Conceptual Study of Enzymatic Wax Ester Synthesis in The Batch Reactor at Steady State and Isothermal Conditions.

by

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a	total substrate concentration of a
C_s	concentration of substrate
E_0	Total concentration of enzyme
ΔG_{cat}	Activation energy of the catalyzed reaction
ΔG_{inact}	Activation energy of the thermal inactivation process
h	Planck's constant
H_{eq}	Change in enthalpy for E_{act}/E_{inact} transition
ΔH_m	Change in binding enthalpy of substrate to enzyme
ΔH_i	Change in binding enthalpy of inhibitor to enzyme
k_B	Boltzmann's constant
k_1	Apparent forward rate constant
k_{-1}	Apparent backward rate constant
k_{cat}	Turnover number
k_{inact}	thermal inactivation rate constant
K_m^A	Michaelis constant for fatty acid
K_m^B	Michaelis constant for fatty alcohol
K_i^A	Inhibition constant for fatty acid
K_i^B	Inhibition constant for fatty alcohol
Keq	Equilibrium constant
K_M	Michaelis constant
N_A	Number of mole of fatty acid
N_{A0}	Initial number of mole of fatty acid
r_A	Rate of reaction of fatty acid
R	Gas constant
R^2	High correlation coefficients
S	Substrate concentration
S_0	Initial substrate concentration
t_R	Retention time

T	absolute temperature
T_{eq}	temperature when E_{act}/E_{inact} equilibrium is at its midpoint
V	Volume of batch reactor
V_{max}	maximum rate achieved by system

ABSTRAK

Wax ester adalah salah satu bahan terpenting dengan aplikasi berpotensi dalam industri makanan, kosmetik, dan farmaseutikal. Eterifikasi enzimatik lebih dipilihi daripada tindak balas kimia kerana tindak balas ringan dan lebih mesra alam. Model matematik berdasarkan keseimbangan mol dalam reaktor kumpulan dan persamaan kadar enzimatik Ping Pong Bi-Bi telah dibentukkan untuk mengkaji parameter kinetik, parameter termodinamik dan parameter operasi esterifikasi enzimatik dengan penggunaan POLYMATH (versi 6.0). Model ini disahkan dengan data eksperimen yang diperoleh dari sumber literatur sementara nilai parameter tertentu dianggarkan. Data eksperimen dan teori menunjukkan kesepakatan yang baik ($0{,}88 \leqslant R^2 \leqslant 0{,}98$) membuktikan model dan parameter yang dianggarkan tepat untuk mewakili proses sebenar. Menurut hasil simulasi, nilai K_m dan ΔG_{cat} yang lebih rendah membawa kepada hasil pengeluaran yang lebih tinggi kerana mengimplikasikan tenaga yang lebih rendah diperlukan untuk pengikatan substrat dan menjadi pemangkin tindak balas. Sebaliknya, nilai K_i dan ΔG_{inac} yang lebih tinggi disyorkan untuk enzim kerana menunjukkan enzim kurang dipengaruhi oleh perencat dan ketahanan terhadap penyahaktifan pada suhu yang lebih tinggi. Keadaan esterifikasi optimum adalah suhu 50°C, kepekatan enzim 1.0×10^{-5} mol/L dan nisbah asid lemak-alkohol lemak. Profil enzim dilakukan untuk mengkaji corak penghambatan. Ia mendapati bahawa, alkohol lemak menunjukkan kesan penghambatan yang lebih teruk terhadap asid lemak terhadap kadar tindak balas. Secara keseluruhan, kerja saya dalam simulasi cukup memadai dan telah meningkatkan pemahaman saya mengenai pelaksanaan proses pengeluaran pada skala industri dari skala makmal. Secara keseluruhan, kerja saya dalam simulasi cukup mencukupi dan telah meningkatkan pemahaman saya mengenai pelaksanaan proses pengeluaran pada skala industri dari skala makmal.

ABSTRACT

Wax ester is one of the most important ingredients with its many potential applications in the food industry, cosmetic industry, and pharmaceutical industry. Enzymatic esterification is preferable over chemical reaction due to its mild reaction and being more environmentally friendly. A mathematical model based on the mol balance in the batch reactor and Ping Pong Bi-Bi enzymatic rate equation was developed to study the kinetic parameter, thermodynamic parameter and operational parameter of enzymatic esterification in batch reactor. POLYMATH software (version 6) was used for the simulation of this mathematical model. The model was validated with experimental data obtained from literature sources while the values of certain parameters were estimated. From the validation, the experimental and theoretical data shows a good agreement ($0.88 \leqslant R^2 \leqslant 0.98$) which proves that the model and the estimated kinetic and thermodynamic parameters are accurate to represent the actual process as R^2 greater than 0.8. According to the findings and simulation results, a lower value of K_m and ΔG_{cat} leads to a higher production yield as it indicates lower energy is required for substrate binding and to catalyze the reaction. On the other hand, a higher value of K_i and ΔG_{inac} are recommended for an enzyme because it indicates the enzyme is less affected by the inhibitor and resistance to deactivation at a higher temperature. Based on POLYMATH, optimum esterification conditions were 50°C temperature, 1.0×10^{-5} mol/L enzyme concentration and 1:2 fatty acid – fatty alcohol mole ratio. Profiling of enzyme is performed to study the inhibitory pattern. It found that, fatty alcohol shows more severe inhibition effect over fatty acid toward rate of reaction. Overall, my work on simulation is sufficiently adequate and has improved my understanding on the implementation of the production process on an industrial scale from laboratory scale.

CHAPTER 1 INTRODUCTION

1.1 Research Background

Wax ester is a high molecular weight lipid, with a combination of one fatty acid and one fatty alcohol with more than 12 carbon atoms. **Figure 1.1** show the typical chemical structure of wax ester. There are various type of wax esters and they are typically grouped into liquid and solid forms. This classification is based on their melting properties at room temperature which also depend on their saturation of fatty acids and alcohols. Wax esters derived from natural resources are non-toxic and show good biodegradability. The hydrophobic characteristic of wax ester is due to long carbon chains belonging to esterified fatty acids and alcohols. Due to this characteristic, it is always used as thickening agents, emollient agents, lubricants and also find application in cosmetic and skin care products (Aguieiras et al., 2019; Ungcharoenwiwat et al., 2016) The demand for wax ester is expected to grow at a growth rate of 3.9% in the forecast period 2020 to 2027 (Wax et al., 2020). Demand for non-petroleum wax types such as synthetic waxes is growing due to environmental concern and depletion of petroleum waxes.

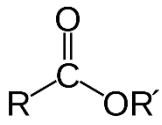

Figure 1.1:Chemical structure of wax ester

Natural sources of wax esters include those derived from animals and vegetables such as carnauba wax, jojoba oil, beeswax and spermaceti wax (Aguieiras et al., 2019). However, natural wax esters extracted from plant and animal sources are expensive and scarce. Thus, scale-up synthesis of wax esters from renewable raw materials by chemical or enzymatic synthesis has become important. Comparing both methods, chemical-catalysed method has many drawbacks such as high energy consumption as reaction occurred at elevated temperature, degradation of

esters produced and need for corrosive acids. Chemical-catalysed reaction consumed 34% more energy compare to enzyme-catalysed reaction (Petersson et al., 2005). The product yield via using chemical-catalysed method also lower than that catalysed by enzymatic method. This is proven by research conducted by Ishak et al. (2016).

According to the research by Ishak et al. (2016), kojic acid ester synthesized from kojic acid and acetonitrile via enzymatic catalysed method can achieved conversion up to 82 % with a molar ratio of acid to alcohol in 2:1. Compared to enzymatic methods, the yield of kojic acid ester is 67% in chemical catalysed method using dimethylformamide (DMF) as solvent which is much smaller than enzymatic method. The lower yield of wax ester may be due to the production of contaminants during chemical catalysed processes. Multistep separation process required to obtain wax ester will increase the operational cost. Besides that, separation of DMF from product is also necessary since it is toxic and not permitted for use in the food industry(Ishak et al., 2016). Since there is less side product formed in the enzymatic process, the separation method is much simple compare to chemical catalytic method. Moreover, a higher operation temperature is required in chemical catalysed methods compared to enzyme catalysed method. For example, kojic acid ester is produced at 110°C using chemical solvents as catalysts while lipase fulfils the same function at 44°C (Ishak et al., 2016).

Hence, enzyme-catalysed method is a sustainable process as it offers mild reaction conditions and is an environment friendly process. The specificity of the enzymes used in enzymatic catalysed methods enable production of high quality products (Sin et al., 2005). Esterification and transesterification are the main reaction catalysed by lipase to produce synthetic wax ester. The general chemical equation of esterification and transesterification are shown below.

Esterification reaction:

$$RCOOH + R'OH \rightleftharpoons RCOOR' + H_2O$$

Transesterification reaction:

$$RCOOR + R''OH \rightleftharpoons RCOOR'' + R'OH$$

Even though the enzyme catalytic method is more environmentally friendly and generates a higher yield of wax ester, but a higher cost is required during the operation. There are two reasons which contribute to this high cost of enzymatic wax ester production. Firstly, the cost of the enzyme is high. Secondly, the lifespan of the enzyme is limited and may shorten due to the deposition of free fatty acid from raw material onto the biocatalyst pores, which may hinder the interaction between substrate and lipase. This shortens the lifespan of the lipase enzyme (Aguieiras et al., 2019).

To conclude, wax ester has many potential applications and shall be a key component in cosmetic and skin care products to improve their emulsifying properties and water absorption capability in the next decades. It also will be widely used as a covering agent or entrapment of medicine in the pharmaceutical sector. Enzyme-catalysed reaction is more favoured to produce wax ester due to its lower energy consumption, higher yield of ester and lower degree of wastewater production. It is more favourable to utilize an enzyme catalysed method to produce the product, as chemical methods will utilize solvents that may pollute the environment. Its operation at a higher temperature will also corrode the equipment. However, more effort is required to minimize the denaturation of the enzyme to enable the enzyme to be reused more often or prolong the lifespan of the enzyme.

1.2 Problem Statement

Due to a high demand for wax esters in various industry, production of wax ester is developed in large scale through chemical-catalysed and enzyme-catalysed methods. However, the prevailing chemical route to produce wax ester may harm the environment as well as requiring a lot of energy and cause corrosion of equipment. Development of enzyme-catalysed method is suitable and more environmentally friendly to produce wax ester for commercial use.

The performance of enzyme-catalysed reaction is affected by reaction time, enzyme concentration and substrate molar ratio (Gunawan et al., 2005). The batch reactor is preferred for enzymatic reaction because it is easier to operate and mixtures can react longer to reach a high conversion. The study on enzyme catalysed wax ester is mostly working with small volumes in laboratory scale operations. The feasibility of such a process is difficult to assess using small scale results compared to an industrial scale production. Hence, simulation study is often adopted to justify the feasibility of a reaction process on a larger scale.

Hence, the main objective of this research is to develop a mathematical model in order to study the effect of each parameter on lipase-catalysed wax ester synthesis in a large-scale batch reactor. A computational software (POLYMATH) shall be used to solve the proposed mathematical model. Typically, the rate of reaction of enzyme-catalysed process obeys the Michaelis Menten equation. The kinetic parameters of the equation tell us about the kinetic behaviour or specifically the maximum achievable rate of reaction (V_{max}) and the degree of binding of both the substrate and inhibitor to the active site of the enzyme (K_m and K_i). The values of the thermodynamic parameters (ΔG_{cat} and ΔG_{inac}) are unique for a specific enzyme which is often foreseen as its potential during the catalytic reaction. A wide range of kinetic parameter and thermodynamic parameter values are often reported in literatures depending on the source the

enzyme was extracted from, method of preparation and reaction media. Understanding the kinetic behaviour of enzyme catalysed reaction could help to evaluate the feasibility of enzyme-catalysed wax ester synthesis in the batch reactor. It can be evaluated in terms of the yield of wax ester and rate of reaction at certain operating conditions. These parameters need to be evaluated properly to ensure that the enzyme prepared is adequate for the reaction and leads to a profitable process.

1.3 Research Objective

The main aim of this research is to study the feasible of lipase-catalysed wax-ester synthesis in a batch reactor based on kinetic parameter values. The following are the deliverable objectives.

i. To develop a mathematical model for lipase-catalysed wax ester synthesis in batch reactors.
ii. To explore the relationship between each parameter towards the yield of wax ester.
iii. To propose the most adequate enzyme profile (kinetic parameter values) for the reaction.

CHAPTER 2 Literature Review

2.1 Wax Ester Application

Wax esters are mostly used in cosmetics, pharmaceuticals, and surfactants. Long chain esters derived from fatty acids and alcohols contain carotenoids, retinoids and tocopherols which enable them to act as antioxidants as components of cosmetic formulations. Wax ester plays an important role in improving the viscosity of lubricants, emulsifying properties and water absorption capabilities in cosmetic products as well as act as binders, stabilizers and conditioners. Hence, it is useful in make-up products, creams, and lotions. It is also used in sunscreens, baby products or body creams due to its water-resistance properties and mineral oil solubility (Ahmad and Ahsan, 2020). In addition, wax ester is also applied in pharmaceutical products as binding and covering agent (Rudolph, 2020). For example, beeswax and carnauba wax are used as surfactants and emulsifiers to increase drug entrapment efficiency (Tada et al., 2014). Wax ester is also an important raw material in the production of penicillin to avoid the formation of foam during bioprocesses. In the food industry, ester-type gum bases are used worldwide as food additives. Short chain esters are used as flavouring agents in dairy products as it gives a sweet fruit smelling aroma. It helps to provide specific textures and surface coatings for chocolates and fruits (Tada et al., 2014). Antioxidant properties of ester helps to preserve food quality for a longer time via wax coating, preventing water loss from fruit or food.

2.1 Enzyme-catalyzed wax ester synthesis

The synthesis of wax esters at a reduced cost and in a continuous manner allows production to meet its high demand. A viable option to synthesis them is via transesterification or esterification using lipase and different raw materials in a batch mode (Aguieiras et al., 2019; Hayes and

Kleiman, 1996; Tomke and Rathod, 2020; Ungcharoenwiwat and H-Kittikun, 2020). It is rare to find articles reporting a continuous mode of synthesis (Dias et al., 2018; Itabaiana et al., 2013). To improve the yield of wax esters synthesized with the aid of catalyst, it is crucial to study its rate expression and reaction mechanism. This is mainly because understanding of said mechanics may contribute vital information for the designing an optimum process.

Several types of wax esters have been synthesized via enzyme catalysed route using commercial immobilized enzyme such as DHSA, Novozym 435, Lipozyme and others for different raw material. For instance, synthesis of octyl-9,10-dihydroxystearate in large scale using DHSA in batch mode stirred tank with one multi-bladed propeller was performed by Awang and his co-worker. Esterification percentage was 90% at a temperature of 50°C, a reaction time of 3 h, catalyst condition of 10 w/w% and an agitation speed of 300 rpm (Awang et al., 2007).

Radzi et al.(2006) has proposed a large-scale production of liquid ester from oleic acid and oleyl alcohol using Novozym 435. The highest wax ester production of 96.7% was achieved using 104 g of Novozym 435 at 49.7°C with 388 rpm agitation speed (Radzi et al., 2006). Novozym 435 also used by Abdul Rahman et al. (2011) in esterification of succinic acid with oleyl alcohol. It yielded in 85% conversion at optimal conditions 41.1°C, using 20 mg of Novozym 435 (Abdul Rahman et al., 2011).

Optimal condition for wax ester synthesis from palm and soybean fatty acid distillates by biocatalyst fermented via fungal strain Rhizomucor miehei (IDAC) and babassu cake as substrate was 8.8 U/g of biocatalyst at 50 °C in a magnetic stirrer tank with a temperature-controlled water bath. Percentage yields of SFAD-cetyl, SFAD-oleyl and PFAD-oleyl esters obtained at these optimum reaction conditions was 80% (Aguieiras et al., 2019).

A research of wax ester derived from palm oil with oleyl alcohol using Lipozyme was conducted by Sin and co-worker. They found that, with molar ratio 3:1 at 50.4℃ in a bioreactor with impeller at speed 242.1 rpm, optimum productivity of wax ester can be achieved, which is 92.3% yields (Sin et al., 2005). Similar research was also done by Gunawan and Suhendra (2010). The optimum percentage yield of ester from refined, bleached and deodorized (RBD) palm oil transesterification and RBD palm kernel oil transesterification were 83% and 87% respectively at optimum condition (Gunawan and Suhendra, 2010).

The highest jatropha oil-based wax ester production of 89% and 86% were achieved using 10 U of immobilized lipase EQ3 (*Burkholderia* sp. EQ3) and Lipozyme RM IM (*Rhizomucor miehei*) in isooctane and hexane respectively (Ungcharoenwiwat et al., 2016). Synthesis of wax esters by coconut oil and oleyl alcohol at molar ratio 1:3 at 30℃ for 12 h achieved 88% conversion using 10 U of Lipozyme RM IM (Ungcharoenwiwat and H-Kittikun, 2020).

2.1.1 Michaelis–Menten Model

Michaelis-Menten equation is frequently used to describe the kinetic behaviour of enzyme-catalysed reactions. The equation tells us that the rate of reaction is strongly dependent on the concentration of substrate at a specific amount of enzyme and reaction conditions. More complex kinetic models are also available which consider substrate inhibition, substrate activation, random pathways of reaction step or allosteric effects (Cornish-bowden, 2013). Regardless, kinetic model incorporated with inhibition effects and enzyme deactivation is sufficient for enzyme-catalysed synthesis of fine chemical such as esters.

Michaelis and Menten equation uses initial rate of reaction to justify the kinetic behaviour. It helps to simplify the complexity of steady state kinetic study as there are over 800 enzymes with

complex curves for various reasons (Mary and Ojh, 1977). The advantages of initial rate equation include:

i. It simplifies the complexity of process such as inhibition by accumulated products, loss of catalytic activity, spontaneous mutarotation of products and inversion which is the use of polarimetric methods to follow the reaction.
ii. Considers a forward direction of enzymatic reaction. A reverse reaction is only considered after there is accumulation of product.
iii. Initial rate equations are easier to derive.
iv. At time equal to zero, there is no drift of pH.

K_M was first introduced in Haldane's book (Cornish-bowden, 2013). It is a numerical value corresponding to the substrate concentration at which the initial rate will be half of the maximum rate. A lower value of K_M means that the rate of reaction shall approach V_{max} with a lower concentration of substrate (Marangoni, 2013) With the introduction of K_M, the equation is written in a form resembling the following:

$$v = \frac{Va}{K_m + a} \qquad 2.1$$

where v = rate of formation of product,

V = the limiting rate,

a = the total substrate concentration of a,

K_M = the Michaelis constant.

However, rather than express the rate of enzymatic reaction in terms of amounts of products changes, kinetic equation relates the rate of formation of product to the concentration of substrate, which is preferable.

The Eq 2.2 is obtained with a series of derivation.

$$v = \frac{V_{max} C_S}{K_M + C_S} \quad 2.2$$

where v = rate of formation of product

V_{max} = the maximum rate achieved by system during saturating substrate concentration

C_s = the concentration of substrate

K_M = the Michaelis constant or affinity constant of enzyme toward specific substrate.

A simple model of enzyme action can be described as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

V_o is the initial velocity of reaction while v_{max} occurs when all the enzyme is tied up with the substrate, which can be known as the concentration of enzyme substrate complex equal to the total enzyme concentration. Both can be expressed as:

$$v_o = k_{cat}[ES] \quad 2.3$$

$$v_{max} = k_{cat}[E]_{total} \quad 2.4$$

At a steady state, the rate of formation and rate of consumption of enzyme-substrate complex are the same. Thus, it can be described as:

$$k_{-1}[\mathrm{ES}] + k_{cat}[ES] = k_1[\mathrm{E}][\mathrm{S}] \quad 2.5$$

The equation can be solved for [ES] to gives;

$$[ES] = \frac{[E]_{total}[S]}{K_M + [S]}] \quad 2.6$$

Where, the Michaelis's constant (K_M) and total enzyme concentration ($[E]_{total}$) are given by:

$$K_M = \frac{k_{-1} + k_{cat}}{k_1} \quad 2.7$$

$$[E] = [E]_{total} - [ES] \quad 2.8$$

Substitute Eq 2.8 into Eq 2.5, we will get Eq 2.3, which is the equation that is generally used nowadays to evaluate performance of an enzyme.

The Michaelis-Menten model was developed with several assumptions (Marangoni, 2013).

1. Substrate binding reaction is at equilibrium in the form of fast formation and break down of enzyme-substrate complex.
2. The conversion product back to substrate is negligible because very little product is accumulated during the reaction time.
3. Enzyme is stable during reaction.
4. Initial rate is used as reaction velocities because enzyme remain stable at the early stage and it is at optimum saturation.
5. Since, the progress curve is linearly when conversion is below 20%, hence, the reaction velocity is directly proportional to total enzyme concentration.
6. Michaelis-Menten equation had been modified to account the effect of inhibition.

Inhibitors are classified into competitive, non-competitive, and uncompetitive inhibitors. Competitive inhibitor has no effect on V but increases the apparent value of K_m. Its effect can be classified as V/K_M. A non-competitive inhibitor will decrease the apparent value of V but has no effect on K_M (Cornish-bowden, 2013).

2.1.2 Bi-substrate Kinetic Model

Wax ester synthesis from lipase catalysed esterification or transesterification is a two substrates reaction which involve fatty acid and fatty alcohol. The general two-substrate enzyme-catalysed reactions of the type are shown below.

$$A + B \leftrightharpoons P + Q$$

There are three types of mechanisms of enzymatic reaction that have been identified. There is random sequential bi-bi mechanism, ordered sequential bi-bi mechanism and ping pong bi-bi mechanism. The detail mechanism for each type is described in the following sub-sections.

i. Random sequential bi-bi mechanism

There is rapid binding of either substrate A or B to the enzyme to form enzyme substrate complex ([EA] or [EB]). Another substrate will bind to the complex to form a ternary complex ([EAB]). Both substrates must present in the active site of enzyme to transform the substrate into the product. The substrates and products are bound and then released in randomly and no preferred order. (Ulusu, 2015). The mechanism of enzyme is shown in **Figure 2.1**. Rate equation by total enzyme concentration is shown below.

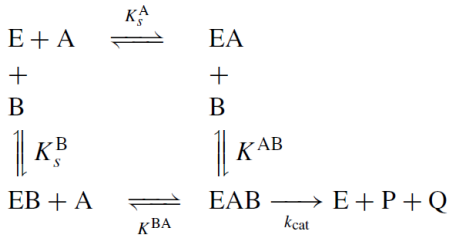

Figure 2.1: Scheme of random sequential bi-bi mechanism (Marangoni, 2013).

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_S^A K^{AB} + K^{AB}[A] + K^{BA}[B] + [A][B]} \qquad 2.9$$

ii. Ordered sequential bi-bi mechanism

All substrates are attached to the enzyme before the first product is released. Unlike random sequential mechanism, substrate A must bind to the enzyme first to form an enzyme-substrate complex ([EA]), followed by substrate B to form a ternary complex ([EAB]). The addition of substrate and formation of product must be in a specific and defined order or sequence (Ulusu, 2015). For example, if substrate B binds to enzyme first before substrate A, a dead-end inhibition complex will form, leading to no reaction. Reaction for this mechanism can be diagrammed as **Figure 2.2.**

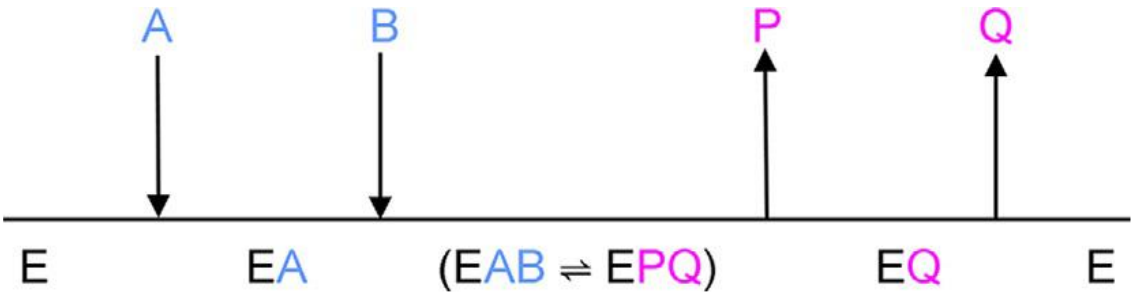

Figure 2.2: Schematic diagram of ping pong bi-bi mechanism (Roskoski, 2015)

$$E + A \overset{K_S^A}{\leftrightarrow} EA + B \overset{K^{AB}}{\longleftrightarrow} EAB \xrightarrow{k_{cat}} E + P + Q$$

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_S^A K^{AB} + K^{AB}[A] + [A][B]} \qquad 2.10$$

iii. Ping-pong bi-bi mechanism

Similar to ordered sequential bi-bi mechanism, substrate A binds to the enzyme first, then only can substrate B bind to the enzyme. The difference among both mechanisms is the enzyme will change to an intermediate form between the additions of two substrates. The intermediate forms because the first substrate transfers a piece of its fragment to the enzyme which will alter the function of the enzyme and enable the binding of the second substrate. The second substrate binds to the intermediate by picking up the transferred piece after the release of the first product (Roskoski, 2015). There is a fast leaving group product (Zhang et al., 2019). The second product forms after isomerization substrate B binds to the intermediate. Rate equation by total enzyme concentration is described in Eq 2.11. Reaction for this mechanism can be diagrammed as **Figure 2.3.**

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E' + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'B \xrightarrow{k_4} E$$

$$\frac{v}{V_{max}} = \frac{[A][B]}{\frac{k_4}{k_2}K_m^A[B] + K_m^B[A] + [A][B]\left(1 + \frac{k_4}{k_2}\right)} \quad 2.11$$

Figure 2.3: Schematic diagram of ping pong bi-bi mechanism (Roskoski, 2015)

2.1.3 Kinetic Model Wax Ester Synthesis

Generally, lipase-catalysed reaction can be described with the ping-pong bi bi mechanism (Stergiou et al., 2013). The kinetic model is vital when designing for a model to be scaled-up to achieve higher productivity and cost reduction. According to Jaiswal and Rathod (2019), the kinetic evaluation revealed that esterification of cetyl alcohol and capric acid using Fermase CALB™10000 followed the ping pong bi-bi mechanism (Jaiswal and Rathod, 2019). Capric acid binds to lipase first, then the first product, which is a water molecule, is released from the enzyme-acid complex. The enzyme is reformed to another structure which differ slightly to the initial conformation and binds with alcohol. The unstable complex releases the desired product, the cetyl caprate ester and enzyme with its initial conformation after isomerization.

Another similar justification of kinetic mechanism of esterification of butyric acid and cinnamyl alcohol reported by Waghmare and his co-worker. They found that, ping-pong bi-bi mechanism with inhibition by both substrates shows a good experimental fit to kinetic data from both experimental and simulated results. In ping-pong bi-bi model, isomerization takes place twice. The substrate fatty acid will bind to the enzyme surface to form an acid-lipase complex. The first isomerization occurs when acyl-enzyme intermediate forms and a water molecule is released. The fatty alcohol will bind to the acyl-enzyme complex to form another complex, so that the

second isomerization occurs. The final product ester is released and the lipase is ready for another cycle of reaction (Waghmare et al., 2017).

A dead-end complex form when there is an irreversible binding of either the alcohol first binding to the enzyme or the acid binding to the acyl-enzyme intermediate. When there is an inhibition of acid substrate, it will inhibit the transfer of acyl moiety to alcohol, thus halting the reaction (Chowdary and Prapulla, 2005). A scheme of ping pong bi-bi mechanism with dead end complex is shown in **Figure 2.4**.

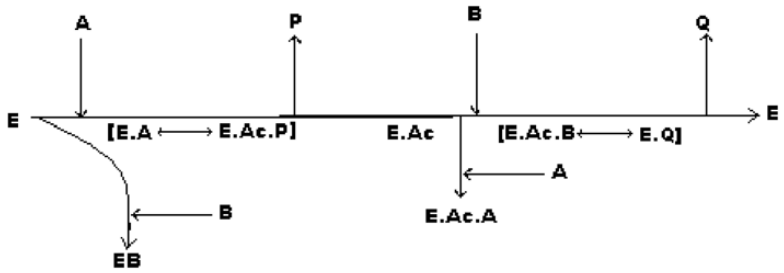

Figure 2.4: Ping pong bi–bi mechanism with dead end complex (Chowdary and Prapulla, 2005)

In Khan et al., (2016), it is proposed that an ordered bi-bi mechanism with dead end formation better describes the reaction of palmitic acid and n-butyl alcohol via Fermase CALB™10000. The mechanism described that the biocatalyst reacts with palmitic acid to form an enzyme substrate complex, then the complex further reacts with n-butyl alcohol. The complex then splits into wax ester, water and lipase (Khan et al., 2016). Parikh et al. (2019) also reported that lipase catalysed synthesis of propyl caprate is incorporated into an ordered bi-bi mechanism according to Lineweaver–Burk plot. Enzyme will first bind to capric acid to form an enzyme substrate complex. Then the alcohol binds to the complex to undergo isomerization to form the desired products, which are propyl caprate and water. The enzyme will be released at this point with the product. A dead end complex may form during the binding of capric acid to enzyme, if there are no other molecules to bind to the enzyme, halting the reaction (Parikh et al., 2019). **Figure 2.5** shows the scheme of ordered bi-bi mechanism with dead end inhibition.

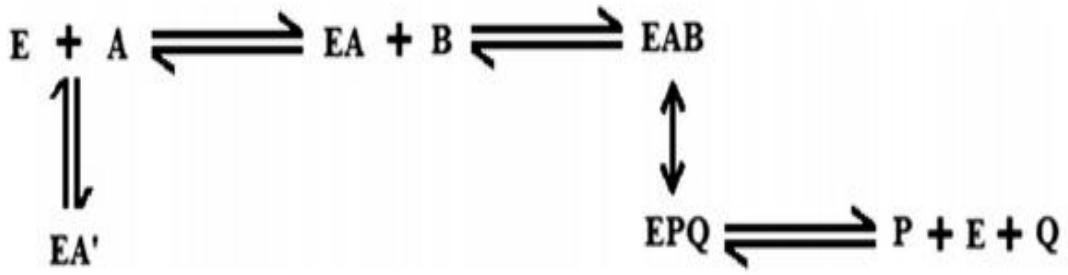

Figure 2.5: Ordered bi–bi mechanism with dead end complex (Parikh et al., 2019)

2.1.4 Kinetic and Thermodynamic Parameter Values

Ping pong bi-bi and ordered bi-bi model are always being used to evaluate the reaction behaviour of the lipase-catalysed wax ester synthesis. Both models have been proposed by several researcher as shown in table below. The values of the kinetic parameters reported in the literatures is summarized in **Table 2.1**.

Table 2.1: Kinetics parameters of lipase-catalyzed wax ester synthesis

Lipase Sp	Wax Ester	K_m^A (mol/L)	K_m^B (mol/L)	K_i^A (mol/L)	K_i^B (mol/L)	K_i (mol/L)	References
Fermase CALB 10000	Cinnamyl butyrate	0.0004	0.1549	0.1263	0.0001	-	(Waghmare et al., 2017)
Fermase CALB™10000	Cetyl caprate ester	0.0119	8.45	0.0202	0.000355	-	(Jaiswal and Rathod, 2019)
Candida rugosa	Methyl oleate	0.033	0.034	0.71	0.003	-	(Zaidi et al., 2002)
Candida rugosa	Ethyl oleate	0.17	0.18	0.53	0.058	-	(Zaidi et al., 2002)
Candida rugosa	Oleyl oleate	0.71	0.42	0.66	0.60	-	(Zaidi et al., 2002)
Candida rugosa	Oleyl butanoate	0.76	0.28	0.52	0.23	-	(Zaidi et al., 2002)
Lipase Fermase	Propyl caprate	0.0020005	0.00056	0.00092	0.00056	-	(Parikh et al., 2019)
Novozym 435	Cetyl oleate	0.701	0.688	0.0163	0.0448	-	(Garcia et al., 2000)
Novozym 435	hexyloctanoate	17.09	1.205	0.173	0.355	-	(Lopresto et al., 2014)
Novozym 435	isopropyl esters	0.3411	0.8282	0.0653	0.0022	-	(Garcia et al., 1999)
Novozym 435	isoamyl butyrate	0.00303	0.00306	6.55	1.05	-	(Bansode et al., 2017)
Lipozyme RM IM	Stearyl oleate	0.011447	0.315	-	-	0.871	(Goma-Doncescu and Legoy, 1997)
Lipozyme	Ethyl oleate	0.120	0.190	-	-	0.040	(Chulalaksananukul et al., 1990)
Lipozyme RM IM	Oleyl oleate	0.203	0.031	-	-	0.536	(Gofferjé et al., 2014)
R. miehei	Ethyl oleate	1.49	1.48	-	-	0.409	(Oliveira et al., 2001)
Lipozyme TLIM	Palm oil ester	0.3147	0.9483	-	-		(Khan et al., 2016)

Notes: A is fatty acid. B is fatty alcohol

When the carbon chain of a fatty alcohol is longer, K_M^B increases and results in a decrease of activity. An alcohol with a longer chain is less nucleophilic and has a larger alcohol moiety. Effectiveness of the nucleophilic attack of alcohol on acyl decreases when there is an increase of carbon atoms of fatty alcohols. Hence, a slower reaction rate occurs when the distance to reaction group increases (Jaiswal and Rathod, 2019). Besides that, the reactivity of long chain fatty alcohol also decreases due to the formation of cis-isomer.

For fatty acids, the longer the carbon chain, the smaller the value of K_M^A, and the larger the value of K_i^A. This may be due to the hydrophobicity, electrophilic character of acyl moiety and steric effect of fatty acid or known as carboxylic acid.

The enthalpy of substrates binding to enzymes to form either enzyme-substrate complexes or dead-end compounds can help to determine the rate of enzymatic reaction. The value of binding enthalpy from various research paper are shown in **Table 2.2**.

Table 2.2: Enthalpy of binding to form compound.

Lipase Sp	**Wax Ester**	**ΔH_m^A (kJ/mol)**	**ΔH_m^B (kJ/mol)**	**ΔH_i^A (kJ/mol)**	**ΔH_i^B (kJ/mol)**	**References**
Trypsin	β-Lactoglobulin	21	28	60	60	(Olsen et al., 2000)
α-Chymotrypsin	ethyl acetate	9.6	6.9	5.3	21	(White and Wharton, 1990)
Candida antarctica	α-monobenzoate glycerol	25.9	33.1	76.7	146	(Tamayo et al., 2012)

The activity of enzymes is influenced by the temperature of their catalytic and inactivation processes, which is caused by thermal inactivation. It can be described in terms of an enzyme's catalytic constant, k_{cat} and thermal inactivation rate constant, k_{inact}. Their relationship is shown as equation below.

$$V_{max} = k_{cat}[E_o]\exp(k_{inac}t) \qquad 2.12$$

where V_{max} = maximum velocity of enzyme;

k_{cat} =enzyme's catalytic constant;

$[E_o]$ = total concentration ofenzyme;

k_{inact} = thermal inactivation rate constant;

t = time.

Both k_{cat} and k_{inact} are temperature dependent and characterized by activation energy, ΔG_{cat} and activation energy for an irreversible inactivation reaction, ΔG_{inact} respectively. They can be calculated in the equation as shown below.

$$k_{cat} = \frac{k_B T}{h} \exp\left(-\frac{\Delta G_{cat}}{RT}\right) \quad 2.13$$

$$k_{inact} = \frac{k_B T}{h} \exp\left(-\frac{\Delta G_{inact}}{RT}\right) \quad 2.14$$

where k_B = Boltzmann's constant;

R = Gas constant;

T = absolute temperature;

h = Planck's constant;

ΔG_{cat} =activation energy of the catalysed reaction;

ΔG_{inact} =ctivation energy of the thermal inactivation process.

Denaturation of enzyme is not only temperature dependent but also time dependent. Hence, the enzyme can only be used several times before needing immobilization. To protect enzymes from thermal inactivation, the Equilibrium Model had been developed to study the effect of temperature on enzymes via the term active-inactive transition and thermal deactivation. It is described by an equilibrium constant, K_{eq}, in which the active enzyme is in equilibrium with the inactive form of the enzyme due to irreversible thermal inactivation to denatured state. K_{eq} can be described in terms of enthalpy of equilibrium, H_{eq} and temperature equilibrium, T_{eq}. Their relationship is given by:

$$K_{eq} = exp\left[\frac{\Delta H_{eq}}{R}\left(\frac{1}{T_{eq}} - \frac{1}{T}\right)\right] \quad 2.15$$

where ΔH_{eq} is the enthalpic change associated with the conversion of active to inactive enzyme,

T_{eq} is the temperature which concentration of E_{act} and E_{inact} are equal or at the mid-point of transition between the two forms. In other words, half of the enzyme is active.

At higher operating temperatures, the enzyme will show higher kinetic energy to enhance the reaction to get a better conversion, but the stability of enzyme will be reduced (Daniel and Danson, 2013) A large portion of enzymatic activity will be lost at temperatures above T_{eq}. This is due to the fact that reversible active-inactive equilibration has occurred by the time a reliable data point has been determined.

T_{eq} and ΔH_{eq} is also affected by the substrate and E_{act}/E_{inact} transition. Hence, their value will vary when different substrates are used and at different operating temperature. Generally, there are around 50% of enzyme with a ΔH_{eq} lower than 150 kJ/mol and only 15% of enzymes have ΔH_{eq} greater than 400 kJ/mol. ΔH_{eq} can be considered as a measure of sensitivity of enzyme's activity to temperature because an enzyme will have a sharp and relatively narrow optimum temperature range if it has a large ΔH_{eq}, whereas a small ΔH_{eq} results in an enzyme with a wide optimum temperature range. Hence the activity of the latter is relatively less sensitive to changes in temperature. In other words, if the enzyme has a ΔH_{eq} greater than 400 kJ/mol, it is very sensitive towards the changes of temperature in terms of product yield.

As mentioned beforehand, the thermal stability of an enzyme depends on its equilibrium temperature and enthalpy of equilibrium. It is important to select a proper environment for enzymatic esterification which not only influences the productivity of enzyme but also ensure the thermal stability of enzyme to optimize the lifespan of the enzyme.

The thermodynamic value of each enzyme is similar. The thermodynamic values of some enzymes are listed in **Table 2.3**.

Table 2.3: Thermodynamic value of lipase-catalyzed was ester synthesis

Organism	Enzyme	Activation energy of the catalysed reaction, ΔG_{cat} (kJ/mol)	activation energy of thermal inactivation process, ΔG_{inact} (kJ/mol)	Enthalpy of enzyme transition, ΔHeq (kJ/mol)	Equilibrium Temperature, Teq (K)	References
Thermus sp. RT41a	Alkaline phosphatase	72	99	305	90	(Daniel and Danson, 2013)
Caldocellulosiruptor sacchrolyticus	β-Glucosidase	88	103	149	74	(Daniel and Danson, 2013)
Wheat gern	Acid phosphatase	79	95	142	64	(Daniel and Danson, 2013)
Moritella profunda	Dihydrofolate reductase	67	93	104	55	(Daniel and Danson, 2013)
G. stearothermophilus	DHFR	67	97	96	53.9	(Daniel et al., 2008)
B. psychrophilus	IPMDH	72	100	123	56.7	(Daniel et al., 2008)
C. *saccharolyticus*	β-GLU	81	98	154	73.9	(Daniel et al., 2008)
B. *taurus*	MDH	53	85	826	67.4	(Daniel et al., 2008)
B. *subtilis*	IPMDH	73	94	255	52.8	(Daniel et al., 2008)
P. *dulcis*	β-GLU	95	100	55.9	61.5	(Daniel et al., 2008)

2.2 Reaction parameters

There are some parameters which need to be considered in enzymatic esterification or transesterification, which include reaction temperature, enzyme concentration and fatty acid to fatty alcohol molar ratio, to obtain the optimum conversion of substrate and yield of product. This is also important to ensure economic feasibility for industrial application.

2.2.1 Reaction Temperature

There are some variables that will affect the enzymatic catalysed reaction. First, enzyme is highly sensitive to temperature. For palm oil transesterification using lipase, the increment of temperature until its optimum temperature, which is around 40-50°C, results in a high degree of yield of wax ester (Alves et al., 2016). When there is a lower mass transfer resistance at higher temperature, diffusion of substrate molecule to enzyme surface to form acyl enzyme complex occurs more frequently due to an increase of solubility of substrate in the mixture (Waghmare et al., 2017). Apart from solubility, a decrease in total viscosity favours the forward reaction and gives a higher conversion of product (Tomke and Rathod, 2020). At elevated temperature, there are more successful collision of particles among substrate and enzyme because of greater kinetic energy to accelerate the movement of particles to increase the number of collisions per unit time, and improves the homogeneity between substrate molecules to form wax ester (Tomke and Rathod, 2020). Similar results were also reported by Gunawan and Suhendra (2010), which they are observed from the increment value of k_1 and K_e (Gunawan and Suhendra, 2010). However, beyond 50°C, k_1 decreases which may be due to enzyme denaturation or the inactivation of lipase molecules. This results in a slight increment of conversion of substrate to ester or even a decrease in conversion percentage.

2.2.2 Substrate molar ratio

Since wax ester is a combination of fatty alcohol and fatty acid, both are important raw material to synthesise wax ester. As mentioned before, both fatty acid and fatty alcohol are assumed to be competitive inhibitors in transesterification, thus a different result is obtained with different molar ratios. The enzyme favours fatty acid compared to fatty alcohol as the Menten constant of fatty alcohol is greater than fatty acid (Waghmare et al., 2017). Generally, ester synthesis catalysed by lipase can be strongly improved by an increase of reactants albeit requiring a longer reaction time. The ester conversion percentage increases when the molar ratio of acid to alcohol is 1.1.25 and 1.5. Even at higher conversions, k_1 and K_e values reduce when the alcohol concentration increases. This may be due to an increase of viscosity in the reaction. Hence, more reaction time is required to reach the maximum conversion of wax ester. It is noted that the yield percentage will decrease when molar ratio of acid to alcohol increases to 1: 3 or more. Activity of lipase will be inhibited as excess alcohol distorts the essential water layer that stabilizes the lipase (Gunawan and Suhendra, 2010). On the other hand, excessive acidification will cause the conversion of ester to decrease due to the strong acidification of the environment, which may affect the performance of lipase (Alves et al., 2016). The three-dimensional structure of lipase suffers a conformational change when the pH value is too low at a microenvironment biocatalyst. Hence, it results in the inactivation of lipase.

2.2.3 Enzyme Concentration

Theoretically, if there is a greater lipase concentration, there will be more active sites available to catalyse the reaction to produce ester, if there are no other limiting factors such as substrate concentration and inhibitors. This is because there is more catalytic sites available to be ready of formation of acyl-enzyme complex (Waghmare et al., 2017). However, optimum enzyme