INFLUENCE OF PROCESS PARAMETERS ON FARNESYL LAURATE PRODUCTION BY ENZYMATIC ESTERIFICATION IN PACKED-BED REACTOR

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by

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LIST OF SYMBOLS

Å	Angstrom unit	
A	First substrate (alcohol / farnesol)	
A_x	Column cross section area	cm^2
[A]	Initial concentration of farnesol	mol l^{-1} g^{-1}
α	Alpha (axial distance from center point which makes the design rotatable)	
a_m	Area of mass transfer	cm^2 cm^{-3}
a_w	Thermodynamic water activity	
B	Second substrate (free fatty acid / lauric acid)	
BE	Enzyme lauric acid complex	
BEB	Dead end enzyme lauric acid complex	
[B]	Initial concentration of lauric acid	mol l^{-1} g^{-1}
β,γ	Constant for Ordered Bi Bi mechanism	
b_0	Constant coefficient	
b_i	Coefficient for the linear effect	
b_{ii}	Coefficient for the quadratic effect	
b_{ij}	Coefficient for the interaction effect	
C_0	Substrate concentration in the bulk	mol l^{-1}
C_s	Substrate concentration at the surface of the immobilized enzyme	mol l^{-1}
Ds/Dz	Concentration gradient along the column length	mmol $l^{-1}cm^{-1}$
E	Free enzyme	
E'	Modified form of enzyme	
EA	Effective enzyme farnesol complex	
EB	Effective enzyme lauric acid complex	

EAB	Effective enzyme farnesol lauric acid complex	
EA↔E'P	Transition state of reaction	
E'B↔EQ	Transition state of reaction	
EAB↔EPQ	Transition state of reaction	
ε	Error	
ε_v	Void of fraction	
$K_{m,A}$	Michealis Menten constant for farnesol	mol l^{-1} g^{-1}
$K_{m,B}$	Michealis Menten constant for lauric acid	mol l^{-1} g^{-1}
K_i	Inhibition constant	mol l^{-1} g^{-1}
$K_{s,A}$	Dissociation constant for substrate A	mol l^{-1} g^{-1}
k_m	Mass transfer coefficient	m s^{-1}
k_r	Reaction rate constant	s^{-1}
Log P	Partition coefficient of a given compound in the octanol and water two phase system	
n	Sample size	
P	First product (farnesyl laurate)	
Q	Second product (water)	
R^2	Coefficient of determination	
R_m	Mass transfer rate	mol cm^{-3} min^{-1}
RSD	Refractive Standard Deviation	%
S	Lauric acid concentration at the bulk liquid	mmol l^{-1}
S_0	Lauric acid concentration at height of packing equal to zero	mmol l^{-1}
S_i	Lauric acid concentration at the external surface of the immobilized enzyme	mmol l^{-1}
V	Substrate flow rate	ml min^{-1}
V_{max}	Maximum rate of reaction	mol $l^{-1}g^{-1}$ min^{-1}

v	Initial rate of reaction	mol $l^{-1}g^{-1}$ min^{-1}
x_i,x_j	Factors (independent variables)	
Y	Response (molar conversion of lauric acid)	%
Y_b	Molar conversion of lauric acid for batch study	%
Y_c	Molar conversion of lauric acid for continuous study	%
Z	Packed-bed height	cm

LIST OF ABBREVIATIONS

ANN	Artificial Neural Network
ANOVA	Analysis of variance
CCD	Central composite design
CCFC	Central composite face centered design
CCID	Central composite inscribed design
CCRD	Central composite rotatable design
CV	Coefficient of variance
DOE	Design of experiment
FID	Flame ionization detector
FTIR	Fourier Transform Infra-Red Spectroscopy
GC	Gas chromatography
H_2O	Water
hr	Hour
ID	Inner diameter
IUN	Interesterification units
KBr	Pottasium bromide
Lipozyme RM IM	Immobilized enzyme of *Rhizomucor miehei*
Lipozyme TL IM	Immobilized enzyme of *Thermomyces lanuginose*
l	Liter
mM	miliMolar
ns	Not significant
Novozym 435	Immobilized enzyme of *Candida antartica* B

PLU	Propyl laurate unit
PBR	Packed-bed reactors
RCWB	Recirculating water bath
RSM	Response surface methodology
rpm	Rotation per minute
SD	Standard deviation
rpm	Rotation per minute
s	Significant
3D	Three dimensional

PENGARUH PARAMETER PROSES KEATAS PENGHASILAN FARNESIL LAURAT OLEH PENGESTERAN BERENZIM DALAM REAKTOR LAPISAN TERPADAT

ABSTRAK

Pengesteran berenzim menggunakan lipase telah terbukti mempunyai potensi dalam penghasilan ester. Di dalam penyelidikan ini, tindakbalas pengesteran dalam pelarut organik bermangkinkan lipase tersekatgerak telah dikaji secara sistem kelompok dan di dalam reaktor selanjar lapisan terpadat untuk sintesis farnesil laurat. Langkah pengoptimuman bagi proses pengesteran untuk kedua-dua sistem dikaji menggunakan perisian rekabentuk ujikaji dengan metadologi permukaan sambutan berdasarkan rekabentuk komposit putaran berpusat. Didapati Lipozyme RM IM daripada sumber *Rhizomucor miehei* adalah biokatalis paling efektif dan *iso*-oktana telah dipertimbangkan sebagai pelarut paling sesuai untuk proses pengesteran ini dengan memberikan penukaran molar asid laurik yang tinggi semasa proses penyaringan.

Di dalam kajian proses pengesteran secara kelompok, parameter tindakbalas yang dikaji adalah suhu, kepekatan lipase, nisbah molar bahan tindakbalas asid laurik kepada farnesol dan kelajuan pengadukan. Keadaan optimum dalam kajian ini masing-masing adalah 0.92 g kepekatan lipase yang dioperasikan pada suhu 45 ^{o}C dan 150 rpm kelajuan pengadukan dengan 1.4 nisbah molar bahan tindakbalas asid laurik kepada farnesol. Penukaran optimal asid laurik sebanyak 94.81 ± 0.84% dengan aras keyakinan 95% telah dicapai selepas 5 jam. Ujikaji terhadap kesan aktiviti air permulaan telah dijalankan untuk menentukan kandungan air optimum atau aras penghidratan yang diperlukan untuk proses pengesteran berlaku di dalam

sistem kelompok. Didapati Lipozyme RM IM aktif pada aktiviti air permulaan 0.11 dengan memberikan penukaran molar asid laurik yang terbaik iaitu sebanyak 96.80%.

Kinetik untuk proses pengesteran farnesol dengan asid laurik yang dimangkinkan oleh lipase juga dikaji. Model berdasarkan Bi Bi Bertertib bersama perencatan oleh asid laurik didapati sepadan dengan data kadar permulaan dan pemalar kinetik yang ditentukan dengan analisis regresi garis tidak lurus. Pemalar-pemalar kadar yang diperolehi adalah; V_{max} = 5.80 mmol l^{-1} min^{-1} g $enzim^{-1}$, $K_{m,\ farnesol}$ = 0.70 mmol l^{-1} g $enzim^{-1}$, $K_{m,\ asid\ laurik}$ = 115.48 mmol l^{-1} g $enzim^{-1}$, K_i = 11.25 mmol l^{-1} g $enzim^{-1}$ dan R^2 = 0.9892.

Dalam operasi selanjar menggunakan reaktor lapisan terpadat dengan penambahan turus molekul penapisan, keadaan optimum untuk ketinggian lapisan terpadat dan kadar aliran bahan tindakbalas masing-masing adalah 18.18 cm dan 0.9 ml min^{-1}. Penukaran molar asid laurik sebanyak 98.07 ± 0.82% telah diperolehi di bawah keadaan ini. Kajian pemindahan jisim luaran dalam sistem reaktor lapisan terpadat juga telah dikaji. Model terhad tindakbalas dan terhad pemindahan jisim digunakan untuk mengkaji samada pengesteran dalam reaktor lapisan terpadat dipengaruhi oleh terhad tidakbalas atau terhad pemindahan jisim. Keputusan menunjukkan persetujuan yang baik diantara model pemindahan jisim dan data daripada eksperimen yang diperolehi daripada operasi lipase tersekatgerak di dalam reaktor lapisan terpadat. Dalam kes ini penukaran asid laurik adalah dalam terhad pemindahan jisim.

INFLUENCE OF PROCESS PARAMETERS ON FARNESYL LAURATE PRODUCTION BY ENZYMATIC ESTERIFICATION IN PACKED-BED REACTOR

ABSTRACT

Enzymatic esterification using lipase has proven to be potential in the production of esters. In the present work, esterification reaction in organic solvent catalyzed by immobilized lipase was studied in batch and continuous packed-bed reactor system for the synthesis of farnesyl laurate. The optimization in esterification process for both systems was carried out using design of experiment software with response surface methodology based on central composite rotatable design. Lipozyme RM IM origin from *Rhizomucor miehei* was found to be the most effective biocatalyst and *iso*-octane was considered the most suitable solvent for this esterification process since they give high molar conversion of lauric acid during screening processes.

In batch esterification process studies, the reaction parameters investigated were temperature, lipase loading, substrate molar ratio of lauric acid to farnesol and agitation speed. The optimum condition in this study was 0.92 g of lipase loading operated at 45 ^{o}C and agitation speed of 150 rpm with the substrate molar ratio of lauric acid to farnesol of 1.4 respectively. The optimal conversion of lauric acid of 94.81 ± 0.84% with 95% confidence level was achieved after 5 hours. Investigation on the effect of initial water activity (a_w) was carried out in order to determine an optimum amount of water or hydration level needed for esterification process to occur in batch system. Lipozyme RM IM was active at 0.11 initial water activity since it gives best molar conversion of lauric acid value of 96.80%.

Kinetics of lipase-catalyzed esterification of farnesol with lauric acid was also investigated. A model based on Ordered Bi Bi with inhibition by lauric acid was found to fit the initial rate data and the kinetics parameters were evaluated by non-linear regression analysis. The kinetic constant obtained are; V_{max} = 5.80 mmol l^{-1} min^{-1} g $enzyme^{-1}$, $K_{m,\ farnesol}$ = 0.70 mmol l^{-1} g $enzyme^{-1}$, $K_{m,\ lauric\ acid}$ = 115.48 mmol l^{-1} g $enzyme^{-1}$, K_i = 11.25 mmol l^{-1} g $enzyme^{-1}$ and R^2 = 0.9892.

In continuous operation using packed-bed reactor with addition of molecular sieve column, an optimum conditions of packed-bed height and substrate flow rate were 18.18 cm and 0.90 ml min^{-1}, respectively. 98.07 ± 0.82% of molar conversion of lauric acid was obtained under this condition. An external mass transfer studies in packed-bed reactor system have also been studied. A reaction limited model and a mass transfer limited model were used in order to investigate if the esterification in the packed-bed reactor was influenced by reaction limited or mass transfer limited. The results showed very good agreement between mass transfer model and the experimental data obtained from immobilized lipase packed-bed reactor operation, showing that in this case the lauric acid conversion was mass transfer limited.

CHAPTER 1

INTRODUCTION

1.1 The Flavour and Fragrance Industry

Farnesyl esters are classified as sesquiterpenic esters which are organic compounds that present a great interest and importance in the food, cosmetic and pharmaceutical industries as flavour and fragrance substances. By definition, flavour is the sensory notion of a food or other substance, and is determined mainly by the sense of taste. Flavour compounds are sold primarily to the food and beverage industries for use in a wide range of consumer products, including soft drinks, confectionery, bakery goods, desserts and prepared foods. Fragrances are designed to emit a pleasant odour and are mostly used as consumer products such as soaps, detergents, cosmetic creams, lotions and powders, lipsticks, deodorants, hair preparations, candles, air fresheners and all purpose cleaners (Aarkstore Market Research Reports, 2010).

According to Aarkstore Market Research Reports, the global flavour and fragrance market was valued at approximately US$16 billion in 2008. Business insights anticipate that the value of global flavour and fragrance market will grow at a current annual growth rate of 2.5% during 2008 to 2013 to reach a total value of approximately US$18 billion in 2013 (Aarkstore Market Research Reports, 2010). Consumption of flavour and fragrance products in 2006 is estimated at well over US$15 billion worldwide, composed of 13% aroma chemicals, 10% essential oils and other natural extracts, 29% fragrance compositions, and 48% flavour compositions. Historically, flavour and fragrance production has been dominated by

the United States, Japan, and Western Europe in particular, France, United Kingdom, Germany and Switzerland and accounted as much as 74% of total consumption (Sri Consulting, 2007). An analysis conducted by ReportLinker reported that in the year 2008, the global flavour and fragrance industry is expected to remain stable in 2009 and 2010, but will grow again in the subsequent years (ReportLinker, 2009).

There are more than 30 large companies that contribute to the flavour and fragrance industry. Table 1.1 shows the top 10 ranking of companies or leaders with estimated sales volume in millions in flavour and fragrance industry for the year of 2004 until 2008. The rank is based on US$ equivalents and estimated sales volume is a final estimate as of October, 2009 (Leffingwell & Associates, 2009).

Table 1.1: 2004-2008 Estimated sales volume in millions for flavour and fragrance industry leaders (Leffingwell & Associates, 2009)

Rank	Company	Year									
		2004		2005		2006		2007		2008	
		US$	Market Share (%)	US$	Market Share (%)	US$	Market Share (%)	US$	Market Share (%)	US$	Market Share (%)
1	Givaudan	2346.9	13.3	2108.9	13.2	2387.9	13.3	3647.0	18.4	3828.7	18.9
2	Firmenich	1782.1	10.1	1752.1	11.0	2052.1	11.4	2512.8	12.7	2474.1	12.2
3	International Flavors & Fragrances	2033.7	11.5	1993.4	12.5	2095.4	11.6	2276.6	11.5	2389.0	11.8
4	Symrise	1540.3	8.7	1360.2	8.5	1623.0	9.0	1860.8	9.3	1837.4	9.1
5	Takasago	985.1	5.6	898.3	5.6	955.7	5.3	1112.0	5.6	1365.6	6.7
6	Sensient Flavors	499.2	2.8	516.4	3.2	535.4	3.0	572.0	2.9	591.0	2.9
7	T.Hasagawa	490.4	2.8	405.7	2.5	394.4	2.2	448.1	2.3	500.3	2.5
8	Frutarom	196.8	1.1	243.8	1.5	287.2	1.6	368.3	1.9	473.3	2.3
9	Mane SA	345.1	2.0	311.4	1.9	380.0	2.1	448.7	2.3	462.9	2.3
10	Robertet SA	275.7	1.6	245.1	1.5	291.8	1.6	352.1	1.8	422.0	2.1

1.2 Enzymatic Synthesis in the Preparation of Flavours and Fragrances

The preparation of flavours and fragrances by isolating them from natural resources began in ancient times and majority of these products were prepared by chemical synthesis, or by extraction from plants. However, enzymatic synthesis is one area of biocatalysis which is increasingly being used in the manufacture of specialty chemicals, particularly molecules used in the flavour and fragrance industries, and as a component of foods and personal care products (Brenna, 2003; Schrader *et al.*, 2004). Moreover, the increasing sensitivity of the ecological systems supports the choice of environmentally friendly processes and consumers have developed a preference for 'natural' or 'organic' products, thus developing a market for flavours of biotechnological origin (Cheetam, 1997; Serra *et al.*, 2005).

Enzymes such as lipases are most explicitly used in the preparation of flavour and fragrance compounds. Lipases were the favourite biocatalyst because they show high selectivity including stereo-selectivity and give products of high purity and improved quality (Hilal *et al.*, 2006; Kraai *et al.*, 2008). Furthermore, they are easily available on large scale and remain active in organic solvents (Jaeger and Eggert, 2002; Serra *et al.*, 2005). Traditionally, enzymatic syntheses have been carried out in aqueous systems. However, enzymatic catalysis in organic solvents has significantly broadens the conventional aqueous-based biocatalysis. A key advantage in these type of reaction systems are, reduction in the enzyme substrate and/or product inhibition, the solubilization of hydrophobic compounds, the possibility of shifting thermodynamic equilibria towards the desired reaction (Oliveira *et al.*, 2001).

Enzymatic synthesis in the modern biotechnology has put more emphasis on immobilization of enzyme onto solid supports. This is one way to overcome the drawbacks such as enzyme denaturation or inactivation by pH, temperature and organic solvents. Moreover, recovery of the enzyme for reuse is usually difficult, which limits their use due to high cost. Immobilization increases the mechanical robustness of the catalysts as their thermal stability and it enables easy separation of the immobilized catalyst from the reactant-product stream (Florentin *et al.*, 2010).

1.3 Feedstock for Farnesyl Laurate Production

Farnesol and lauric acid are the starting materials required for the production of farnesyl laurate via esterification process as feedstock. Farnesol and lauric acid are both derived from natural resources and thus it was considered attractive to study the enzymatic synthesis of farnesyl laurate in organic media since non-aqueous enzymology has potential applications in the industry compared to the conventional chemical synthesis of the esters. Until today, lauric acid has limited use in the esterification research using farnesol.

Farnesol or trimethyl dodecatrienol is one of sesquiterpenes and fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Besides, farnesol is also a natural pesticide for mites and is a pheromone for several other insects. Its use worldwide is in 1-10 metric tones per annum (Lapczynski *et al.*, 2008). Farnesol is a natural organic compound found as a colourless liquid. It is insoluble in water, but miscible with oils. It is present in many essential oils such as citronella, neroli, cyclamen, lemon grass, tuberose, rose,

musk, balsam, and tolu. Esterification between farnesol and lauric acid can be represented in Figure 1.1.

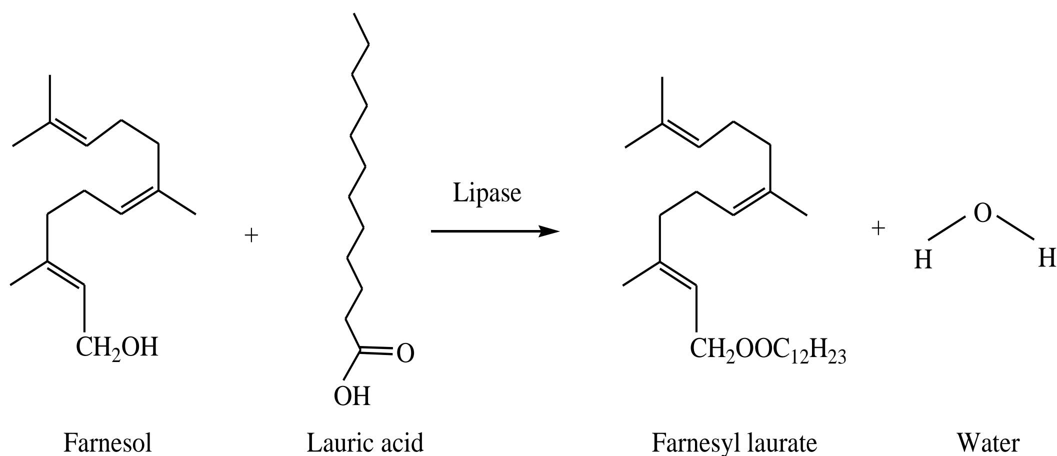

Figure 1.1: Esterification between farnesol and lauric acid (Leray, 2000)

Lipases can accept a broad range of natural substance, thus, lauric acid was chosen as an acyl donor in the esterification of farnesyl laurate. Lauric acid or dodecanoic acid is a white powdery solid with a faint odor of bay oil or soap. It can be obtained from renewable raw materials such as oils and fats (Serri, 2006). Approximately 50% of coconut oil and palm kernel oil are lauric acid. Lauric acid is inexpensive, has a long shelf-life, and is non-toxic and safe to handle. Lauric acid is also believed to possess antimicrobial properties and is frequently exploited by pharmaceutical companies (Chua and Sarmidi, 2004). The exploitation of synthetic capability of lauric acid will promote sustainable chemistry.

1.4 Problem Statement

Terpene ester as well as sesquiterpene ester has been used as the desirable flavouring and fragrance compounds. Organic esters are used in a variety of industries such as perfumery, flavour, pharmaceuticals, plasticizer, solvents, and intermediates. Esters are one of the most important natural fragrances. The traditional extractions from plant materials, steam distillation and direct biosynthesis by fermentation are the three generally used methods for flavour and fragrance production. However, these methods exhibit a high cost of processing and low yields of desired esters for a large amount of raw material (Abbas and Corneau, 2003; Ganapati and Trivedi, 2003; Ganapati and Lathi, 2004). In addition, steam distillation requires high energy consumption (Al-Marzouqi and Rao, 2006). Therefore, better processes with minimum processing cost need to be developed.

Chemical routes are being less favoured owing to the attendant problems such as poor reaction selectivity leading to undesirable side reactions, low yields, pollution and high cost of manufacturing. Thus enzymatic, particularly lipase synthesis flavour ester is gaining much attention from the researchers to enhance currently used method in the production of commercial ester compounds (Serra, 2005). The main problem of the enzyme catalyzed process is the high cost of the lipases used as catalyst. However, high operational stability of the immobilized lipase was reported in several studies (Chen and Wu, 2003; Polizzi *et al.*, 2007), making its recycle possible in a batch system, or its long use in a continuous system by regeneration of support, which reduces the incidence of catalyst cost. Furthermore, continuous synthesis of ester using immobilized lipase would be

advantageous for mass production although most of the synthesis have been carried out in batch reactor (Pioa *et al*., 2004).

Solubility is another problem in enzymatic approaches for flavour and fragrance production primarily in aqueous system because many flavours and fragrances are composed of relatively high molecular weight compounds that are poorly soluble or insoluble in water. Problems arise when both poor diffusion of these substances and mass transfer limitations are associated due to the reaction media that composed high amount of water (Gabelman, 1994; Krishna and Karant, 2002). Owing to the obvious advantages offered by the non-aqueous system, the use of organic solvents as a reaction medium may overcome this problem, thus, enhancing flavour and fragrance production. This system would increase the solubility of non-polar substrates and products, limit enzymes deactivation and shifting thermodynamic equilibria to favour ester synthesis over hydrolysis (Krishna and Karant, 2002).

Lipase catalyzed esterification in organic solvents has the advantage to remain active and catalyzed a wide range of esterification reactions. Therefore, after considering all the circumstances, esterification of farnesol with lauric acid using Lipozyme RM IM as the catalyst in *iso*-octane system was investigated as the present study. In this *iso*-octane system, one step esterification reaction was conducted in a batch process to get the optimum condition for the production of farnesyl laurate. High yields of product can be obtained since the solubility problem can be eliminated in *iso*-octane system. Both farnesol and lauric acid are soluble in *iso*-octane. Furthermore, this study aimed to develop an optimal continuous production of

farnesyl laurate in a packed-bed reactor to investigate the possibility of large scale production further in order to suit the growing demand in biotechnology.

1.5 Research Objectives

The main objective of this research project is to synthesize farnesyl laurate ester using enzymatic approach particularly by immobilized lipase. This research project aims to achieve the following specific objectives:

1. To determine the best fatty acid, solvent and immobilized lipase which gives the highest conversion in the esterification of farnesyl ester during screening processes.
2. To study the effect of process parameters (temperature, lipase loading, agitation speed and substrate molar ratio) on the esterification of farnesyl ester by using immobilized lipase in batch system as the basis to conduct continuous study (packed bed height and substrate flow rate) in packed-bed reactor and optimization using Response Surface Methodology.
3. To determine the kinetic parameters by evaluating two different mechanisms based on Ping Pong Bi Bi and Ordered Bi Bi mechanism for farnesyl ester synthesis using immobilized lipase in batch system.
4. To study the effect of external mass transfer by varying parameters such as packed-bed height and substrate flow rate in a continuous packed-bed reactor.

1.6 Organization of the Thesis

This thesis is divided into five chapters as follows;

Chapter 1 gives the introductory of this research project. This chapter starts with the market demand in flavor and fragrance industry reported by market researcher. It also gives brief overview of the flavour and fragrance production which leads to the development of enzymatic synthesis. The problem statement and objectives of this research project are also stated clearly in this chapter.

Chapter 2 describes the literature review from other researchers and methods applied in the present days for the industrial production of esters specifically in enzymatic-catalyzed esterification in non-aqueous media. It is followed by a discussion on advantages of using immobilized lipase in the esterification process. Reviews on kinetic, mass transfer and modeling of enzymatic esterification using statistical method are also covered.

Chapter 3 describes the methods and analysis required for the esterification process. It also explained on the chemical requirements and equipments used throughout the whole process of this study. The subsequent topics describe clearly the methodology of this research project-synthesis using immobilized lipase, optimization, kinetic and mass transfer studies.

Chapter 4 presents the results obtained from experimental runs and discusses on every effect of parameters on the synthesis of farnesyl laurate ester.

Chapter 5 concludes the research project. Recommendations for future work related to this research project are also given.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzymatic-catalyzed Esterification

Enzyme catalyzed esterification reactions have found many applications, ranging from the modification of vegetable oils for human consumption to the production of optically pure chemicals (Lortie, 1997). Historically, enzymatic catalysis has been carried out primarily in aqueous systems. However, water is a poor solvent for nearly all reactions in preparative organic chemistry. To displace the equilibrium in favor of synthesis, rather than hydrolysis, these reactions are performed in non-aqueous or microaqueous media dated back to the beginning of last century (Krishna and Karanth, 2002).

Esterification reactions between polyhydric alcohols and free fatty acids are catalyzed by lipases in water-poor organic solvents under conditions of low water activity or even solvent free systems. Further discussion on organic media and its suitability for enzymatic reaction will be described later in Section 2.2.1. Although ester synthesis can be done chemically with acid or base catalysis, the use of enzyme technology since many years ago offers the advantages of mild operating conditions, low energy requirement, accept a broad range of substrates, biodegradability, reduced side reactions, and specificity (Okumura *et al*., 1979, Marlot *et al*., 1985).

2.1.1 Introduction to the Enzymatic Esterification Process

Esterification is the reverse reaction of hydrolysis. This reaction is only possible and useful in a microaqueous reaction system where hydrolysis can be

minimized and controlled with limited amounts of water in the system. In water-abundant reaction systems, hydrolysis is the main reaction and it is difficult to make useful reaction for esterification. A large number of applications have been studied on the basis of extensive studies of microaqueous enzymology. This gives a variety of possibilities to exploit the enzyme technology for ester synthesis.

Esterification is the simple reaction between an organic acid and an alcohol. The reaction is depicted in Figure 2.1. Water is one of the direct products from the reaction and it has important effects on the shifting of reaction equilibrium. It has to be continuously removed from the reaction system in order to minimize the reverse hydrolysis reaction. Xu (2003) has summarized the common methods to remove water from the reaction system in laboratory experiments or industrial applications as shown in Table 2.1.

Water in the reaction system is in one way the reaction by-product, which should be removed in order to force the reaction to the product side. On the other hand, a certain dynamic water environment should be maintained in order to maintain high enzyme activity. This is especially important for the long-term use in an industrial plant.

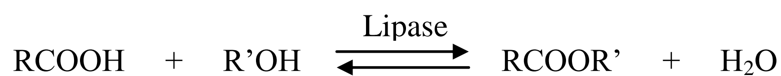

Figure 2.1: A simple description of enzymatic esterification.

Table 2.1: The common methods to remove water from the reaction system in laboratory experiments and industrial processes (Xu, 2003)

Method	Description	Applications
Vacuum evaporation	The system is applied with vacuum and optimum vacuum should be used to maintain a certain water activity in the reaction system. A too high vacuum may lead to a low enzyme activity.	Industrial scale
Membrane pervaporation	A membrane pervaporation system with vacuum is applied to remove water from the reaction system. A suitable water activity in the reaction system can be maintained by an automatic control system with a humidity sensor.	Industrial scale
Azeotropic solvent distillation (external drying agents)	A distillation system is applied and the distilled solvent or alcohol can be dried in a separate connected chamber with a drying agent. After drying the solvent alcohol can fed back to the reaction system.	Industrial scale
Drying agent (internal or external)	Drying agents such as a molecular sieve or silica gel are added to the reaction system to remove water. It is very useful for large-scale plant operations.	Industrial scale
Air or N_2 bubble	Bubbling by dry air or nitrogen can be also used to remove water. It is useful in small-scale experiments.	Laboratory scale
Solvent engineering	A hydrophilic solvent is used to extract water and keep water away from the reaction.	Industrial and laboratory scale
Salt pair (internal)	Salt pairs with certain water activity are used to maintain the water activity of the system.	Laboratory scale
Water activity control (external)	Vapor phases in the reaction system are circulated with the vapor phase of the saturated salt solution or salt pair. Water content or activity of the reaction system can be thus regulated.	Industrial and laboratory scale

2.1.2 Lipases and Its Application

Enzymes are considered as nature's catalysts. Most enzymes today are produced by the fermentation of biobased materials (Louwrier, 1998). There are six major groups of enzymes that can be categorized into oxido-reductase, transferase, hydrolases, lyases, isomerase and ligase. Of all of the enzymes, hydrolases are mostly employed for industrial biotransformation because of their biotechnological potential (Benjamin and Pandey, 1998).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are part of the family of hydrolases and most frequently used enzymes in organic chemistry including for the enantioselective hydrolysis, transesterification, esterification and interesterification reaction (Effenberger *et al*., 1997; Konigsberger *et al*., 1999; D'Antona *et al*., 2002; Jin *et al*., 2003; Brady *et al*., 2004; Long *et al*., 2005; Ong *et al*., 2005). Lipases are widely used because of their ready availability, low cost of production, and enormous utility in organic synthesis. Lipases from many different microbial sources are commercially available. Each of these lipases demonstrates its distinct substrate specificity which is known to be less stringent compared to other enzymes (de Zeote *et al*. 1994). In addition, lipases offer the usual advantages by enzymes, such as mild operating conditions, biodegradability, and so forth as mentioned earlier in Section 2.1. Thus, lipases have tremendous potential as industrial catalysts.

There are several applications of lipases. Its versatility makes lipases the enzymes of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Houde *et al*., 2004; Fariha *et al*., 2006). The lipase catalyzed transesterification in organic solvents is an emerging

industrial application such as production of cocoa butter equivalent, human milk fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acids (PUFA) and production of biodiesel from vegetable oils (Jaeger and Reetz, 1998; Nakajima *et al*., 2000). Akoh (1993) also reported that *Mucor miehei* (IM 20) and *Candida antartica* (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent and transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols.

Lipases can be used as biocatalyst in the production of useful biodegradable compounds. For example, 1-Butyl oleate was produced by direct esterification of butanol and oleic acid to decrease the viscosity of biodiesel in winter use. Lipases can catalyse ester syntheses and transesterification reactions in organic solvent systems has opened up the possibility of enzyme catalyzed production of biodegradable polyesters (Linko *et al*., 1998). In the textile industry, polyester has certain advantages including high strength, stretch resistance, stain resistance, machine washability, wrinkle resistance and abrasion resistance. Furthermore, lipases have found to be important applications in detergent industry as an addition to detergents which are used mainly in household and industrial laundry and in household dishwashers. To improve detergency, modern types of heavy duty powder detergents and automatic dishwasher detergents contain one or more enzymes, such as protease, amylase, cellulose and lipase (Ito *et al*., 1998).

Lipases have also been used for addition to food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known as flavour and fragrance components (Macedo *et al*., 2003). Earlier, lipases of different

microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Seitz, 1974; Fariha *et al*., 2006). In pharmaceutical industry, lipases can be used to resolve the racemic mixtures and to synthesize the chiral building blocks. Chiral intermediates and fine chemicals are in high demand from the pharmaceutical and agrochemical industries for the preparation of bulk drug substances and agricultural products. For example, lipase from *Candida antartica* (Novozyme 435) has been used for the kinetic resolution of racemic flubiprofen by the method of enantioselective esterification with alcohols (Zhang *et al*., 2005) and lipase from *Candida cylindracea* has been used for resolving racemic mixture of baclofen that is normally used in the therapy of pain and as a muscle relaxant (Maulidhar *et al.*, 2001).

Besides pharmaceutical industry, lipases have found significant used in cosmetics industry. Unichem International (Spain) has used *Rhizhomucor miehei* lipase as a biocatalyst for the production of isopropyl myristate, isopropyl palmitate and 2-ethylhexylpalmitate for use as an emollient in personal care products such as skin and sun-tan creams, bath oils etc. Wax esters (esters of fatty acids and fatty alcohols) have similar applications in personal care products and also being manufactured enzymatically using *Candida cylindracea* lipase in a batch bioreactor (Fariha *et al*., 2006).

2.1.3 Lipase Biocatalysis in the Production of Esters

The benefits of employing lipase biocatalysis in ester production are obvious. Consequently, lipase biocatalysis has been intensively studied during recent years. The number of available lipases has increased considerably since the 1980s. This is mainly as a result of the enormous achievements made in the cloning and expression of enzymes from microorganisms, as well as an increasing demand for these biocatalysts. Instead of ester synthesis, extensive research proved that lipases are very effective biocatalysts for the synthesis of optically pure compounds. Moreover, they often exhibit very good stability, are active in a wide range of organic solvents and do not need cofactors (Bornscheuer and Kazlauskas, 1999; Liase *et al.*, 2000).

Reaction media for esterification processes have been conducted either in solvent free systems (aqueous media) or solvent systems. It has been demonstrated that in organic solvent systems, due to low water activity, the equilibrium can be shifted so that esterification reaction can occur. Besides reaction media, lipase modifications such as immobilization have several advantages to the esterification processes. Some of the advantages of immobilized lipases are simplified product recovery, increased the enzyme reusability, significant higher productivity in continuous operation as compared to the batch operation and improved in stability (Gabelman, 1994). In addition, Table 2.2 summarized the esterification process for various types of lipases with different types of alcohols and fatty acids either in solvent free system or solvent medium. Further discussion on immobilization of lipases will be described later in Section 2.1.4.

Table 2.2: A literature survey of lipase-catalyzed ester synthesis

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	**Acid**				
Geraniol Menthol	Butyric acid Lauric acid	*Geotricum candidum* *Aspergillus* sp. *Mucor miehei* *Hog pancreas* *Alcaligenes* *Candida cylingracea* *Rhizopus arrhizus* *Rhizopus delamar* *Pseudomonas*	Heptane / diisopropyl ether / hexane / benzene	Immobilized on hydrophilic support by adsorption / entrapment. Support : Adsorption – spherosil XOBO15,XOBO75 from Rhone Poulene. Celite, porous glass, alumina, titania from corning. Entrapment - polyurethane	Marlot *et al*., 1985
Geraniol Isoamylic alcohol	Acetic acid Propionic acid Butyric acid	*Alcaligenes* *Aspergillus* *Aspergillus niger* *Candida rugosa* *Geotricum candidum* *Fungal lipase* *Mucor miehei* *(Gist-Brocades)* *Mucor miehei* (*Novo,Lipozyme)* *Penicillium cyclopium A* *Penicillium cyclopium B* *Rhizopus arrhizus* *Porcine pancreas(Rohm)* *Porcine pancreas* *(koch-light)*	*n*-heptane	Physical state of these preparations was a dried mycelium/crude powder both of variable granulometry. *Mucor miehei* was immobilized on a solid support. *Aspergillus niger* was in a liquid form,immobilized by adsorption on porous glass beads (Spherosil XOBO75). Geranyl butyrate: *C.rugosa*(90%), *M.miehei*(96%),*M.miehei* (lipozyme,93%), *Porcine pancreas* (rohm,85%), *R.arrhizus* (97%) conversion in 24hrs.	Langrand *et al*.,1988

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	Acid				
Geraniol	Butyric acid	*Rhizopus oryzea* (CBS 112-07)	Pentane (90%) *n*-heptane (91%) Toluene (41%) Diethyl ether (21%) Tetrahydrofuran (2%)	Lyophilized whole cells of *R.oryzea*. The reaction is adequately performed in solvents with high log P (pentane or *n*-heptane) even with water content remarkably higher than the substrate concentration. Maximum activity is at 55-60°C (95%).	Molinari *et al*., 1995
Citronellol	Butyric acid	*Candida rugosa* *Pseudomonas Fluorescens* *Rhizopus japonicus*	Solvent free system and *n*-hexane	Highest conversion 98% without additional organic solvent.	Wang and Linko, 1995
Geraniol	Propionic acid Butyric acid Valeric acid	*Esterase 30 000 (85%)* *(Mucor miehei)* *Rhizopus arrhizus (<10%)* *Lipozyme (M.miehei, 66%)* *Piccantase B (80%)* *Esterase 193 (63%)*	Solvent free system	*Esterase 30 000* and *R.arrhizus* was used in powder form. Lipozyme was immobilized onto a macro porous anion-exchange resin. Conversion by esterase of geranyl butyrate (85%), geranyl valerate (85%), geranyl propionate (<40%).	Karra-Chaabouni *et al*., 1996
Benzyl alcohol	Lauric acid	*Pseudomonas*	Benzene	Ester yield 80% after 72 hrs using free and immobilized lipase in stirred tank bioreactor.	Fukunaga *et al*., 1996

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	**Acid**				
1-phenylethanol	Vinyl acetate	*Pseudomonas fluorescens*	*t*-butylmethyl ether	Eupergit C250L was the best support for immobilization of lipase used.	Ivanov and Schneider, 1997
Geraniol Geranyl acetate Various acetate and butyrates	Short-chain acids	*Fusarium oxysporum* (well suited for the production of short chain geranyl esters)	Ethylene glycol diacetate Isopropyl acetate *n*-hexane	Esterase – in dried powder form. 85-95% conversion of geranyl acetate. Geranyl and butyric acid – no inhibition of *F.oxysporum* esterase activity by butyric acid was observed even at high acid concentration.	Stamatis *et al*., 1998
Ethanol tripalmitin	Oleic acid triolein	*Rhizopus niveus* *Mucor miehei*	Microaqueous, biphasic (*n*-hexane-water) and surfactant-enriched biphasic system	Biphasic system was more efficient than in microaqueous for esterification but not for interesterification.	Tweddell *et al*., 1998
Geraniol Citronellol	Fatty acid vinyl esters	*Celite-adsorbed lipase of Trichosporon fermentans*	*n*-hexane	With fatty acid vinyl esters as acyl donors, the lipase catalysed the synthesis of geranyl an citronellyl esters with carbon chains shorter than C_6 in with yields of more than 90%.	Nakagawa *et al*., 1998

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	**Acid**				
Geraniol Nerol Hexanol 2-hexanol Citronellol Cis-3-hexanol	Propionic acid Butyric acid Valeric acid Caproic acid	*Esterase 30 000 (71.4%) (Mucor miehei)* *Candida cylingracea (83.1%)* *Pseudomonas fluorescens (80%)* *Rhizopus arrhizus (11%)* *R.niveus (3.4%)* *R.javanicua (4%)*	Solvent free system.	No immobilization, lipases used in powder form without further purification. Yield increase when acid chain lenght increase (C_2-C_6). *M. miehei* : - geranyl butyrate (60%), geranyl acetate and geranyl propionate (30%), geranyl valerianate (74%) and geranyl caproate (85%) conversion.	Karra-Chaabouni *et al*., 1998
Monohydric alcohol (1-octanol)	Dihydroxystearic acid (DSHA)	*Rhizomucor miehei* (Lipozym IM) *Candida antartica* (Novozym 435)	DMF Acetone Diethyl ether Chloroform Toluene Pentane Hexane Heptane Octane Nonane Decane Dodecane Hexadecane	The percent conversion was higher in organic solvents with log P (the logarithm of the partition coefficient of solvent in octanol/water system) from 2.0 to 4.0. Increasing the mole ratio of alcohol to acid above 2.0 did not increase the percent conversion of ester.	Awang *et al*., 2000

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	**Acid**				
Benzyl alcohol	Octanoic acid	*Candida rugosa*	Diisopropyl ether Chloroform Toluene Cyclohexane *n*-heptane Iso-octane	Cyclohexane was the best solvent. Reaction follows Ping Pong Bi Bi mechanism.	Matsumoto *et al*., 2001
Hexanol Geraniol 2-octanol	Acetic acid Butyric acid	Dry mycelium of 4 strains of *Rhizopus oryzea* : - *R.liquefaciens* (CBS 260.28) - *R.delamar* (CBS 328.47) - *R.javanicus* (CBS 391.34) - *R.oryzea* (CBS 112.07)	*n*-heptane	Non immobilized enzyme. Dry mycelium of 4 strains of *Rhizopus oryzea* proved effective for efficiently catalyzing the synthesis of hexylacetate and butyrate, geranylacetate and butyrate. The esterification of racemic mixture of 2-octanol and butyric acid proceeded with high enantioselectivity (>97%) using CBS 260.28 and CBS 112.07.	Gandolfi *et al*., 2001
Geraniol Citronellol	Acetic acid	*Candida antartica* (entrap-immobilized in cellulose acetate-TiO_2 gel fiber by sol gel method)	Heptane	Conversion reached 85% for geranyl acetate and 75% for citronellyl acetate after 80 hrs. The activity of the fiber-immobilized lipase was not affected by bulk water content or the produced water.	Ikeda and Kurokawa, 2001

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	**Acid**				
Methyl alcohol *n*-propyl alcohol *n*-butyl alcohol *n*-hexyl alcohol *n*-octyl alcohol *n*-decyl alcohol Lauryl alcohol Myristyl alcohol Cetyl alcohol	Lauric acid	Lipolase 100L (*Thermomyces lanuginosus/Aspergillus oryzea)*	*Iso*-octane	*n*-butyl reacted with lauric acid with highest reaction rate. Esterification reaction using reactor with 4 baffles and 6 blades turbine impeller.	Shintre *et al*., 2002
Lauryl alcohol	Lauric acid	*Candida rugosa*	Chloroform Toluene Hexane Cyclohexane Octane *Iso*-octane No-solvent	95% of substrate conversion. *Iso*-octane exhibited the best reaction media.	Wu *et al*., 2002
Ethanol	Acetic acid 1-propionic acid Butyric acid Hexanoic acid Valeric acid Heptanoic acid Octanoic acid	*Rhizopus chinesis* *Rhizomucor miehei* *Candida rugosa* *P. Pancreas* *C. Lipolytical* *Candida antartica* *P. nitens* *Mucor javanicus* *R. arrhizus* *A. niger* *Pseudomonas* sp.	Ethanol Acetone Tetrahydrofuran Pentanol Benzene Cyclohexane Hexane Heptane Octane Nonane Dodecane	Esters conversion more than 80%, ethyl hexanoate gives the highest conversion in heptane. *Rhizopus chinesis* is the best catalyst. Stability until 840-981 hrs (half life period).	Xu *et al*., 2002

Table 2.2: Continued

Substrate		Enzyme/Lipase	Reaction media	Description	Reference
Alcohol	Acid				
Butyl alcohol	Lauric acid	*Candida rugosa*	*Iso*-octane	Ester yield after 48 hrs were around 30-40mM (~80%). Enzyme stability until 30 days.	Nagayama *et al*., 2002
Geraniol	Butyric acid	*Mucor miehei (Gist-Brocades) – powder form* -for dehydrated substances, the enzyme was dried over P_2O_5 under vacuum for 24h at room T, the acid and the alcohol were dried by adding molecular sieves that had been freshly reactivated by heating.	Solvent free system.	Study the role of water activity. Conversion yield after 75 hrs at 37^0C : -both substrates and enzyme not dried (71%) -substrates dried, enzyme not dried (75%) -substrates not dried, enzyme dried (72%) -both substrates and enzymes dried (73%)	Karra-Chaabouni *et al*., 2002
Geraniol	Acetic acid	*Candida cylindracea* lipase was entrapped in organic hybrid sol-gel polymers (formed within nonwoven fabric) made from tetramethoxysilane (TMOS) and alkyltrimetoxysilanes.	hexane	Protein immobilization efficiency was 91% and the specific activity of the immobilized enzyme was 2.6 times that of the free enzyme. Excellent thermal stability for immobilized enzyme.	Chen *et al*., 2002