substrate complex EA (K_S^A), the equilibrium dissociation (K_S) or steady-state Michaelis (K_M) constants for the formation of the ternary enzyme–substrate complex EAB (K^{AB}).

c) Ping-pong Bi Bi mechanism

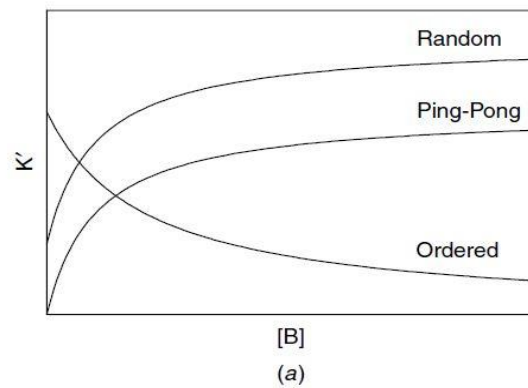
The enzyme starts by reacting with substrate A, then releases product P and forms the enzyme species E'. Then, substrate B reacts with E' and form second product Q and free enzyme E by breakdown the $E'B$ complex. There no ternary complex formation for this mechanism. The general steady-state scheme for ping-pong bi bi mechanism expressed as:



…(2.16)

d) Differences between random, ordered and ping-pong mechanisms

The differentiations between these three mechanisms can be achieved by careful scrutiny of K' versus substrate concentration patterns. The dependence of the apparent Michaelis constant (K') on the fixed substrate concentration of these three mechanism which shown in the figure below.



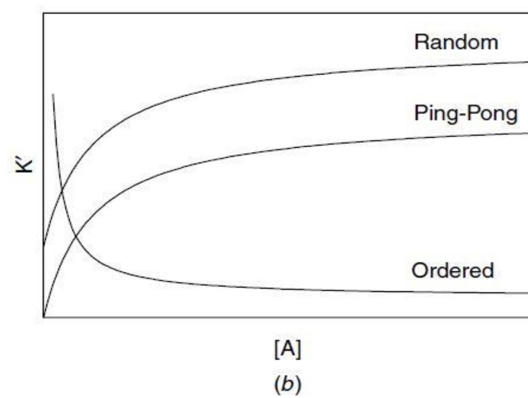


Figure 2.2: Dependence of the apparent Michaelis constant (K′) on the concentration of fixed substrate for random-sequential, ordered-sequential, and ping-pong mechanisms (Marangoni, 2013).

CHAPTER 3

METHODOLOGY

3.1 Research Methodology

The methodology of development of mathematical model for enzyme reactor is shown in **Figure 3.1**. There are five important steps to follow to run the simulations that enables to predict the conversion and rate of reaction of enzymatic reaction. To develop a mathematical model, the mole balance of packed bed reactor and rate equation for enzymatic reaction is required. The published experimental data is required to run the simulation. The kinetic parameters and the thermodynamic parameters are also estimated based on the literature data.

3.2 Develop Mole Balances for Packed Bed Reactor (PBR)

At steady state, mole balance for PBR in differential form expressed as:

$$-\frac{dF_A}{dW} = -r'_A \quad \text{... (3.1)}$$

While in integral form expressed as:

$$W = F_{AO} \int_0^X \frac{dX}{-r'_A} \quad \text{... (3.2)}$$

Where differential form equation for PBR in terms of concentration of C_A expressed as Equation 3.3, when substituting $F_A = C_A v_o$ into Equation 3.1:

$$-\frac{dC_A}{dW} = \frac{-r'_A}{v_o} \quad \text{... (3.3)}$$

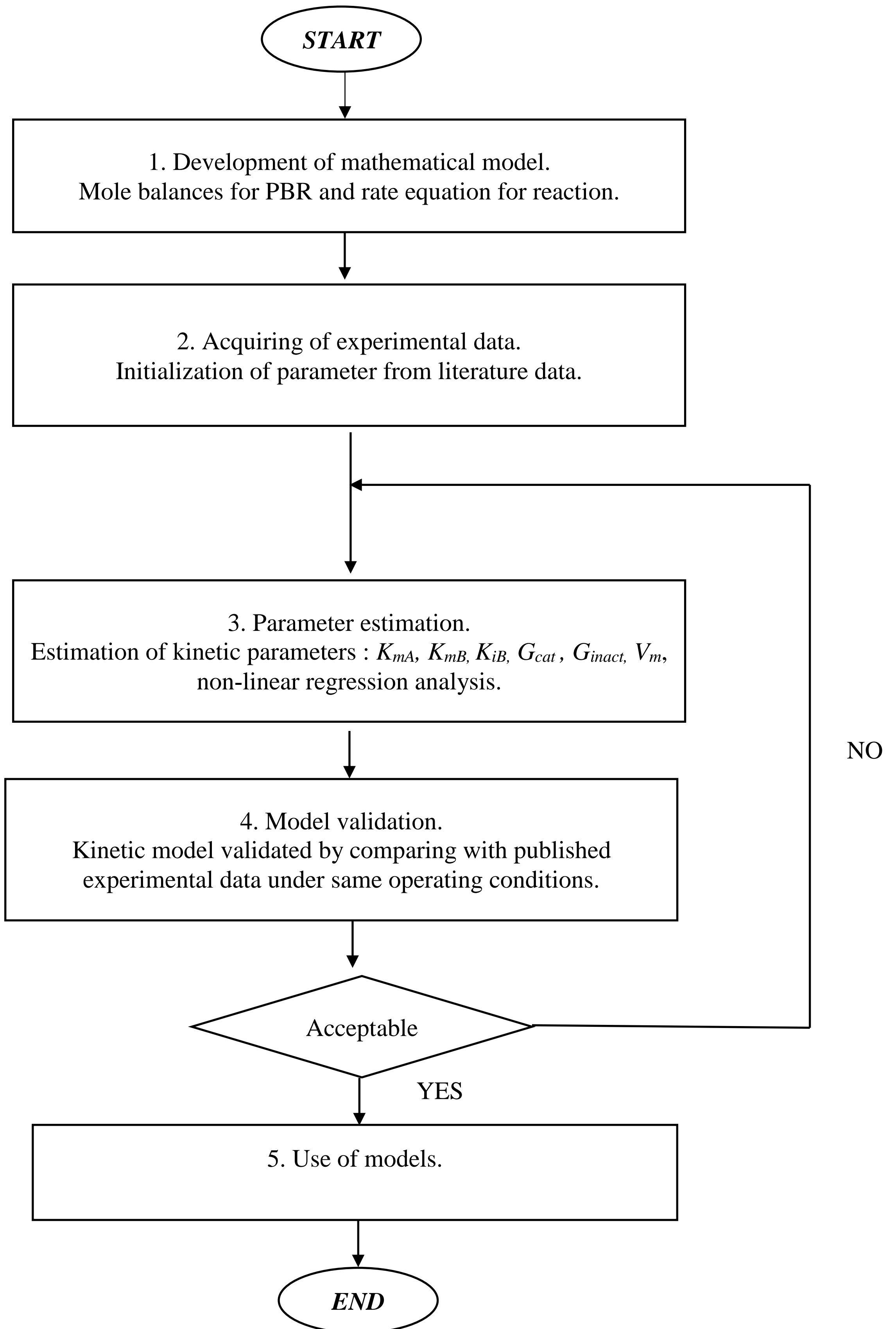
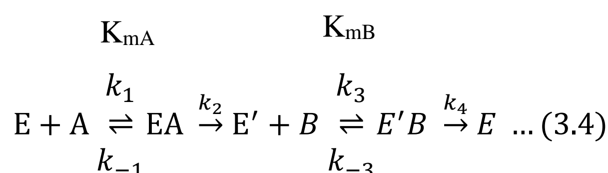


Figure 3.1: Strategy of development of mathematical model for enzymatic packed bed reactor (Vasi & Findrik, 2011)

3.3 Develop Rate Equation (Ping-pong Bi Bi)

The enzyme (E) combines with substrate (A) first, followed by the release of product (P) and the formation of the enzyme species (E’). Next, substrate (B) binds to (E’) and the free enzyme E and the second product (Q) is formed by breaking down the (E’B) complex. No ternary complex formed for ping-pong mechanisms (Marangoni, 2013).

At general steady-state, ping-pong bi bi mechanism:



Rate equation:

$$\mathrm{v} = k_4[E'B] \quad \ldots (3.5)$$

Steady state Michaelis constants:

$$K_{mA} = \frac{k_{-1} + k_2}{k_1} = \frac{[E][A]}{[EA]} \quad \ldots (3.6)$$

$$K_{mA} = \frac{k_{-3} + k_4}{k_3} = \frac{[E'][B]}{[E'B]} \quad \ldots (3.7)$$

Mass balance for enzyme:

$$[E_T] = [E] + [EA] + [E'] + [E'B] \quad \ldots (3.8)$$

The Ping-Pong Bi–Bi mechanism is suggested for lipase-catalysed esterification, transesterification and hydrolysis reactions occurred with two substrates. Based on the

proposed mechanism, the reaction rate was given by Equation (3.9) (Kinetic model I) when there was not any inhibition effect (Gu et al., 2014):

$$v = \frac{V_{max}\, C_A\ C_B}{C_A\, C_B + K_{mA}\, C_B + \ C_A\, K_{mB}} \quad \ldots (3.9)$$

where v is the initial reaction rate (mol/Lh), V_{max} is the maximum reaction rate (mol/Lh), C_A is the initial methyl caffeate concentration (mol/L), C_B is the initial 1-propanol concentration, K_{mA} and K_{mB} are the Michaelis constants for substrates (A) and (B) respectively.

The ping-pong bi–bi mechanism, with substrate B as inhibitor is given by Equation (3.10) (Kinetic model II) as (Gu et al., 2014):

$$v = \frac{V_{max}\, C_A\ C_B}{C_A\ C_B + K_{mA}\ C_B(1 + \frac{C_B}{K_{iB}}) + \ C_A\ K_{mB}} \quad \ldots (3.10)$$

where v is the initial reaction rate (mol/Lh), V_{max} is the maximum reaction rate (mol/Lh), C_A is the initial methyl caffeate concentration (mol/L), C_B is the initial 1-propanol concentration, K_{mA} and K_{mB} are the Michaelis constants for substrates (A) and (B) respectively, K_{iB} is the inhibition constant for B.

3.4 Estimation of Kinetic Constants and Operating Parameters

The kinetic parameters and operating parameters for the simulation is taken from various sources in **Table 3.1** and **Table 3.2** respectively. The range of operating parameters for referred to (Wang et al., 2013) is tabulated in **Table 3.3**.

Table 3.1: Kinetic parameters for lipased catalysed reactions

Substrates	Substrate / Enzyme (mass ratio)	K_{mA} (mmol.L^{-1})	K_{mB} (mmol.L^{-1})	V_{max} (mmol.min^{-1}g^{-1})	K_{iB} (mmol.L^{-1})	References
Caffeic acid + 2-phenylethanol	1:90	432.10	1279.00	16.95	482.40	(Wang et al., 2014)
Caffeic acid + 2-phenylethanol	1:18	42.9	165.7	0.89	146.2	(Gu et al., 2014)
Phenyl benzoate + hydrazine		2837	1263	2340	4084	(Yadav & Borkar, 2010)

Table 3.2: Operating parameters for lipased catalysed reactions

Substrates	Temperature (°C)	Concentration C_A (mM.L^{-1})	Concentration C_B (M.L^{-1})	References
Caffeic acid + 2-phenylethanol	60	15-40	0.16-0.64	(Wang et al., 2014)
Caffeic acid + 2-phenylethanol	80	10-65	0.04-0.8	(Gu et al., 2014)
Phenyl benzoate + hydrazine	50	10	0.02	(Yadav & Borkar, 2010)

Table 3.3: Range of operating parameters for synthesis of propyl caffeate (Wang et al., 2013)

Operating Parameter	Range	Units
Volume of reactor	3.33×10^{-8}	L/sec
Temperature	25 – 75	°C
Substrate concentration	0.0052 - 0.0412	mol/L
Substrate Molar Ratio	1:10 – 1:80	No unit
Weight of catalyst	0.09	g
Reaction time	2.5	h

3.5 Equilibrium Model for Effect of Temperature on Enzyme Activity

Enzyme activity affected by temperature has been considered, the enzyme denatures, losing its activity irreversibly at higher temperatures after reach a certain point. In this model, the change in enzyme activity occurs when the temperature increases is simply the combined result of the effect of temperature increasing k_{cat} and k_{inact} on two step conversion ($E_{act} \rightarrow X$), then loss of activity is depends with time, expressed V_{max}, as (Daniel & Danson, 2013):

$$V_{max} = k_{cat} \,.\, [E_o] . e^{-k_{inact} \cdot \, t} \quad \dots (3.11)$$

where V_{max} = maximum velocity of enzyme; k_{cat} = enzyme's catalytic constant; [E0] = total enzyme concentration; k_{inact} = thermal inactivation rate constant; t = assay duration.

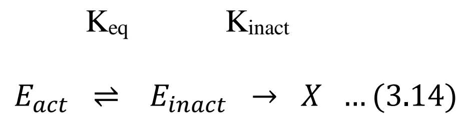
The variation of the two rate constants in Eq. (3.11) with temperature is given by :

$$k_{cat} = \frac{k_B \, T}{h} e^{-(\frac{\Delta G_{cat}}{RT})} \quad \dots (3.12)$$

$$k_{inact} = \frac{k_B\, T}{h} e^{-(\frac{\Delta G_{inact}}{RT})} \quad \ldots (3.13)$$

where kB = Boltzmann's constant; R = Gas constant; T = absolute temperature; h = Planck's constant; ΔG_{cat} = activation energy of the catalysed reaction; ΔG_{inact}= activation energy of the thermal inactivation process.

The Equilibrium Model is introduced a new temperature-dependent behaviour of enzymes which an intermediate inactive (but not denatured) form of the enzyme that is in rapid equilibrium with the active form, and it is the inactive form that undergoes irreversible thermal inactivation to the denatured (irreversibly inactive) state (X):



$$E_{act} \overset{K_{eq}}{\rightleftharpoons} E_{inact} \overset{K_{inact}}{\rightarrow} X \quad \ldots (3.14)$$

where E_{act} is the active form of the enzyme, which is in equilibrium with the inactive form, E_{inact}. K_{eq} is the equilibrium constant describing the ratio of E_{inact}/E_{act}; k_{inact} is the rate constant for the E_{inact} to X reaction; and X is the irreversibly-inactivated form of the enzyme.

The variation of enzyme activity with temperature by using the Equilibrium Model can be expressed by:

$$V_{max} = \frac{k_{cat} \,.\, [E_o].\, e^{\frac{k_{inact}\,.\,K_{eq}\,.\,t}{1+K_{eq}}}}{1 + K_{eq}} \quad \ldots (3.15)$$

Where

$$K_{eq} = e^{\frac{\Delta H_{eq}}{R}(\frac{1}{T_{eq}} - \frac{1}{T})} \quad \ldots (3.16)$$