MODELING AND OPTIMIZATION OF ISOAMYL ACETATE PRODUCTION IN ENZYME CATALYZED ESTERIFICATION

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by

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LIST OF ABBREVIATIONS

US	United State
EC	European Commission
RSM	Response Surface Methodology
CCD	Central Composite Design
CCRD	Central Composite Rotatable Design
DoE	Design of Experiment
ANOVA	Analysis of Variance
DF	Degree of freedom
CV	Coefficient of Variances

LIST OF SYMBOLS

A	Acetic acid	
B	Isoamyl alcohol	
P	Isoamyl acetate	
W	Water	
r	Reaction rate	$mol.l^{-1}.h^{-1}$
V	Volume	L
n	Number of moles	Mol
t	Reaction time	H
ΔU	Internal energy	$J.mol^{-1}$
ΔE_k	Kinetic energy	$J.mol^{-1}$
ΔE_p	Potential energy	$J.mol^{-1}$
Q	Heat added to the system	$J.mol^{-1}$
W	Work	$J.mol^{-1}$
ΔH	Enthalpy	$J.mol^{-1}$
p	Pressure	Pa
W_{sh}	Shaft work	J
ΔH_R	Heat of reaction	J
T	Temperature	K
X	Ester conversion	
$\hat{c}_p$	Heat capacities	$J.mol^{-1}$
s_i	Stoichiometric constant of component *i.*	
ΔH_{fi}	Standard heat of formation of component *i*	$J.mol^{-1}$
R	Gas constant	$J.mol^{-1}.K^{-1}$
a	Antoinne's contant	

b	Antoinne's contant	
c	Antoinne's contant	
d	Antoinne's contant	
r_{max}	Maximum reaction rate	$mol.l^{-1}.h^{-1}$
$[S]$	Concentration of substrates	$mol.\ l^{-1}$
K_M	Kinetic constant	$mol.\ l^{-1}$
E	Enzyme	
E_a	Activation energy	$J.mol^{-1}$
$[i]$	Concentration of component *i*	$mol.\ l^{-1}$
K_{mA}	Kinetic constant for acetic acid	$mol.\ l^{-1}$
K_{mB}	Kinetic constant for isoamyl alcohol	$mol.\ l^{-1}$
K_{iA}	Kinetic constant for inhibition by acetic acid	$mol.\ l^{-1}$
n_i	Number of mole for component *i*	Mol
n_{Ai}	Number of mole for acetic acid at initial state	Mol
n_{Bi}	Number of mole for isoamyl alcohol at initial state	Mol
$[A]_i$	Concentration acetic acid at initial state	$mol.\ l^{-1}$
$[B]_i$	Concentration isoamyl alcohol at initial state	$mol.\ l^{-1}$
K_d	Denaturation constant	
r_{e0}	Initial reaction rate	$mol.l^{-1}.h^{-1}$
MR	Molar ratio	
E_d	Deaactivation energy	$J.mol^{-1}$

PEMODELAN DAN PENGOPTIMUMAN PENGHASILAN ISOAMYL ACETATE DALAM PENGESTERAN BERMANGKIN ENZIM

ABSTRAK

Isoamyl acetate adalah bahan penting yang digunakan dalam pelbagai industri termasuk industri perisa dan wangian. Pengeluaran kompaun ini menggunakan enzim sebagai biomangkin telah menarik perhatian ramai kerana kelebihannya iaitu parameter operasi yang sederhana, tindakbalas sampingan terhad dan pemulihan produk yang mudah. Dalam pengesteran bermangkin enzim, asid asetik dan alkohol isoamyl digunakan sebagai substrat kepada *Candida antarctica* diamobilisasi lipase untuk menghasilkan isoamyl acetate. Dalam tindak balas langsung pengesteran, sistesis ester dipengaruhi oleh air yang berlebihan yang boleh mengubah keseimbangan termodinamik tindak balas terhadap hidrolisis. Sebaliknya, semasa peringkat awal tindak balas, air mengaktifkan enzim dan meningkatkan aktiviti enzim seterusnya meningkatkan kadar tindak balas. Oleh itu, jumlah kandungan air telah menjadi parameter kritikal dalam pengesteran bermangkin enzim. Selain ini, beberapa parameter dianggap mempengaruhi proses iaitu suhu, beban enzim, kepekatan substrat dan masa tindak balas. Untuk memahami proses dan hubungan parameter ini dipilih, perkembangan model adalah penting. Proses pengoptimuman dilakukan melalui "satu-faktor-pada-satu-masa" dan metodologi permukaan respon (*RSM*). Keadaan optimum yang diperolehi daripada "satu-faktor-pada-satu-masa" telah dibandingkan dengan RSM dan keputusannya dalam lingkauan jarak. Pekali penentuan, R^2 dari model adalah 0.9448. Kemudian, model RSM itu disahkan dan ralat adalah ± 5% yang boleh diterima. RSM dianggap berfaedah kerana jumlah ujikaji yang sedikit dan model yang diperoleh boleh diulang semula. Oleh itu,

kandungan air dioptimumkan menggunakan RSM. Model yang diperolehi daripada tindak balas tambahan yang dipercayai dengan R^2 dari 0.9925 dan 0.9779. Hasil produk maksimum adalah 94% pada keadaan optimum; suhu pada 36.5ºC, alkohol/asid nisbah molar 0.94, enzim/substrat nisbah sebanyak 0.75 g/l dalam tempoh 6 jam. Berdasarkan kajian kinetik enzim, dua model matematik telah dicadangkan. Model pertama menunjukkan hubungan antara hasil ester dan nisbah molar substrat, manakala model kedua menunjukkan hubungan antara kadar tindak balas dan subu. R^2 untuk kinetik enzim dan suhu model pergantungan adalah 0.906 dan 0.992 masing-masing. Model yang dikembangkan ini menunjukkan peratus kesilapan sehingga 17%.

MODELING AND OPTIMIZATION OF ISOAMYL ACETATE PRODUCTION IN ENZYME CATALYZED ESTERIFICATION

ABSTRACT

Isoamyl acetate is an important substance which is used in various industries including flavour and fragrance industry. The production of this compound using enzymes had attracted many attentions due to its advantages of moderate operating parameters, limited side reactions and easy product recovery. In enzyme catalyzed esterification, acetic acid and isoamyl alcohol are used as substrates to the *Candida antarctica* immobilized lipase to produce isoamyl acetate. In direct esterification reaction, the ester synthesis is affected by excess water which can change the thermodynamic balance of reaction towards hydrolysis. On the other hand, during the initial stage of the reaction, water activates the enzymes and increases enzyme activity thus increases the reaction rates. Therefore, the amount of water content has been a critical parameter in enzyme catalyzed esterification. Beside this, few parameters considered to affect the process were temperature, enzyme loading, concentration of substrates and reaction time. In order to understand the process and the relationship of these selected parameters, process optimization and model development are essential. The processes optimizations were done via "one-factor-at-a-time" and response surface methodology (*RSM*). The optimum conditions obtained from "one-factor-at-a-time" were compared with *RSM* and the results were in range. The coefficient of determination, R^2 of the *RSM* model was 0.9448. Then, the error obtained from model validation was less than $\pm 5\%$ which is acceptable. *RSM* was considered advantageous due to less number of experiments and the model obtained was replicable. Therefore, the water content was optimized using *RSM*. The models

obtained from additional response were reliable with R^2 of 0.9925 and 0.9779. The maximum product yield was 94% at optimized conditions; temperature at 36.5ºC, alcohol/acid molar ratio 0.94, enzyme/substrate ratio of 0.75 g/l within 6 hours. Based on enzyme kinetic study, two mathematical models were proposed. The first model shows the relationship between ester yield and substrates molar ratio, meanwhile the second model shows relationship between reaction rate and temperature. The R^2 for enzyme kinetic and temperature dependence model were 0.906 and 0.992 respectively. These developed models showed percentage error up to 17%.

CHAPTER 1

INTRODUCTION

This chapter introduces background information and current issues of the research study. Then, it covers the research motivation in Problem Statement Section. The solutions to problem statements generate the research objectives. Afterward, the Scope of Study Section explains the ranges of work involved to achieve the research objectives. Lastly, the Organization of the Thesis Section elaborates a general description of this thesis in whole.

1.1 Background of Study

Flavour and fragrance have been recognized as an important industry due to its application in various products. In 2006, Asia Pacific is reported to conquer 27% of the world flavours market or US$ 1,738.6 millions. This is the world second largest flavours market after North America (IAL Consultants, 2007). With the increasing of population each day, the demand for flavour especially food flavour is also increasing. Moreover, people nowadays crave for heavily flavoured products, which contribute to a rapid growth of food flavor industry (Koncept Analytics, 2009).

Food flavours mainly used in food products in order to obtain the desired aroma and taste. The application of food flavour material is widespread in processed and convenience food, bakery and dairy, candy and confectionary, ice creams, beverages, savory and snack foods. In Malaysia, popular flavors includes banana, barley, blueberry, cappuccino, cheese, corn/sweetcorn, egg & milk, local coffee,

mango, mocha, orange, pineapple, coconut-milk, taro, rose, white rose, whole milk and yam flavor.

As consumers were particularly selective in the food flavour, they were also concerned about the natural ingredients of food flavours. Although the price of natural food flavours from the extraction process are quite high, it seems to be the future trend due to its high demand. Synthetic flavour, which is cheaper, tends to have less market in food flavouring industry. According to the 'Code of Federal Regulations' (1990), the term 'natural flavour' means '…the essential oil oleoresin, essence or extractive, protein hydrolysate, distillate of any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, juice fruit, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products or fermentation products thereof, whose significant function in food is imparting flavouring rather than nutrition'. The EC Flavour Directive (88/388/EEC) defines natural flavours as '…flavouring substances or preparations which are obtained by appropriate physical processes from material of vegetal or animal origin'. Products that occur in nature but are produced via a chemical (a non natural) process are called 'nature-identical'. (Vandamme and Soetaert, 2002)

Traditionally, the natural flavour and fragrance is produced using the technology of extraction from plant material. It is known to be an expensive process since it requires large bulk of raw materials for a low yield of product. Moreover, the availability of the crops merely depends on unstable weather condition and plant disease problem. Instead of this, people find suitable land to plant the crops that have

significant commercial value such as vanilla, lavender, etc. Alternatively, biotechnology routes in producing natural flavours are introduced.

Biotechnology of flavour production offer less drastic process conditions and are consequently less damaging to the environment than the chemical processes. Though, some processes show difficulties of recovery from the bioreaction mixture and substrates inhibition (Willaert et al., 2005). The processes are categorized based on the biocatalyst used; plant cell cultures, microorganisms and isolated enzymes.

Both plant cell cultures and microbial cultures have complex reactions which have many by-products. However, by using enzyme, the reactions are already specified to certain substrates which limit its side reactions. The higher product conversion is an advantage that simplifies the product recovery (Ghamgui et al., 2006). In addition, enzymatic esterification is carried out at moderate temperature and pressure compared to chemical syntheses (Hari Krishna et al., 2001). Therefore, enzymatic esterification offers the best possible route for industrial purposes of isoamyl acetate production.

1.2 Problem Statement

The production of isoamyl acetate using enzymatic esterification has been studied rigorously due to its high market demand in various industries including food flavour. Enzyme catalyzed esterification provides natural labeled products, low operating conditions, and the biocatalyst can be recycled. The isoamyl acetate yield obtained in previous literatures is between 90 – 95% (Krishna et al., 2000; 2001). Other researchers have improved the yield up to 100% using different biocatalysts, solvents and acyl donors (Romero et al., 2005a; Feher et al., 2008).

In enzyme-catalyzed process in organic solvents, one of the main concerns is the amount of water presence (Zaks and Klibanov, 1985). Water content is reported to have several effects on the enzymes, reaction and product yield. Small amount of water activates enzyme and hence increasing initial reaction rates. However, excess amount of water will cause problems. As a by-product of the direct esterification, water has greatly influenced the thermodynamic balance of the reaction which leads to hydrolysis (Gayot et al., 2003). When the equilibrium shifted towards hydrolysis instead of synthesis then the product yield will be decreased. Therefore, water content should be controlled to have a high product of ester.

In solution to this, a model that represents the process is required. This model consists of enzyme kinetics and material balances. In order to realize this, advance steps are taken which includes selection of parameters that effects the process and optimization study of the process conditions. Then, the optimized condition is used to propose a model. Lastly, a statistical analysis with experimental data is essential in order to validate the model.

1.3 Research Objectives

The objectives of this research are:

i. To identify the critical parameters and optimum conditions of the isoamyl acetate enzymatic esterification.

ii. To optimize the isoamyl acetate production using Response Surface Methodology (RSM).

iii. To develop a mathematical model of the isoamyl acetate enzymatic esterification process incorporating with the selected parameters.

1.4 Scope of Study

In this research, the isoamyl acetate enzymatic esterification was studied using isoamyl alcohol, acetic acid in *n*-hexane as solvent and in the presence of *Candida antarctica* immobilized lipase. The experiments were carried out in shake flasks under various parameters. These parameters were temperature, alcohol/acid molar ratio, enzyme/substrate ratio and reaction time. Optimization study was done to improve the product yield with the optimum conditions obtained. Meanwhile, the kinetic study was done to have a better understanding on the reaction mechanism of the enzyme. Lastly, the model of isoamyl acetate enzymatic esterification process was developed to replicate the process.

The optimization process was done using ‘one factor at a time’. In comparison of ‘one factor at a time’ approach, design of experiment (DoE) coupled with response surface methodology (RSM) was proposed. In RSM, a central composite design (CCD) was chosen to investigate the interaction between parameters studied. The optimum conditions were obtained which was then verified

by the experimental data. Selected responses were yield of isoamyl acetate percentage and water content percentage.

Enzyme kinetic study was done using the Michaelis Menten equation and the kinetic constants were determined. The inhibition studies were carried out to determine inhibition type. The reaction rate equation obtained from the kinetic study will be a part of models used in process modeling. The first principle approach was used in modeling. Fittings of model to the experimental data were done to complete the model equation.

1.5 Organization of the Thesis

This thesis is divided into five chapters as follow:

Chapter 1 covers the introductory part of the thesis. The research background of isoamyl acetate production is explained. The problem statement, research objectives, scope of study and thesis organization are also presented in this chapter.

Chapter 2 describes a review of isoamyl acetate production. The application and production of isoamyl acetate are explicated in details. Previous literatures related to present findings are highlighted and theories applied for the research are covered in this chapter.

Chapter 3 describes the materials/chemicals, equipments and experimental procedures of the research undertaken. The optimization methods are also elaborates in details. This chapter explains the development of mathematical modeling of the process which includes mass and energy balances and enzyme kinetic study.

Chapter 4 presents the results and discussions of the research findings. The results are based on shake flask study of isoamyl acetate enzymatic production and further optimized by Response Surface Methodology. The results of mathematical modeling are also presented and discussed in details.

Chapter 5 describes a summary of the research conducted. Some recommendations are provided for future direction.

CHAPTER 2

LITERATURE REVIEW

This chapter covers the research background of the present work. The previous studies from recognized research works will be stated with reviews and comparison. These literatures are the guidelines of the current research works. Most of all aspects in this project will be explained. This chapter starts with the application of isoamyl acetate in various industries. The enzymatic esterification process is elaborated in terms to substrates, enzymes and solvents used. Then, the attention focuses on studied parameters; temperature, alcohol/acid molar ratio, enzyme/substrate ratio, reaction time, water content and mixing rate. Justifications of selected ranges of studied parameters are given. For the optimization process, general theories of RSM are reviewed. The last part enlightens the readers on basic knowledge of material balance and enzyme kinetic study to develop mathematical models.

2.1 Isoamyl Acetate and Its Application

Isoamyl acetate is synonym to isopentyl acetate, 3-methylbutyl acetate and amylacetic ester. The compound has a strong fruity aroma similar to banana and pear. It is also called as ‘banana oil’ or ‘pear oil’. The ester is a clear, colorless liquid and hardly soluble in water. The chemical and physical properties of isoamyl acetate are shown in Table 2.1.

Table 2.1: Chemical and physical properties of isoamyl acetate (Wypych, 2008).

Chemical/physical properties	Values
Empirical formula	$C_7H_{14}O_2$
Molecular weight	130.19 g/mol
Boiling temperature	142°C
Freezing temperature	-79 °C
Specific gravity	0.87
Solubility in water	2 g/kg
Vapor density	4.5 at 20 °C
Viscosity	0.79 mPa.s at 25 °C
Auto-ignition temperature	379 °C
Flash point	25 °C

Apart from its major role as food flavor, isoamyl acetate is also popular as a solvent for varnishes, paints and nitrocellulose lacquers. The compound is also used in rayon, dyes, artificial pearls, artificial leathers, films manufacturing and extraction of penicillin (HowStuffWorks, 2009). Hence, it is also used in cosmetic industry such as nail polish. In aromatherapy, banana oil commonly used in scenting candles, potpourri, soaps, massage oils and bath oils. Isoamyl acetate is also used to test the effectiveness of respirators or gas masks and acts as honey bee pheromone to attract large group of honeybees (Wikipedia, 2011). The diverse uses and applications of isoamyl acetate made it highly in demand in various sectors.

2.2 Production of Isoamyl Acetate

Isoamyl acetate compound can be produced via chemical synthesis and biotransformation. Chemical catalyzed esterification of isoamyl acetate usually uses sulfuric acid (H_2SO_4) as catalyst. Other types of catalysts were explored such as $FeCl_3$, $CuSO_4$, ferric tri-dodecane sulfonate, $FeCl_3/MnO_2$, KH_2PO_4, sulfonated polystyrene, $TiSiW_{12}O_{40}/TiO_2$ (Pang, et al., 2008). However, these chemical catalysts are not efficiently used for industrial purposes because of the low product yield, high

cost in catalyst preparation, high wastage and not environmental friendly. Most of all, the product produced using chemical catalyst is not 'natural' labelled.

In solution to the chemical catalyzed esterification, biotechnological routes are introduced. There are three routes differentiated by its sources which are plant cell cultures, microbial and enzymes. The plant cell culture is one of the alternative flavour biosynthesis which is able to provide the source of product continuously. However, it weak points lay on the slow growth of plant cell culture (Xu, 2007) and low product yield, which made it an expensive process (Scragg, 2007). The flavours produced from plant cell cultures are given by Table 2.2.

Table 2.2: Flavours produced from plant cell cultures.

Plant cell culture	Flavour	Reference
Haematococcus pluvialis (microalgae)	Vanilla	Tripathi et al., 2002
Alliums (onion, garlic, chives and leak)	Garlic	Hughes et al., 2005
Oryza sativa	Basmati	Bhattacharjee et al., 2002
Theobroma cacao	Cocoa	Jones et al., 2002
Coffea Arabica L.	Coffee	Santana-buzzy et al., 2007

On the other hand, microbial cultures have been long known to produce aroma compounds in food such as alcoholic beverages, soy sauce and vinegar. Microorganism has complex reactions which made it able to produce a wide range of flavour products. Therefore, these flavour compounds need further downstream processes which increases the cost in industrial scale up. Examples of microorganisms producing flavour are tabulated in Table 2.3.

Table 2.3: Flavours produced from microorganisms.

Microorganism	Flavour	Reference
Ceratocystis fimbriata CBS 374-83	Fruity flavour	Soares et al., 2000
Kluyveromyces marxianus ATCC 10022	Fruity flavour	Medeiros et al., 2000
Saccharomyces cerevisiae	Ethyl ester	Saerens et al., 2008
Geotrichum fragans	Fruity flavour	Damasceno et al., 2003

Enzyme catalysed reactions showed a very promising future for food flavour production as enzymes has high substrate specificity, regio- and enantioselectivity (Longo and Sanroman, 2006). These characteristics not only remove undesired side reactions but also produce the specific flavour compound in simple reaction step. Moreover, the enzymes can be recycled, thus minimizing the reaction residues (Romero et al., 2007). Most popular enzymes used were lipases as presented in Table 2.4.

Table 2.4: Flavours produced from isolated enzymes.

Enzyme	Flavour Compound	Reference
Candida Antarctica	Isoamyl acetate	Romero et al., 2005b
Rhizomucor miehei	Terpinyl acetate	Liaw and Liu, 2010
Candida rugosa	Butyl esters	Santos et al., 2007
Mucor miehei	Hexyl acetate	Shieh and Chang, 2001
Staphylococcus simulans	Isoamyl acetate	Ghamgui et al., 2006

2.3 Enzymes for Industrial Applications

Enzymes are biocatalysts that have excellent characteristics. It is a protein molecule with high molecular weight ranging from 10,000 to 2,000,000 (Worthington Biochemical Corporation, 1972). The potential uses of enzyme in industrial application have been recognized by many (Posorske, 1984; Hasan, et al., 2006). There are list of industries such as detergents, starch and fuel, textile, pulp and paper, fats and oils including food and beverages (Kirk, 2002). In food industry, flavors production from enzyme catalyzed process has been reviewed by Christen and Lopez-Munguia (1994).

Common types of enzymes used in food flavour are hydrolases and oxidoreductases (Christen and Lopez-Munguia, 1994). Lipases (Triacyl glycerol

hydrolase E.C.3.1.1.3) belong to hydrolases type and its particular function is hydrolysis of ester, however it is also capable to form ester bonds under reverse hydrolytic conditions which allow them to catalyze various other types of reactions including esterification. Though, the key point for using lipases is the importance and demand for products prepared by natural and environmental friendly. Therefore, lipases are considered as enzymes of high commercial potential due to their flexibility in application (Divakar and Manohar, 2007).

In isoamyl acetate enzymatic esterification, there are a few lipases have been used by previous studies as shown in Table 2.5. The table also shows the techniques of immobilization applied to lipases. Immobilized enzymes are always preferred than free enzymes because of many advantages such as recyclability, better control of the reaction, easy recovery, possibility of continuous flow-system and greater stability (Mosbach, 1976).

Table 2.5: Lipases and its immobilization method used in enzymatic esterification of isoamyl acetate.

Lipases	Immobilization method	References
Candida antarctica	Macroporous acrylic resin	Güvenç et al., 2002; Romero et al., 2005a, 2007
Rhizomucor miehei	Macroporous anion exchange resin	Krishna et al., 2001 Ghamgui et al., 2006
Rhizomucor miehei	Formaldehyde phenyl anion exchange resin	
Mucor miehei	Duolite anion exchange resin	Krishna et al., 2000
Staphylococcus simulans	$CaCO_3$	Ghamgui et al., 2006
Thermomyces Lanuginosus	Porous silica granulates	Romero et al., 2005b

The enzymes used in enzymatic esterification and yield of isoamyl acetate based on previous studies are tabulated in Table 2.6.

Table 2.6: Lipases and the yield of isoamyl acetate based on previous studies.

Lipases	Yield of Isoamyl Acetate (%)	References
Candida antarctica	>90	Vija et al., 1997
	95.5	Gubicza et al., 2000
	80	Güvenç et al. 2002
	100	Romero et al., 2005a, 2007
	192	Romero et al., 2005b
	75	Güvenç et al. 2007
	100	Feher et al., 2008
Rhizomucor miehei	>90	Krishna et al., 2001
Mucor miehei	>80	Razafindralambo et al., 1994
	96.4	Krishna et al., 2000
Staphylococcus simulans	64	Ghamgui et al., 2006

The number of recyclability of an enzyme is an important factor for consideration in industrial scale production as it can reduce cost of raw material. *Rhizomucor miehei* can be recycled up to 10 cycles (Krishna et al., 2001). *Candida antarctica* over more than 10 cycles (Gubicza et al., 2000; Feher et al., 2008) and *S. simulans* can be reused up to 4 cycles (Ghamgui et al., 2006). In several studies, they had compared the ability of enzymes towards esterification reaction. A study on free solvent system has proved that Novozym 435 from *Candida antarctica* was more efficient than Lipozyme RM IM from *Rhizomucor miehei* (Güvenç et al., 2002). This is agreed by Romero and his co-researchers (2007) who continued on a kinetic study on isoamyl acetate enzymatic esterification using Novozym 435 using an organic solvent.

Based on previous studies, *Candida antarctica* immobilized lipase was chosen to be used in the present study. This is because it provides the high yield of

isoamyl acetate and the highest number of cycles that can be used. However, in this study a commercial lipase has been used. According to the manufacturer, the lipase has been produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and adsorbed on a macroporous resin. It is also known as Novozym 435.

2.4 Substrates of Isoamyl Acetate

In direct esterification of isoamyl acetate, the common substrates used are isoamyl alcohol and acetic acid. Acetic acid is widely known as it sour taste in vinegar. It is a simplest chemical yet very important in various industry including the production of synthetic fibers and fabrics. Acetic acid is known as an acyl donor as it contributes acyl derivatives. Other promising acyl donor is acetic anhydride (Romero et al., 2007). In other enzyme catalyzed process such as transesterification, acyl donor used are ethyl acetate (Rizzi et al., 1992) and glycerol triacetate (Wolfson et al., 2010).

The strong inhibition effect of acetic acid has been observed in several studies. Razafindralambo and his co-researchers (1994) had reported that the acid inhibition on lipases occurred because of the local pH decreases and enzyme active site is modified. Therefore, they have proposed to reduce the amount of acid and increase the amount of enzyme in the media. In addition to that, Romero and his co-researchers (2007) also provided the same explanation about acid inhibition effects; however they had suggested a different way out. To maintain the enzyme integrity and activity, a buffer salt (Na_2HPO_4) is added. This has facilitated buffering of the microaqueous interface giving an increase in the extent of esterification up to 79% from 32% without the buffer salt (Romero et al., 2005b). However, it is only showed

a positive effect on esterification of isoamyl alcohol and acetic acid. In contrast, when using acetic anhydride as substrate, it did not improve the conversion.

The other substrate used is isoamyl alcohol which can be found in fusel oil, a by-product of ethanol fermentation from molasses. The isoamyl alcohol is primarily used in solvent industry. Nowadays, the main application of isoamyl alcohol is in pharmaceutical and flavoring industry. Isoamyl alcohol on the other hand, is observed to not exhibit inhibitory effects on lipases (Romero et al., 2007). Contradict to what reported by Chowdary and his co-researchers in 2000 where alcohols are terminal inhibitors of lipases. In his study the isoamyl alcohol indicates inhibitory effects on *Rhizomucor miehei* and explains that the branched substrates are difficult to esterify.

2.5 Solvents for Enzymatic Esterification

Solvents used to dissolve substances into one solution. According to Krishna and Karanth (2002) there are 10 types of solvent system in enzymatic syntheses which are aqueous, water: water miscible, water: water immiscible, nonaqueous, anhydrous, supercritical fluids, reversed micelles, solvent-free systems, gas-phase and ionic liquids. Each type of solvent has their advantages and disadvantages relative to the process.

The most common solvents used in isoamyl acetate enzymatic esterification are nonaqueous organic solvents. This is because it offers great stability of enzymes (Zaks and Klibanov, 1985), hence it influence enzymes activity. Moreover, enzymes are insoluble in organic solvent, which makes it easier to recover from the process

stream (Carrea and Riva, 2000). This is in line with the ability of recycled/reusable enzymes which stated in earlier Section of 2.3. Therefore, the organic solvent has been used in many studies such as *n*-hexane (Romero et al., 2005b; Eisenmenger and Reyes-De-Corcuera, 2010; Dheeman et al., 2011) and *n*-heptane (Krishna et al., 2000; 2001). The selection of suitable solvent is guided by the log *P* concept. *P* is the partition coefficient of the solvent in an octanol/water biphasic system or also means by polarity. Hydrophilic solvents with log $P < 2$ often lead to enzyme inactivation. On the other hand, log $P \geq 4$ are compatible with enzymes (Adlercreutz, 2008).

Beside organic solvents, supercritical carbon dioxide has become an attractive alternative solvent. Supercritical carbon dioxide ($SCCO_2$) offers cheap, inflammable and nontoxic properties compared to other supercritical fluids. It has a near-ambient critical temperature (31.1^{o}C) and moderate critical pressure (73.8 bar) (Srivastava et al., 2002). Romero and his co-researchers (2005a) also studied the supercritical carbon dioxide and concluded that its advantage lies on the easy coupling of reaction and separation processes. The conditions in $SCCO_2$ appear to be more suited for commercialization because of the high conversions obtained with low enzyme and substrate concentration (Srivastava et al., 2002).

Due to toxicity of organic solvent, many have studied the free solvent system. In solvent free system, the reactant itself acts as the solvent. The product recovery for this free solvent system would be easier since fewer components would be present in the end of the reaction. Besides, the removal of solvents from the production step offers significant cost savings and minimizes environmental impact (Güvenç et al., 2002). On the other hand, Feher and his co-researchers (2008) have studied on ionic

liquid as a solvent. Even though the ionic liquid is expensive, it can be recycled and the product yield achieved 100% conversion. However, to compare with the cost of using organic solvent which is cheap with the product yield obtained is rather high, made it an efficient solvent to be used. Therefore, n-hexane is selected as solvent used in this present study.

2.6 Studied Parameters

There are few aspects commonly studied in achieving efficient biocatalysis such as medium engineering, substrate engineering and biocatalyst engineering (Adamczak and Krishna, 2004). However, in this study the medium (solvent), substrates and biocatalyst (enzymes) have been fixed as mentioned in earlier subsections. Therefore some variables from operating conditions that represent these aspects are reviewed. A set of four parameters are selected which are temperature, alcohol/acid molar ratio, enzyme/substrate ratio and reaction time. Then, a high product yield of isoamyl acetate percentage is the ultimate objective. In addition to that, a response of water content as by-product of esterification reaction is also studied.

2.6.1 Temperature

The optimum temperature of a given enzymatic reaction depends on the enzyme source, the type of immobilization and the nature of the substrates (Ghamgui et al., 2006). Temperature effect on the reaction rates is described using Arrhenius expression:

$$k = k_0 exp\left\{-\frac{E_a}{RT}\right\}$$

Where k = rate constant, k_0 = pre-exponential constant, E_a = activation energy, R = gas constant, and T = absolute temperature. For enzyme catalyzed isoamyl acetate, the activation energy reported as Table 2.7.

Table 2.7: The activation energy of enzyme catalyzed isoamyl acetate.

Enzyme	Activation energy (kJ.mol^{-1})	Reference
Candida antarctica	11.3	Romero et al., 2005b
Candida antarctica	8.5	Wolfson et al., 2010
Rhizopus oryzae	7.35	Kumari et al., 2009

At first, an increase in temperature would have a positive effect on the kinetic constant. However, a high temperature can disrupt enzyme tertiary structure, loosing its catalytic activity (Romero, et al., 2005b). Enzyme used in this enzymatic reaction is Novozym 435, a heat tolerant enzyme that can survived up to 100^{o}C. However it inactivates faster at those temperature. The determination of optimum temperature is important as it will affect the productivity along with the operational cost. Therefore, the selection of temperature range should be wise.

Table 2.8 summarized the range of temperature used by previous studies of isoamyl acetate esterification. The optimum temperatures obtained from their studies are also listed. As shown in Table 2.8, the lowest studied temperature is 30^{o}C; meanwhile, the highest is 120^{o}C. However, in order to study how the enzyme reacts in lower temperature than 30^{o}C, we start the temperature range at 20^{o}C. In the meantime, the maximum temperature range had to be lower than the boiling point of solution, which is 70^{o}C (see Appendix B1). Therefore, in present study, we chose the temperature range of 20 to 60^{o}C.

2.6.2 Alcohol/Acid Molar Ratio

In this research, the effects of substrates are studied by varying the molar ratios of alcohol/acid concentration. Concentration of acyl donor (acetic acid) is the most critical as it effects the product concentration. In the same time a high concentration of acid inhibits enzyme and deactivates them. Table 2.9 shows the range of substrates concentrations used in previous studies.

In order to determine the suitable concentration of acetic acid, a preliminary study has been done using ranges mentioned in Table 2.9. A minimum range is applied as to avoid inhibition and in the same time to achieve a high yield. From the results of the preliminary study, the selected acid concentration is 0.1 M. Meanwhile the isoamyl alcohol concentration was determined by studying the previous alcohol/acid molar ratio used. These data are provided in Table 2.9.

In Table 2.9, some studies have been done acid/alcohol molar ratio, however for the standard summary of alcohol/acid molar ratio which is used in this dissertation; the numbers have been changed to have the same molar ratio. The optimization of substrates molar ratio is done because of the need to minimize the concentration of substrates used. This contributes to the minimum quantity of substrates, which is economical.

Taking the lowest molar ratio in previous literature as 0.2, as the starting point. Then, again a preliminary study has been done to choose the appropriate range of molar ratios. The results from preliminary study have suggested that the maximum molar ratio for this research is 2.0. As for molar ratio 0.2 to 1.0 demonstrates the excess of acetic acid while molar ratio of 1.0 to 2.0 demonstrates the excess of isoamyl alcohol, which is a good condition to study. Therefore, the molar ratio of alcohol/acid is varies from 0.2 to 2.0.

2.6.3 Enzyme/Substrate Ratio

The quantity of biocatalyst or enzyme is often expressed by enzyme/substrate ratio (E/S) with the unit of g/l or g/mol alcohol. However, the use of weight percentage (w/w) is also popular. In Table 2.10, the previous studies with their suggested enzyme loading are listed with a variation of units. Enzyme practically acts as catalyst that increases the reaction rate in a reaction. Therefore the higher amount of enzyme loading results in a high product yield. However, as the product yield is dependent on both of enzymes and substrates, the most suitable proportion of these two are needed. With the minimum ratio of enzyme/substrate, it is targeted to fully use these substances in a reaction process.

Hence, this research used enzyme/substrate ratio as another important parameter to be optimized. As tabulated in Table 2.10, there were wide ranges of enzyme loading which is difficult to determine the best ranges used. In addition to that, enzyme loading is varied to the type of enzyme used. Consequently, a preliminary study was done to select the best range for enzyme/substrate ratio. The chosen ranges are 5 mg to 11 mg on the basis of 10 ml of working volume. Therefore, enzyme/substrate ratios are from 0.5 to 1.1 g/l.

2.6.4 Reaction Time

A study of reaction time is important to reduce the reaction time to the minimum. This is not only to save time, but also to save energy used in the process production. Previous studies of ranges and optimum reaction time are listed in Table 2.11.

Table 2.11: Previous studies of reaction time in enzyme catalyzed isoamyl acetate.

Lipases	Range of reaction time (h)	Optimum reaction time (h)	Ester yield	References
Rhizopus sp.	6 – 72	48	80%	Macedo et al., 2003
Rhizomucor miehei	0 – 24	6	80%	Guvenc et al., 2002
Mucor miehei	4 – 48	24	80%	Razafindralambo et al., 1994
Rhizomucor miehei	24 – 72	72	95%	Krishna et al., 2000
Candida antarctica	5 – 30	24	98 - 100%	Feher et al., 2008
Candida antarctica	0 – 48	2	100%	Romero et al., 2007

For this research, a preliminary study of the longest reaction time reported (72 h) is done to determine the best studied range. However, the product yield shows data fluctuations with the higher of reaction time above 10 h. This probably because of the reaction was shifting back and forth from ester synthesis to hydrolysis. Therefore, the selected range of reaction time for this study is 1 to 10 h.

2.6.5 Water Content

Water is a by-product of esterification reaction. It has been recognized as a critical parameter in direct enzymatic esterification reaction. At higher water content, water had a negative effect on the thermodynamic balance, shifting the equilibrium towards hydrolysis. Feher and his co-researchers (2008) have reported that initial water content up to 4% (w/w) has a positive effect on the enzyme activity by reducing the contact with the deactivating acetic acid molecules. Above that value, the initial reaction rate and the final yield decreased gradually and approached to zero at 10% (w/w). However, catalytic activity in non-conventional media strongly depends on the amount of water associated with the enzyme forming its native conformation (Zaidi et al., 2002).

Ghamgui and his co-researchers (2006) have suggested that a small amount of water coats the enzyme and probably minimizes its contact with acetic acid molecules which are known as an inhibitor of the lipase activity. Water content also has an effect on the thermodynamic balance of the reaction. However, esterification reaction is inhibited due to a large amount of water that leads to hydrolysis. Therefore it is important to control the water content during the enzymatic esterification reaction. There are various method used to remove the water such as