Conceptual Study of Enzymatic Sugar ester synthesis in the PBR at Steady-State and Isothermal Conditions

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by

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LIST OFABBREVIATION

E_0	Total concentration of enzyme
ΔG_{cat}	activation energy of the catalysed 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
k_B	Boltzmann's constant
k_{cat}	Turnover number
k_{inact}	thermal inactivation rate constant
K_m	Michaelis constant
K_i	Dissociation constant
Keq	Equilibrium constant
K_M	Michaelis constant
[A]	Concentration of substrate 1
[B]	Concentration of substrate 2
r_A	Rate of reaction
R	Gas constant
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

Enzim Lipase digunakan secara meluas dalam penghasilan gula ester terutama dalam pemprosesan makanan yang menggunakan PBR sebagai reaktor. Jumlah hasilan gula ester akan dipengaruhi oleh aktiviti enzimatik yang terutamanya dipengaruhi oleh tenaga, suhu dan jumlah kepekatan substrat yang digunakan. Parameter yang ideal adalah masalah utama untuk menghasilkan penghasilan gula ester dalam kadar yang banyak dan efisyen. Persamaan yang digunakan untuk simulasi diperoleh berdasarkan persamaan teori untuk PBR dan juga berdasarkan reaksi dua substrat (Varde & Fogler, 2001). Dua parameter dibandingkan untuk mendapatkan hasil optimum gula ester iaitu suhu dan nisbah molar substrat. Hasil simulasi dapat meberi nilai R^2 lebih hampir pada 1 jika dibandingkan dengan hasil experimen kerana dapat meningkatkan ketepatan data yang digunakan oleh eksperimen. Menurut Harwell dan Rakan sekerjanya, hasil yang diperoleh oleh eksperimen dapat diubahsuai dengan menggunakan kaedah simulasi dan mampu memperoleh hasil yang lebih bagus (Harwell et al., 2017). Tren pada graf untuk kesan suhu terhadap hasil ester gula menunjukkan bentuk yang serupa dengan hasil yang diperoleh melalui kedua-dua kaedah dimalana suhu tertinggi dicatatkan pada 40 ºC tetapi sedikit berbeza untuk kesan nisbah molar kepekatan substrat terhadap hasil ester gula dimana nisbah molar gula: asid lemak untuk simulasi adalah 1: 1 sementara untuk eksperimen menunjukkan 1: 2. Setelah memperoleh parameter dan pemalar optimum dan ideal bagi ester gula berdasarkan perbandingan kedua-dua kaedah tersebut, kesan parameter kinetik dan parameter termodinamik pula dibincangkan. Dari apa yang diperoleh dalam parameter kinetik, semakin tinggi tenaga pengaktifan pengikatan substrat ke enzim dan semakin rendah tenaga pengaktifan perencatan akan meningkatkan kadar tindak balas reaksi antara enzim dah substrat. Sementara untuk parameter termodinamik, semakin tinggi

tenaga pengaktifan proses pemangkinan, $\triangle G_{cat}$, tenaga pengaktifan semasa keseimbangan, $\triangle H_{eq}$ dan suhu keseimbangan, T_{eq} dan semakin rendah tenaga pengaktifan proses inaktivasi termal, $\triangle G_{inact}$, akan menghasilkan kadar tindak balas yang lebih rendah. Ini menunjukkan bahawa enzim dapat bertindak dengan ideal sekiranya tenaga pengaktifan pengikatan substrat lebih tinggi sementara tenaga pengaktifan perencatan lebih rendah. Selain itu, enzim juga dapat bertindak dengan ideal sekiranya tenaga pengaktifan pemangkinan, tenaga pengaktifan semasa keseimbangan, dan suhu keseimbangan lebih rendah manakala jika tenaga pengaktifan proses pengaktifan proses inaktivasi termal lebih tinggi.

ABSTRACT

Lipase was used widely in the production of sugar ester especially in food processing which used PBR as the reactor. The amount of yield produce is affected by the enzymatic activity which also effected by binding energy, temperature and amount of concentration of the substrate used. Ideal parameters based on experimental and also simulation path are the major problem in order to produce higher amount of sugar esters. The equation used for simulation were obtained based on the design equation for PBR and based on the two substrate reaction(Varde & Fogler, 2001). Two parameters were compared to obtain an optimum yield of sugar ester which are the temperature and the molar ratio of substrate. This parameters are validate and compared between the results obtained from Sebatini experiment in the literature with the simulation by using POLYMATH. From the comparison, Simulation result are much more accurate based on the validation of R^2 methods as it can improve the data that used by the experimental. In agreement with Harwell and Co-workers, The result that obtained by the experimental can be modify by using the simulation methods (Harwell et al., 2017). The trend of the graph for the effect of temperature against yield of sugar ester shows the similar shape which the yield obtained was the highest at 40 ºC but slightly different for the effect of substrate concentration molar ratio against yield of sugar ester as the as it stated that the molar ratio of sugar: fatty acid for simulation should be 1:1 while for the experimental was 1:2 respectively. After obtaining the optimum and ideal parameters and constant for the sugar ester based on the comparison of both sources, the effect of kinetic parameter and thermodynamic parameter were discussed. From what obtained in kinetic parameter, the higher activation energy of substrate bind to enzyme and the lower the inhibition activation

energy will increase rate of reaction. While for the thermodynamic parameters, the higher the activation energy of catalysis process, $\triangle G_{cat}$, activation energy during equilibrium, $\triangle H_{eq}$ and equilibrium temperature, T_{eq} and the lower the activation energy of thermal inactivation process, $\triangle G_{inact}$, will yield lower rate of reaction. This shows that the enzyme can act ideally if the activation energy of substrate binding higher while the inhibition activation energy is lower. Beside that, enzyme also can act ideally if the activation energy of catalysis, activation energy during equilibrium, and equilibrium temperature are lower while if the activation energy of thermal inactivation process is higher.

CHAPTER 1 INTRODUCTION

1.1 Background

Sugar ester (SEs) is known as Sugar fatty acid ester. It is tasteless, odourless, nontoxic and edible. Generally, sugar ester is non-ionic surfactant since it have non-charge polar head from sugar-based compound such as sucrose and non-polar tail from free fatty acid. They usually synthesized from natural occurring compound thus make them a biodegradable surfactant. Figured 1 below show the example of the chemical structure of sugar ester which mainly contain sugar and fatty acid that linked by ester bond.

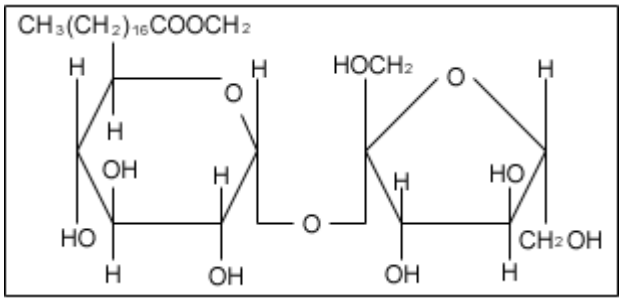


Figure 1.1: Sucrose Monostrate

As sugar ester are good stabilizing and conditioning compound, they are used widely in food pharmaceutical, detergent, agricultural, fine chemical and cosmetics (Zheng et al., 2015). Sugar ester can be used as sweetener in food industries and as it have emulsifying properties, it is very useful in pharmaceutical and cosmetics which can improve the skin texture and tone. The example of useful sugar ester are Fructose-mono-palmitate, Fructose-mono-laurate, sugar-poly-palmiate, sugar-poly-laurate and many more (Siebenhaller et al., 2018).

There are a few process used to synthesize Sugar esters. First one is by emulsion process which by using concept of micro-emulsion. First, the sugar or sucrose is dissolved in a solvent, propylene glycol, potassium carbonate as catalyst and added the soap or fatty acid salt (Osipow & Rosenblatt, 1967). Next, sugar esters also can be

synthesized by melt process by co-melting sugar react with fatty acid ester. Sucrose ester or alkaline cation soap were added as emulsifier. The reaction produced more substituted sugar esters where the sucrose can be degraded due to high temperature (Fitremann et al., 2007). Furthermore, sugar ester can be produced by solvent process which used basic transesterification process in solvent of di-methyl formamide. Alkaline catalyst is used to catalyse the reaction with methyl ester fatty acid (Teng et al., 2020). Last but not least, sugar ester can be produce by enzyme catalysed-reaction. It is a biochemical reaction that have enzyme that catalyse the reaction which will lower the activation energy required for substrate convert to product (Warshel, 1978). It can produce high yield compared to other method mention above.

Emulsion, melt and solvent method mention are chemical reaction. As for enzymatic reaction, it is more ideal to be used to improve the product amount. It can provide alternative pathway reaction for the sugar ester production by lowering the activation energy. The issue with enzyme catalysed-reaction is that the reaction rate can be decrease if there is inhibitor. It can bind reversible or irreversible with the enzyme to reduce the production rate (Goldstein, 1944). This problem can be eventually overcome if we increase the enzyme concentration or increasing concentration of substrate.

Therefore, enzymatic reaction is a sustainable process and can undergo process at mild temperature. It is also an environmental friendly reaction. The type of enzyme used for and reaction can provide the best yield product for that particular reaction. As for production of ester sugar, the best enzyme used is lipase as it can be used during transesterification and esterification process (Camino Feltes et al., 2011).

In conclusion, sugar esters is a compound that is very useful in daily live. Furthermore, sugar esters can be synthesizes by 4 method in which three are chemical

reaction and another one is enzymatic reaction. Enzyme catalyse-reaction is different from chemical reaction in term of substances used to increase the yield of the product. Unlike chemical reaction, enzyme catalysis may have some issue with inhibitor during a reaction but it can be minimize for a better outcome.

1.2 Problem statement

Packed bed reactor is the most viable solution for the enzymatic production of sugar ester as it could be loaded with high amount of immobilized enzyme to improve the yield of product and the bio-catalyst can be recycled. Formation of sugar ester is endothermic reaction, thus sufficient heat should be supplied to meet the energy requirement. As the enzyme is heat sensitive bio-catalyst, exposure to excess heat might reduce it performance due to denaturation. The enzyme need to be maintain at optimum temperature but not too low to prevent lower yield. Therefore, the appropriate range of temperature for a specific enzyme has to be established in order to maintain high efficiency of the production. In order to get the best yield from the reaction it is not an easy job as it required optimum condition to keep the enzyme activation-deactivation process in balance. Moreover, the kinetic parameters of the enzyme determine the performance of the reaction and it's is temperature dependent Therefore, a mathematical model of lipase-catalysed sugar ester synthesis shall be developed by incorporating the effect of temperature on the reaction process in PBR and then the model is solved via Polymath. The model will be validated by comparing the experimental data reported in the literatures. The investigation shall focused on the effect of kinetics parameters on the optimal temperature during sugar ester synthesis with primary objective to get maximum conversion as possible. The appropriate

enzyme characteristic in term of its kinetic parameter shall be proposed for sugar ester synthesis in the packed-bed reactor.

1.3 Objectives

1. To develop and validate the mathematical model for lipase catalysed synthesis of sugar ester in packed-bed reactor using Polymath software.
2. To investigate the effect of temperature and molar ratio on the conversion of sugar ester.
3. To propose ideal enzyme characteristic in term of kinetic parameters and thermodynamic parameter for sugar ester synthesis in PBR.

CHAPTER 2 LITERATURE REVIEW

2.1 Sugar ester application

Sugar ester are a non-ionic surfactant that can be synthesized in a single step of enzymatic reaction by using lipase. As the compound of sugar ester contain sugar based surfactant, therefore, it contain good surface activity and can get from the renewable resources. The non-ionic surfactant of sugar ester are used widely in cosmetics, fine chemistry, and food formulations.

Beside that, the sugar based surfactant are readily accessible feedstock, inexpensive and renewable compound which will not harm the environment when used it (Taylor et al., 2015). Furthermore, sugar ester also used as emulsifying agent. Sugar ester have reduction in interfacial tension and have a very good stability of oil in water or water in oil emulsion which allow them to be good emulsifier. It is used widely in food and pharmaceutical application as a cheap and environmental friendly emulsifier agent.

2.2 Enzyme-catalyzed reaction for sugar ester production

The stability of enzyme catalyst is a concerning issues during the reaction. The enzymatic kinetic model with various type of inhibition will affect the stability of catalytic process. Furthermore, enzyme is temperature sensitive and it stability is greatly affected with slight change of temperature. As for improving the yield, the temperature is not the only factor but the effect of reactant also need to be consider as it have high impact on the yield of sugar ester. The enzyme deactivation will affect the rate of reaction of the process, thus lower the conversion of sugar ester production. There are two main inhibition which are reversible and irreversible where reversible can be minimize while irreversible cannot be minimize the reduction in yield product.

2.3 Kinetic model

Kinetic Modelling are models, which are real-world simplifications, aim to predict a system's behaviour, based on knowledge of the component properties of the systems. The common kinetic model used for enzyme-catalysed reaction is michaelis mentens. . The model was develop by two scientist which known as Leonor Michaelis and Maud Leonora Menten which used to account for enzymatic dynamics. The model is used to describe how an enzyme can improve a reaction's kinetic rate and illustrate how reaction rates depend on the enzyme and substrate concentration. Beside that, michaelis menten equation also can be used as bi-substrate reaction and the only enzymatic bi-substrate model that are verified. Based on U. Zaidan and his members, michaelis menten equation can be used for sugar ester synthesis as lipase enzyme are involved during the esterification. They can determined the effect of the substrate concentration on the rate of reaction (Zaidan et al., 2011)

2.3.1 Michaelis Menten Equation

Based on the equilibrium kinetic model of Michaelis and Menten, the substrate-binding step is assumed to be fast relative to the rate of breakdown of the enzyme-substrate (ES) complex. Thus the concentration of ES is assumed constant and the Michaelis-Menten equation is proposed as given by Equation 2.1(Marangoni, 2013).

$$v = V_{max}\, x \frac{[S]}{K_m + [S]} \quad - Michaelis\ Menten\ Equation \qquad \textit{Equation 2.1}$$

Where, Ks is the equilibrium dissociation constant, Vmax is maximal velocity of the reaction (or maximal rate) and [S] is substrate concentration. The Ks represents the affinity of enzyme and substrate concentration on (Marangoni, 2013). Below are the

assumption made for Michelis Menten model. Substrate binding reaction is at equilibrium at fast formation and break-down of substrate-enzyme complex.

1. The conversion of product back to substrate is negligible, since very little product has had time to accumulate during the time course of the reaction
2. The enzyme reaction is stable along the time course.
3. Initial rates are used as reaction velocities
4. Enzyme concentration are directly proportional with reaction velocity.

A linear plot also can be provide by using Michaelis and Menten equation by converting the equation into lineweaver-Burk plot equation as given by Equation 2.2(Marangoni, 2013). Further studies in the nonlinear equation by Rodriguez and members, the lineweaver-Burk plot is more accurate in estimating V_{max} and finding information of inhibition (Rodriguez et al., 2019).

$$\frac{1}{v} = \frac{K_m}{V_{max}\, x\, [S]} + \frac{1}{V_{max}} \quad -Lineweaver-Burk\ equation \qquad \textit{Equation 2.2}$$

2.3.2 Enzyme inhibition/ deactivation

The catalytic performance of the enzyme can reduce in the presence of inhibitor. Inhibitor is a substances that can slow down the rate of reaction or can prevent a particular chemical reaction or the process which reduce the activity of the particular reactant, enzyme or catalyst. There are irreversible and reversible inhibitors. Irreversible inhibitor can modify the enzyme structure by binding covalently which the inhibition may also not be reversed. As for reversible inhibition, the inhibitor will bind non-covalently which allow the enzyme to revert into it old structure but this can slow down the reaction or the process of the enzyme itself. Reversible inhibition have 4 type which

are competitive, uncompetitive, linear mixed and also non-competitive (Marangoni, 2013).

2.3.2.1 Competitive inhibition

This type of inhibition normally competes with an enzyme's substrate during binding at the active site. Figure 2.1 shows that the mechanism for competitive inhibition. Competitive inhibition increases the K_m which mean the binding affinity of the enzyme decrease.

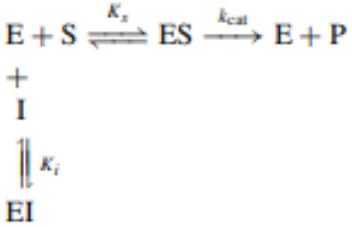


Figure 2.1: Mechanism scheme during competitive inhibition

Rate of reaction of competitive inhibition is describe as below (Marangoni, 2013),

$$v = \frac{V_{MAX}[S]}{K_s^* + [S]} = \frac{V_{MAX}[S]}{\propto K_s[S]} \qquad \textit{Equation 2.3}$$

Where K_s^* is a constant relate to apparent enzyme-substrate dissociation which presence in inhibitor.

2.3.2.2 Uncompetitive inhibition

For uncompetitive inhibitor, the inhibitor will interact during enzyme-substrate complex but not at the active site. This will reduce the V_{MAX} and K_s of the reaction as it increase the affinity of enzyme for substrate. Uncompetitive inhibition also will reduce the V_{max} and K_m as the inhibitor will bind to enzyme and turn into new product which will limit the substrate binding site. Figure 2.2 shows that the mechanism for uncompetitive inhibitor.

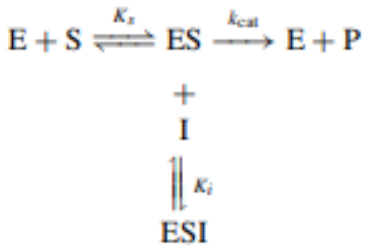


Figure 2.2: Mechanism scheme for uncompetitive inhibition

Rate of reaction of uncompetitive inhibition can be described as below (Marangoni, 2013),

$$v = \frac{V^*_{MAX}[S]}{K^*_s + [S]} = \frac{\left(\frac{V_{MAX}}{\propto}\right)[S]}{\left(\frac{K_s}{\propto}\right) + [S]} \qquad \textit{Equation 2.4}$$

Where V^*_{MAX} and K^*_s are constant related to apparent enzyme maximum velocity and apparent enzyme-substrate dissociation in presence of inhibitor.

$$\propto = 1 + \frac{[I]}{K_i} \qquad \textit{Equation 2.5}$$

Where [I] is the inhibitor concentration while K_i is constant during inhibition.

2.3.2.3 Linear mixed inhibition

For this type of inhibition, the inhibitor can act as uncompetitive or competitive inhibitor as the inhibitor can interact with both free enzyme or during enzyme-substrate complex at site other than of active site. Furthermore, linear mixed inhibition can have same characteristic as other 3 inhibition as it can provide an affinity towards the inhibitor either the enzyme are catalysed by substrate or not. Figure 2.3 shows that the mechanism of linear mixed inhibition.

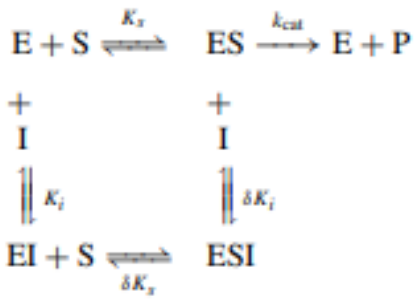


Figure 2.3: Mechanism scheme for linear mixed inhibition

Rate of reaction for linear mixed inhibition are shown as below (Marangoni, 2013),

$$v = \frac{V^*_{MAX}[S]}{K^*_s + [S]} = \frac{\left(\frac{V_{MAX}}{\beta}\right)[S]}{\left(\frac{\propto}{\beta}\right)K_s + [S]} \qquad \textit{Equation 2.6}$$

$$\propto = 1 + \frac{[I]}{K_i} \qquad \textit{Equation 2.7}$$

$$\beta = 1 + \frac{[I]}{\delta K_i} \qquad \textit{Equation 2.8}$$

2.3.2.4 Non-competitive inhibition

Non-competitive inhibition is same with linear mixed inhibition but the value of $\delta = 1$ and $\alpha = \beta$. Thus the mechanism is same with linear mixed inhibition but different a bit in rate of reaction. Lastly, non- competitive inhibition reduce V_{max} in which it reduce the reaction time of the enzyme only. Below are the rate of reaction formula for non-competitive inhibition.

$$v = \frac{V^*_{MAX}[S]}{K_s + [S]} = \frac{\left(\frac{V_{MAX}}{\propto}\right)[S]}{K_s + [S]} \qquad \textit{Equation 2.9}$$

2.3.3 Two substrate reaction

Many enzyme-substrate reaction usually required only mono-substrate. As for sugar ester production, the number of substrate or reactant required usually more than one. Therefore, there were two mechanism can be applied which are ping-pong or sequential. Sequential method have 2 type which were random-sequential bi bi mechanism and ordered sequential bi bi mechanism (Marangoni, 2013). In further study on sequential mechanism by Henry Jakubowski, the substrates might bind to the enzyme in a random fashion (A first then B or vice-versa) or in an ordered fashion (A first followed by B). Both substrate must be inside the active side of enzyme in order to transform into product. For both mechanisms, Lineweaver-Burk plots at varying A and different fixed values of B give a series of intersecting lines. Derivative curves can be solved to obtain appropriate kinetic constants (Mechanism, 2020).

2.3.3.1 Ping Pong bi bi Mechanism and rate of equation

For ping pong mechanism, involve two substrates that do not enter the active site at the same time. Instead, each substrate enters the active site on an individual basis, forming an intermediate complex. The first substrate transforms into the product and usually transfers some group onto the enzyme and modifies it temporarily. Once the first product is released, the second substrate enters and receives the group that was transferred onto the enzyme from the first substrate. The process are same as ordered sequential bi bi mechanism which bind first with 1 substrate and the only different are the second substrate will bind after released of product and enzyme of the first substrate. Figure 2.4 shown that the steady state equation for this mechanism.

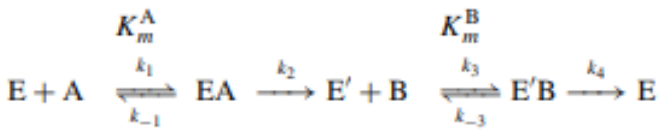


Figure 2.4: General scheme of Ping Pong Bi Bi Mechanism

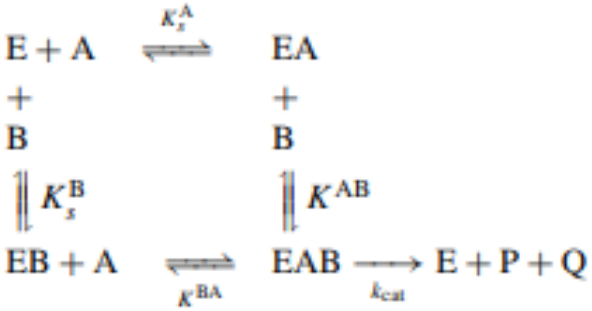
The rate of reaction of ping pong bi bi mechanism are shown as below (Marangoni, 2013),

$$\frac{v}{V_{max}} = \frac{[A][B]}{\frac{k_4}{k_2}K_m^A[B] + K_m^B[A] + [A][B]\left(1 + \frac{k_4}{k_2}\right)} \qquad \textit{Equation 2.10}$$

$$Where, \alpha = \frac{k_4}{k_2} \qquad \textit{Equation 2.11}$$

2.3.3.2 Random sequential bi bi mechanism and rate of equation

There are no particular order in this mechanism during binding of substrates with the enzymes to form ternary complex. The first substrate will bind rapidly with the enzyme and another substrate will bind during the substrate enzyme complex to form ternary complex EAB. The process occur very fast which the reaction almost like the binding of both substrate with enzyme occur simultaneously. The general reaction from this type of mechanism are shown as Figured 2.5.



Figured 2.5: General scheme for Random-Sequential Bi Bi Mechanism

Below are the rate of reaction for random sequential bi bi mechanism (Marangoni, 2013),

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_S^A K^{AB} + K^{AB}[A] + K^{BA}[B] + [A][B]} \quad \textit{Equation 2.12}$$

$$Where, V_{MAX} = k_{cat}[E_T] \quad \textit{Equation 2.13}$$

2.3.3.3 Ordered sequential bi bi mechanism and rate of reaction

For this type of mechanism, the enzyme will bind first with one substrate before bind with another substrate. The process is slower compared to the previous mechanism. Figured 2.6 shown the general reaction mechanism for ordered sequential bi bi mechanism.

$$\mathrm{E} + \mathrm{A} \overset{K_s^A}{\rightleftharpoons} \mathrm{EA} + \mathrm{B} \overset{K^{AB}}{\rightleftharpoons} \mathrm{EAB} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P} + \mathrm{Q}$$

Figured 2.6: General scheme for ordered sequential bi bi mechanism

Below are the rate of reaction for ordered sequential bi bi mechanism (Marangoni, 2013),

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_S^A K^{AB} + K^{AB}[A] + [A][B]} \quad \textit{Equation 2.14}$$

$$Where, V_{MAX} = k_{cat}[E_T] \quad \textit{Equation 2.15}$$

2.4 Kinetic Parameters

Enzymatic kinetic parameter were used to estimate the inhibition of the reaction and also to estimate the effectiveness of the process. The kinetic parameter usually focus on concentration of enzyme in the reaction. Further studies in kinetic parameter by Uswatun H. Z. and members reported that the immobilized enzyme have higher V_{max} and K_i value but lower in K_m compared to free enzyme (Zaidan et al., 2011). In agreement with Siti N. and members studies, it was reported that the immobilized enzyme have higher affinity compared to free enzyme.(Abd Rahim et al., 2013). Table 2.1 shows that the kinetic parameter obtain by using free or attached enzyme during esterification process.

Table 2.1: Kinetic Parameters between immobilized lipase and free lipase during esterification of lactose and capric acid

Parameters (mmol /L .min .mg)	**Free lipase**	**Immobilized lipase**
V_{max}	0.050	0.080
$K_{m(capric\ acid)}$	14.800	14.500
$K_{m(lactose)}$	16410.000	9308.000
K_i	0.078	0.229

On the further study of the effect of immobilized lipase on the kinetic parameter by A.C. Oliveira with his members, shows the similar result as the previous researchers on the V_{max} and K_m value obtain with K_s for free lipase is lower compared to the immobilised lipase. Table 2.2 shows that the effect of lipase during esterification process (Oliveira et al., 2001).

Table 2.2: Kinetic constant obtained for esterification reaction catalysed by free lipase and immobilised lipase.

Parameters	Free Lipase	Immobilised Lipase
	Nonlinear regression	
V_{MAX} (M/h.mg)[a.b]	0.057	80.174
$K_{m(Et)}$ (M)[a]	1.790	1.200
$K_{m(Ol)}$ (M)[a]	1.800	1.160×10^{-8}
$K_{s(Et)}$ (mmol/ hr.mg.M)[a]	0.637	2.900
$K_{s(Ol)}$ (mmol/hr.mg.M)[a]	0.633	3.000×10^{8}

2.4.1 Effect of temperature

Temperature are very important in improving enzymatic reaction. It is concern that the optimum temperature should be applied in order to obtain sustainable amount of product. Based on Sebatini and co-worker, the suitable temperature for the enzymatic reaction was at 40ºC which can be consider very optimum temperature for many type of enzyme (Sebatini et al., 2016). In agreement with Taylor institution study, it stated that at temperature between 35- 45ºC the yield of many type of sugar ester would be more than 90% (Taylor et al., 2015). Beside that, based on Abdulmalek and members, if the sugar ester production required a higher temperature than at optimum temperature, therefore thermostable enzyme can be used in order to avoid degrading of enzyme during high temperature reaction. The experiment were conduct at 50-80ºC which at 65ºC. The amount of yield were not so high through isolated enzyme (Abdulmalek et al., 2020). Table 2.3 shows that the type of reaction with suitable temperature to obtain highest amount of sugar ester.

Table 2.3: Type of reaction with optimum temperature to obtain high amount of sugar ester yield

Type of reaction	Optimum temperature (ºC)	Sugar ester Yield (%)	References
Esterification of glucose and strearic acid	40	86	(Sebatini et al., 2016)
Esterification of glucose and stearic acid	35-45	>90	(Taylor et al., 2015)
Esterification of galactose and linoleic acid	65	65	(Abdulmalek et al., 2020)
Esterification of lactose with oleic acid	40	84.1	(Neta et al., 2012)
Esterification of fructose with ethanol	40	93.2	(Runa et al., 2017)

2.4.2 Effect of reactant

Reactant that react also can affect the enzymatic reaction and production yield. Gumel and co-workers mention that the fatty acid and molar ratio of fatty acid and sugar are the main concern in production yield (Gumel et al., 2011). The suitable molar ratio for the reactant were 3 fatty acid: 1 sugar. Taylor institution reported that the molar ratio should be parallel with solubility of reactant. Increasing amount of fatty acid concentration will increase the production of sugar ester. The longer the chain of fatty acid will reduce the rate of esterification while increasing carbon chain will increase the yield (Taylor et al., 2015). In contrast with Abdulmalek and members reported that the longer the chain of fatty acid give higher conversion of sugar ester. It also stated that the longer chain might be less favour if the phase of fatty acid used was in solid form rather than liquid as the viscosity of solid are higher than liquid which can defect the enzyme , (Abdulmalek et al., 2020).

2.5 Sugar ester production in Packed bed reactor

Enzymatic reaction were carried out in Packed Bed Reactor (PBR). It is an economical yet very useful reactor for enzyme catalytic reaction. It can operate continuously due to long life span of catalyst. Due to continuous mixing, the mass transfer coefficient (kc) will affect the production yield. The larger the surface area and porosity of the reactant, the higher the mass transfer coefficient (Ishak et al., 2016). Mole balance of PBR can be define as below formula,

$$\frac{F_{A0}dX}{dW} = -r_A' \qquad \textit{Equation 2.16}$$

Where rate of reaction can be calculate or get from,

$$-r_A' = kC_A^2 \qquad \textit{Equation 2.17}$$

Concentration of A can be determine as

$$C_A = F_A/v_0 \qquad \textit{Equation 2.18}$$

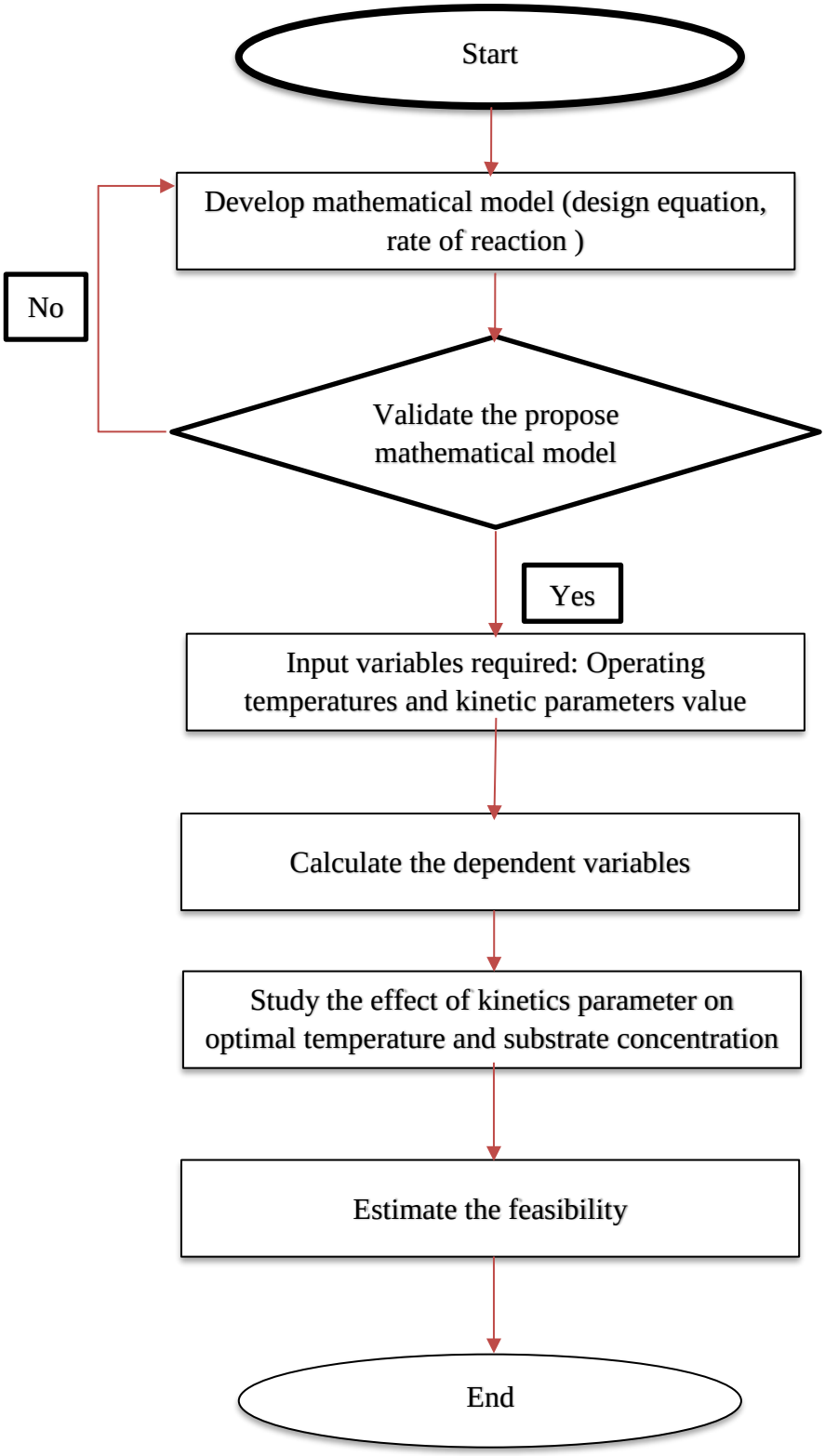
As isothermal there is no temperature changes, therefore, $T=T_o$. So, Equation 2.15 will we finalized as

$$\frac{dX}{dW} = \frac{kC_{A0}}{V_o}\left[\frac{(1-X)}{1+\varepsilon X}\right]^2\left[\frac{P}{P_o}\right]^2 \qquad \textit{Equation 2.19}$$

Where, $F_{A0} = v_o C_{A0}$

CHAPTER 3 METHODOLOGY

3.1 Research flowchart



3.2 Mathematical model development

The mathematical software used were Polymath. The capable to solve multiple ODE equation for present study but limit only for 40 equations only. First of all, a process model of enzymatic reaction for second order reaction in synthesis of sugar ester had been developed. Then, the POLYMATH software was used to calculate the kinetic parameter which required for the process. The calculation estimation of parameters were important for a better understanding of second order enzymatic reaction and provide data to be used later. In POLYMATH, the suitable formula for Packed Bed Reactor (PBR) were developed and selected. From the literature review, appropriate assumption and information will be provided especially for the kinetic parameter and operating condition which have to be consider for the enzymatic reaction model.

General mass balance of Reactor is

In – Out + Generation = Accumulation *Equation 3.1*

Where in packed bed reactor (PBR),

$$F_{jo} - F_j + r_j \triangle W = \frac{dC_j}{dt} \quad \textit{Equation 3.2}$$

F_{jo} is the inlet flow, F_j is the outlet flow, r_j is the reaction rate while $\triangle$W is weight changes during reaction. Where $\frac{dC_j}{dt}$ will be assume 0 as there is no accumulation. After the derivation, we got the final equation of mass balance in PBR which is equal to,

$$r_j' = \frac{dF_j}{dW} \quad \textit{Equation 3.3}$$

Lipase catalysed reaction in PBR is used in this study. The general stoichiometry equation for enzymatic reaction for production of sugar ester are as below where A was sugar B was fatty acid, C was sugar ester and D was alcohol or water depend on the processes:

$$A + B \rightleftharpoons C + E$$

Below are the formula used to determine the rate of reaction in Packed Bed Reactor. As the process are second order, therefore there will be two substrate and two rate of reaction involved.

$$\frac{dF_{A0}}{dW} = -r_A \qquad \textit{Equation 3.4}$$

$$\frac{dF_{B0}}{dW} = -r_B \qquad \textit{Equation 3.5}$$

The enzymatic equation chosen is Michaelis- Menten Enzyme kinetics equation. Below are the equation for the Michaelis menten based on two substrate-reaction. The formula also include the inhibition that occur during the reaction. The type of reaction are ping pong bi bi mechanism with sugar and acid or alcohol are the inhibition substrate (Chowdary & Prapulla, 2005).

$$-\frac{d[A]}{dt} = -r_A = v = \frac{V_{MAX}[A][B]}{[A][B] + K_{mA}B\left[1 + \frac{[B]}{K_i[B]}\right] + K_{mB}A\left[1 + \frac{[A]}{K_i[A]}\right]} \qquad \textit{Equation 3.6}$$

Where, v is initial reaction rate; V_{max} is the maximum reaction rate; A is sugar concentration and B, alcohol/acid concentration; KmA, KmB are binding constants of sugar and acid/alcohol, Ki A, Ki B are inhibitory constants of sugar and acid/alcohol respectively.

For the effect of temperature toward the kinetic parameter and rate of reaction, the rate constant was actually changing with temperature. This can be shown in the arrhenius

equation. Below shows that the effect of temperature toward the kinetic parameter (Ks). The equation also can be alter within the inhibitory constant and binding constant to be temperature independent equation.

$$k_s = Ae^{-\frac{E_a}{RT}}$$. *Equation 3.7*

Arrhenius equation

$$K_{MA} = K_{MAo}e^{-\frac{\triangle HmA}{RT}}$$ *Equation 3.8*

Sugar binding energy temperature dependent equation.

$$K_{MB} = K_{MBo}e^{-\frac{\triangle HmB}{RT}}$$. *Equation 3.9*

Fatty Acid binding energy temperature dependent equation

$$K_{iA} = K_{iAo}e^{-\frac{\triangle HiA}{RT}}$$. *Equation 3.10*

Sugar inhibition energy temperature dependent equation

$$K_{iB} = K_{iBo}e^{-\frac{\triangle HiB}{RT}}$$ *Equation 3.11*

Fatty Acid inhibition energy temperature dependent equation.

This expression shown that the relation of substrate affinity, K_m and K_i with temperature, which for the enzyme- catalysed reactions, it is apparent during the Enzyme-Subtrate complex, ES complex. A and E_a are characteristic constant of the reaction.

Catalytic constant (Catalytic turn over number), k_{cat}, was also affected by temperature. Therefore, based on michaelis menten equation, the rate of reaction will be changed base on the catalytic constant (Peleg et al., 2012). Below are the Arrhenius's correlation for the catalytic constant (Daniel & Danson, 2013);

$$k_{cat} = \frac{k_B T}{h} e^{-\left(\frac{\triangle G_{cat}}{RT}\right)}$$ *Equation 3.12*

The inactivation of the enzyme can be written as a function of temperature by adopting Arrhenius correlation as following (Daniel & Danson, 2013);

$$k_{inact} = \frac{k_B T}{h} e^{-\left(\frac{\triangle G_{inact}}{RT}\right)} \qquad \textit{Equation 3.13}$$

Where,

k_B = Boltzmann's constant, R = Gas constant; T = absolute temperature, h = Planck's constant, $\triangle G_{cat}$ = activation energy of catalysed reaction and $\triangle G_{inact}$ = activation energy of thermal inactivation process.

Thus the variation of enzyme activity with temperature changes can be describe as (Daniel & Danson, 2013),

$$V_{MAX} = \frac{k_{cat} E_0 e^{-\left(\frac{k_{inact} K_{eq} t}{1+K_{eq}}\right)}}{1 + K_{eq}} \qquad \textit{Equation 3.14}$$

Where, K_{eq} is equilibrium constant and E_0 is total concentration of enzyme. K_{eq} is equal to,

$$K_{eq} = e^{\frac{\triangle H_{eq}}{R}\left(\frac{1}{T_{eq}} - \frac{1}{T}\right)} \qquad \textit{Equation 3.15}$$

T_{eq} is temperature at which E_{act}/E_{inact} is at equilibrium while $\triangle H_{eq}$ is change is the enthalpy changes for E_{act}/E_{inact}. E_{act} is activation of enzyme while E_{inact} is inhibition energy. Enzyme constantly activate & deactivate throughout the reaction period. When E_{inact} is large, enzyme tend to deactivate and vice versa (Daniel & Danson, 2013).

3.3 Manual Calculation of R^2

Firstly, take the X and Y value for each point. For example based on the Sebatini. Table 3.1: X and Y value from literature.Table 3.1 below showed that the value of x (Temperature) and y (sugar ester yield) obtained from Sebatini literature result.

Table 3.1: X and Y value from literature

x	y
20	9
30	39
40	86
50	51
60	40

Next, find the mean of y. Then, take the original value of y to substrate with mean of y as shown in Table 3.2 below to get the new value.

Table 3.2:Data for y- mean of y

x	y	y- $\bar{Y}$
20	9	-33
30	39	-2
40	86	41
50	51	6
60	40	-5

Mean, $\bar{Y}$ = 45

After that, squared all of the substrate value and the new added all the value. Then from the graph shown below has equation of $y = 0.74x + 15.4$. the value of y was found by using the value of x from the above data. Then the estimated value from the equation will needed to substrate with the mean value of y. Table 3.3 below shows all the data for the above explanation.