KINETICS OF BIOTRANSFORMATION FOR CITRONELLOL PRODUCTION USING *SACCHAROMYCES CEREVISIAE*

by

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TABLE OF CONTENTS **Page**

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Description
DNA	Deoxyribonucleic acid
ATP	Adenosine triphosphate
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NAD^+	Oxidized form of NADH
$NADP^+$	Oxidized form of NADPH
GRAS	Generally recognized as safe
CCGLB	Continuous-closed-gas-loop bioreactor
e.e. %	Percentage enantiomeric excess
YADH	Yeast alcohol dehydrogenase
rpm	Rotation per minute
hr	Hour
PTFE	Polytetrafluoroethylene
DO	Dissolved oxygen
OD_{680}	Optical density at wavelength of 680 nm
ODE	Ordinary differential equation
DNS	Dinitrosalicyclic acid
U	Units of productivity
VOC	Volatile organic compound
MBS	Membrane-based system
YPD	Medium for fermentation of yeast
ISPR	*In-situ* product removal
HCl	Hydrochloric acid
KOH	Potassium hydroxide

KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Potassium hydrogen phosphate
s	Second
min	Minute
T	Temperature
R	Ideal gas constant
vvm	Volume liquid per volume of reactor per minute
Eqn.	Equation
N	Nitrogen
S	Sulphur
P	Potassium
NaOH	Sodium hydroxide
FID	Flame ionized detector
GC	Gas chromatography
M	Molar
mM	Milimolar
CO_2	Carbon dioxide
atm	Atmospheric
g	Gram
L	Liter
mL	Mililiter
µm	Micrometer
µg	Microgram
nmol	Nanomol
S. cerevisiae	*Saccharomyces cerevisiae*
sp.	Species
v/v	Ratio volume per volume

EMP	Embden Mayerhof Pathway
M-M	Michaelis-Menten
N.A	Not available
TCA	Tricarboxylic acid
MPa	Megapascal
OSN	Organic solvent nanofiltration
ILs	Ionic liquids
i.d	Internal diameter
ATCC	American Type Culture Collection

LIST OF SYMBOLS

Symbol	Description	Unit
μ	Specific growth rate	hr^{-1}
Q_s	Glucose consumption rate	g/L.hr
X	Cell concentration	g/L
t	Time	hr
X_o	Initial concentration of cell	g/L
S	Substrate concentration	g/L
r_i	Mass transfer rate of component *i*	g/L.hr
$k_L a$	Overall mass transfer coefficient	hr^{-1}
C^*	Equilibrium concentration at interface	g/L
i	Component *i*	-
C_i	Concentration of component *i*	g/L
P_i	Partial pressure of component *i*	atm
H	Henry's coefficient	L.atm/mol
$C_{G,g\ in}$	Concentration of geraniol in gas phase at bioreactor inlet	g/L
$C_{G,g\ out}$	Concentration of geraniol in gas phase at bioreactor outlet	g/L
$C_{C,g\ out}$	Concentration of citronellol in gas phase at bioreactor outlet	g/L
$C_{G,l}$	Concentration of geraniol in bulk liquid	g/L
$C_{C,l}$	Concentration of citronellol in bulk liquid	g/L
Q	Substrate flow rate	L/hr
V_g	Volume of gas	L
V_T	Volume of bioreactor	L
V_l	Volume of liquid	L

$C_{G,l}^{*}$	Equilibrium concentration of geraniol at interface	g/L
$C_{C,l}^{*}$	Equilibrium concentration of citronellol at interface	g/L
R	Ideal gas constant	L.atm/mol.K
T	Temperature	K
r	Rate of reaction	g/hr.g_{cell}
K_m	Michaelis constant	g/L
V_{max}	Maximum rate of reaction	g/hr.g_{cell}
U	Units of production	µmol/min

ABSTRAK

Kajian ini dijalankan bagi mengkaji keupayaan keseluruhan sel *Saccharomyces cerevisiae* mentransformasikan monoterpen yang bersifat hidrofobik, iaitu geraniol kepada sitronellol di dalam reaktor gelung gas tertutup berterusan (CCGLB). Geraniol yang mempunyai tekanan wap dan kemeruapan yang tinggi serta kebolehlarutan yang rendah di dalam medium akuas amat sesuai digunakan dalam sistem ini. Kajian kinetik terhadap proses biotransformasi menggunakan dua jenis keadaan sel; iaitu sel tumbuh dan sel rehat telah dijalankan. Kajian awal menunjukkan sistem biotransformasi fasa cecair hanya sesuai dijalankan dengan penggunaan sel rehat di mana perencatan substrat terhadap sel telah diperhatikan apabila sel tumbuh digunakan. Walaubagaimanapun, kedua-dua jenis sel telah berjaya mentransformasikan geraniol di dalam CCGLB. Sistem gelung gas tertutup tersebut telah menghasilkan kepekatan sitronellol yang maksimum iaitu sebanyak 2.38 g/L dengan aktiviti spesifik 7.9 U/g_{cell}. Ia telah dihasilkan dengan menggunakan sel rehat sebagai pemangkin bio pada pH 7, 350 rpm, kadar aliran substrat pada 8 L/min dan kepekatan glukosa sebanyak 50 g/L. Proses tersebut berjaya meningkatkan produktiviti sitronellol sebanyak 5 kali ganda berbanding biotransformasi di dalam mod kelompok (biotransformasi fasa cecair). Bagi proses biotransformasi menggunakan sel tumbuh pula, didapati kehadiran substrat biotransformasi telah mempengaruhi penumbuhan sel di mana penurunan kepekatan sel sebanyak 60% telah direkodkan. Proses tersebut telah menghasilkan sitronellol yang maksimum sebanyak 1.18 g/L pada keadaan kendalian pH 7, 500 rpm dan kadar aliran substrat pada 8 L/min. Sistem CCGLB juga berkebolehan menyingkirkan produk secara *in-situ* di mana produk yang terhasil adalah jernih tanpa memerlukan sebarang proses hilir. Pemodelan sistem biotransformasi

menggunakan sel rehat di dalam CCGLB telah dibina dengan menggabungkan model Michaelis-Menten serta model dua filem. Pemalar-pemalar kinetik yang diperoleh daripada eksperimen mod kelompok adalah 0.015 g/hr.g_{cell} bagi V_{max} dan 17.9 g/L bagi K_m. Model penyelakuan telah dipadankan dengan keputusan eksperimen pada kadar aliran substrat yang berbeza. Dari kerja pemodelan tersebut, pekali pemindahan jisim keseluruhan (k_La) bagi geraniol boleh ditentukan. Nilai yang diperoleh adalah 4, 7 dan 10.8 hr^{-1} bagi kadar aliran substrat 4, 6 dan 8 L/min.

ABSTRACT

The present study describes the biotransformation of hydrophobic monoterpene, geraniol into citronellol by whole cells of *Saccharomyces cerevisiae* in a continuous closed-gas loop bioreactor (CCGLB). Geraniol which has high vapor pressure, high volatility and low solubility in aqueous medium is highly suited to be used in this system. The kinetics of the biotransformation using both states of cells; growing and resting cells were investigated. The preliminary results showed that the liquid-phase biotransformation system was only practical with resting cells where the growing cells were incapable to transform geraniol. However, both growing and resting cells have successfully performed the biotransformation of geraniol in CCGLB. The gas loop led to a maximum citronellol concentration of 2.38 g/L and specific activity of 7.9 U/g_{cell}. This was obtained by the biotransformation using resting cells at pH 7, 350 rpm, substrate flow rate of 8 L/min and glucose concentration of 50 g/L. Process improvements achieved a 5-fold increase in the citronellol production over the shake-flask performance (liquid-phase biotransformation). For the biotransformation using growing cells, it was found that the cell growth was affected by the presence of substrate for biotransformation with about 60% reduction of final cell concentration was observed. The process gives a maximum citronellol formation of 1.18 g/L at conditions of pH 7, 500 rpm and 8 L/min of substrate flow rate. The CCGLB system also brings advantage of *in-situ* product removal where a clear product was obtained without a need of downstream processes. A modeling work of the biotransformation using resting cells in the CCGLB was further developed with a combination of Michaelis-Menten and two-film models. The kinetic constants of V_{max} and K_m were determined from the shake-flask experiments to be 0.015 g/hr.g_{cell} and 17.9 g/L, respectively. The simulations

results were validated to the experimental results at different substrate flow rates. From the modeling work, overall mass transfer coefficient ($k_L a$) of geraniol could be predicted and were found to be 4, 7 and 10.8 hr^{-1} at substrate flow rates of 4, 6 and 8 L/min, respectively.

CHAPTER ONE

INTRODUCTION

1.0 Overview

In recent years, biocatalytic process has become a popular method in organic compound synthesis. A wide range of compounds can be produced in a highly chemo-, regio- and enantioselective manner using such a method through a process called biotransformation. Monoterpenoids are one of the most important starting materials in biotransformation and are widely used as flavors in the fine chemicals sector such as pharmaceuticals and food industries (Bull *et al.*, 1999; Miller and Nagarajan, 2000). In the biocatalytic branch, microbial cells are of the preferential biocatalysts for reduction and oxidation (redox) reactions as they provide an *in-situ* cofactor regeneration system (Carballeira *et al*., 2009). The addition of external cofactors is necessary when dealing with an isolated enzyme system (Liu and Wang, 2007).

Conventionally, whole-cell biotransformation is carried out in an aqueous medium where the cells are most active. However, the substrates involved, which are usually organic, have low solubility in the aqueous phase and the substrates and/or products may cause inhibition to the cells at high concentrations (Leon *et al*., 1998; Marques *et al*., 2010). In order to overcome such problems, a prominent development has been made approaching several ways such as metabolic engineering, reaction medium engineering and also through the introduction of new concepts in bioreactor design. The combined effect of advances in these areas is found to contribute to the success in many biotransformation processes. Such

technologies could promote the biological-based process for use in important sectors which have long been dominated by the ordinary chemical-based process.

1.1 Natural flavor compounds

Flavor compounds are considered ‘natural’ if they were synthesized through fermentation, enzymatic process or extraction from plants (Serra *et al*., 2005). According to the regulations of US Food and Drug Administration Guidelines, the natural product must be identical in physical and chemical aspects to the natural substance that is already known to exist in nature (de Carvalho and da Fonseca, 2006). Product from chemical catalysis is considered synthetic because its chemical property differs to that of the natural substance even though it gives similar taste and odor (Brenna *et al*., 2003). A rising demand for natural products in food processing and drug manufacturing over the last decade has triggered off significant research activities in flavors production using biocatalysis (Berger, 1995; Demyttenaere, 2001). Two main factors owing to such a high demand are the specific isomer (chiral) property of product and the strong preference towards ‘natural’ instead of synthetic product among customers. Chirality is an important feature in flavors as well as in fragrances since two different isomers of the same compound can have quite different odors and tastes. For example, *R*-(+)-limonene gives orange scent while *S*-(-)-limonene gives turpentine scent (Berger, 1995). There are various types of flavors that have been successfully produced through biocatalytic process. A comprehensive overview of the specific biocatalytic reaction involved in the synthesis of flavors has been well presented in the literature (Cheetham, 1993; Berger, 1995; Demyttenaere, 2001; de Carvalho and da Fonseca, 2006).

1.2 Biotransformation

Biotransformation is described as a selective modification of a compound into other specific compound with the use of biological catalysts such as plant cells, microorganisms or isolated enzymes (Kieslich, 1984). The process involves a vast array of enzymatic reactions, which is always referred to as a biocatalytic reaction. Historically, the first biotransformation was discovered by Pasteur in 1862 for acetic acid production using a pure culture of *Bacterium xylinum* (Csuk and Glanzer, 1991). Both whole cells and isolated enzymes have great potentials for biotransformation purposes. The selection is mainly based upon reaction type. Hydrolases-type enzyme such as lipase and esterase are the most predominantly used in catalyzing hydrolysis reaction while the whole cells are of choice in reactions involving electron transfer such as oxidation and reduction as they provide *in-situ* cofactor recycling as the reaction progresses. Industrial application of enzymes in redox reaction has not yet been recognized due to a high cost of enzymes as well as the external cofactors pool present (Turner, 1995; Straathof *et al.*, 2002). Often, the cofactors are more expensive compared to that of the desired products (Liu and Wang, 2007), which makes the choice of isolated enzymes not practical. Nevertheless, several cofactors can now be effectively regenerated using enzymatic method, but they are still scarcely used in industry (Shin *et al.*, 2007).

In comparison to the enzymatic system, whole-cell biotransformation generally perform slower rate of reaction due to the fact that there exist a permeability barrier of cell membrane to substrate and/or product. Fortunately, the drawback has been addressed using molecular engineering approach of recombinant DNA to resolve the problem (Shin *et al.*, 2007). Apart from that, multiple enzymes in the whole cells always lead to a number of side reactions. This phenomenon has also

been controlled which resulted in the increase of optical purity (Chartrain *et al.*, 2001). The pros and cons between these two methodologies are presented as in Table 1.1. The whole-cell biotransformation system is discussed at length in Section 1.3 as this study is particularly focused on the similar type of system.

Table 1.1: Comparisons between whole-cell and enzymatic biotransformation (Ishige *et al*., 2005; Shin *et al*., 2007)

Whole-cell biotransformation	**Enzymatic biotransformation**
Favorable in redox reaction	Requires addition of expensive cofactor(s) for redox reaction
Readily available	Required protein purification steps
Cheaper biocatalyst	Expensive biocatalyst due to extraction and recycling costs
Side reactions may occur due to multi-enzyme system	Clean process
Protected by cell envelope (stable)	Exposure to external environment (rather unstable)

Biotransformation offers a product with outstanding properties of chemo-, regio- and stereospecificity. The chemospecificity refers to a restricted single chemical reaction when several functional groups present at the compound's structure; and thus, avoids side reactions. Regiospecificity gives an indication of substrate molecules to react at the same site of enzymes while stereospecificity indicates the enzyme preference to attack with one of enantiomers from two entities of *R*- or *S*- configurations resulted in a single enantiomer compound, thereby avoiding difficulties of racemic mixtures (Leuenberger, 1990). Such a problem is a key issue recognized in the chemical catalysis which makes it unfeasible choice for the production of compound that requires high selectivity.

Generally, the conditions used in carrying out biotransformation are comparatively mild; with temperature as low as 30 to 40°C and at atmospheric

pressure (Bommarius and Riebel, 2004). In contrary, a chemical catalysis normally requires extreme conditions which results in high energy consumption. Biotransformation is environmentally friendly, in contrast to chemical catalysis due to the usage of heavy metal catalysts (Gotor, 2002). The advantages offered by biotransformation over chemical catalysis are summarized in Table 1.2.

Table 1.2: Comparisons between biotransformation and chemical catalysis

Biotransformation	**Chemical catalysis**
Operated at mild operating conditions	Operated at high temperature and pressure
Product specificity	Product with racemic mixtures
Environmentally friendly	Used heavy metal catalysts
Acknowledged as 'natural'	Acknowledged as artificial/synthetic

The introduction of biotransformation technique in chemical synthesis is unlikely to replace the conventional chemical method completely. In most industries, a close co-operation between these two methodologies is essential for their success. The possible routes for each target compound are influenced by a number of considerations. The availability of starting materials, product specificity, the number of steps involved, environmental considerations, scalability, downstream processing and development time are initially evaluated (Shaw *et al.*, 2003). Therefore, biotransformation in particular, can be a useful tool in cases where product is difficult to obtain via conventional chemical catalysis.

1.3 Whole-cell biotransformation

Nowadays, rapid advances in the life sciences especially in microbiology have greatly increased the usage of whole cells in biotransformation processes. Whole cells can either come from microorganisms such as fungi, yeast, bacteria, or

may be from plant cells culture. In order to understand the catalytic reaction using the whole cells of microorganisms, one must explore their cell physiology as well as its metabolism. An explanation of these topics is detailed-out in the following sub-sections.

1.3.1 Growing and resting cell systems

The whole-cell biotransformation is generally classified according to the culture state as growing or resting phenomenon (Kieslich, 1984). The difference between both systems is of the different ways of media preparation. A simple media preparation of buffer solution or even water with an addition of carbon source is enough to build the resting cell biotransformation system while the growing cells require other nutrient sources, such as nitrogen and amino acid (Chin-Joe *et al*., 2002). Selection of the best system normally relies on the availability and also the cost of microorganisms. Normally, the former was used due to restricted availability of the microorganisms in market. Nevertheless, the best example of commercially applied resting cells is the *Saccharomyces cerevisiae* (baker's yeast). Baker's yeast represents the largest bulk production of any single-cell microorganism throughout the world with several million tons of fresh baker's yeast cells are produced annually for human consumption (Di Serio *et al.*, 2001).

In growing cell biotransformation, substrate can be induced during the inoculation time or in the later phases of the microbial growth. As the biotransformation progresses, the cells will grow and increasing in their numbers. When dealing with such a biotransformation system, substrate concentration is always crucial. High concentration of substrates could inhibit the cell activity. Due to this reason, the biotransformation processes are normally carried out at late

exponential or during stationary phase where the cells and their enzymes are at their maximum density (Kieslich, 1984). On the other hand, cells in resting state are viable, but they are unable to grow as the biotransformation is in progress due to limited amount of nutrient. Generally, the resting cell biotransformation is performed in order to investigate in particular, the biocatalytic reaction without the interference of cell growth which could affect the production of the responsible enzyme (Wang *et al*., 2005).

1.3.2 Cofactor regeneration in the whole-cell system

Microorganisms are capable to catalyze almost all chemical reactions such as isomerization, racemization, oxidation, reduction and hydrolysis. Among these reactions, oxidation and reduction reactions are relatively complex as they require cofactor-dependent enzymes, or known as oxidoreductases (Presečki and Vasić-Rački, 2005). The cofactor pool exists naturally in the whole cells as a result of cellular metabolism, and can be recycled by the addition of co-substrates such as glucose (Buque *et al*., 2002). In order to understand how the microorganism generates these cofactors, one should explore the main centre of the cellular metabolism which is known as the glycolysis process.

Glycolysis involves a degradation of carbon source, which in this work is glucose, into smaller molecules of pyruvate with concomitant production of an amount of energy in the form of ATP, which is the form needed by the cells. Most of pyruvates are further degraded to produce ethanol with the release of carbon dioxide. Simultaneously, the reduced form of cofactor, NAD(P)H is oxidized into $NADP^+$ (Shuler and Kargi, 1992). When a new sub-system is employed, for instance the addition of substrate of biotransformation, the cofactor will interact with the new

sub-system and performs a side reaction. The substrate is reduced into the corresponding product while at the same time, the NAD(P)H is continuously oxidized into $NADP^+$ (Chin-Joe *et al*., 2002). The process continues as long as there is enough glucose to carry out the reaction. A schematic diagram which represents the cofactor recycling in yeast cell with geraniol as the substrate is shown in Figure 1.1.

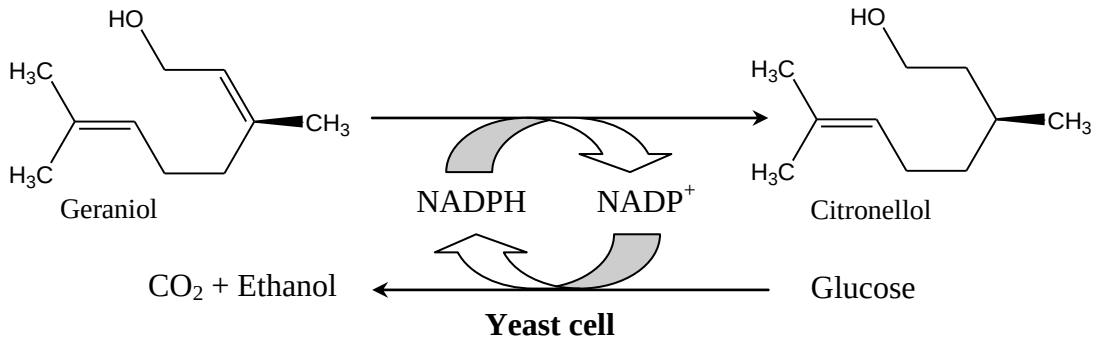


Figure 1.1: Schematic representation of cofactor regeneration during the reduction of geraniol into citronellol by *S. cerevisiae* with glucose as co-substrate.

1.4 Yeast as a source of biocatalyst for biotransformation

Yeast is a well-known microorganism and probably the only group of microorganisms, that has a cultural history dating back to prehistoric time. *Saccharomyces*, *Candida*, *Kluyveromyces* and *Pichia* are among the genus of yeast that have been manipulated as biocatalysts (Li *et al.*, 2010). The yeast can be found in various sugar-rich fruits, in water or in soils. The characteristics of yeast have been extensively discussed in a number of textbooks (Wang *et al*., 1979; Shuler and Kargi, 1992; Wainwright, 1992). In short, the yeast cells are eukaryotes, having cellular organelles such as mitochondria, a nucleus, etc. They are all mostly unicellular and reproduce asexually by budding off daughter cells from a single mother cell. Figure 1.2 shows a parent cell of *S. cerevisiae* that produced new cells

from budding process. The size of yeast is normally larger than bacteria with about 5-12 µm in diameter. A prominent feature of all yeast cells is that the cell wall is mainly made up of polysaccharides which give strength and rigidity to the cell. In the yeast family, *S. cerevisiae* or commercially known as baker's yeast is the most popular and most studied species of yeast. The species was the first eukaryote to have its entire genome sequenced (Vassarotti *et al.*, 1995). *Saccharomyces* means 'sugar fungi', and indeed they grow well in various types of sugar such as glucose, glycerol and acetate (Zhang *et al.*, 2003). *S. cerevisiae* has been used for fermentative food processing since the ancient times; and thus, are considered to be quite safe for industrial use (Servi, 1998).

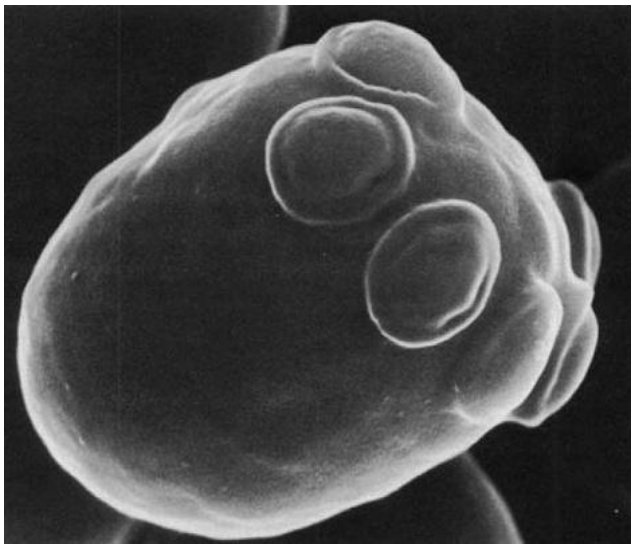


Figure 1.2: Electron micrograph showing *Saccharomyces cerevisiae* parent cell that has produced at least six daughter cells as shown by the circularly arranged bud scars (Hatzis and Porro, 2006).

The uniqueness of baker's yeast is that, it can be easily grown without a sterile condition. As reported by several researchers, baker's yeast is genetically stable and very robust organism, non-toxic, easy to handle and not a strict anaerobiosis (Servi, 1998; Perles *et al.*, 2008). In the field of biotransformation, the

ability of baker's yeast to perform an asymmetric reduction has been first presented by Dumas in 1874, and nowadays, there are a huge number of studies being published which employ the microorganism especially for the reduction of carbonyl and carbon-carbon double bonds compounds. A brief description on the biotransformation using baker's yeast as biocatalyst is presented in the next chapter.

1.5 Biotransformation in non-conventional systems

Biotransformation of organic compounds in non-conventional systems have been extensively explored due to several weaknesses associated in conventional system such as low substrate solubility and substrate and/or product inhibition (Leon *et al.*, 1998). This causes only a small amount of substrate to come into contact with the cells, which could lead to a decrease in the volumetric productivity. Apart from that, a mixture of biocatalyst and product in the reaction medium requires several downstream processes for product separation. A number of non-conventional systems have been proposed to tackle the solubility problem including; water-immiscible organic solvent system (Nikolova and Ward, 1993; Doig *et al.*, 1998b; Leon *et al.*, 1998; Kansal and Benerjee, 2009); ionic liquid system (Itoh and Tomoko, 2007; Sureshkumar and Lee, 2009), supercritical fluid system (Al-Duri *et al*., 2001) and compressed solvent system (Oliveira *et al.*, 2006). An integrated design of bioreactor with *in-situ* product removal (ISPR) technique has also been introduced to eliminate the downstream processes such as the membrane-based system (Collins and Daugulis, 1997; Doig *et al.*, 1998a; Onken and Berger, 1999; Krieg *et al.*, 2000; Carvalho *et al.*, 2001; Valadez-Blanco *et al.*, 2008); solid-gas system (Lamare and Legoy, 1993; Maugard *et al.*, 2001; Yeom and Daugulis, 2001; Létisse *et al.*, 2003; Marchand *et al.*, 2008); and the gas loop system (Steinig *et al.*, 2000; Pescheck *et al.*, 2009).

1.6 Problem statement

Flavor and fragrance compounds can be obtained through three major techniques which are; the physical extraction from plants, chemical catalysis and biocatalysis. A traditional method of physical extraction from plants has been abandoned since such a technique suffers from seasonal variation, low yield of the final product and high production cost due to the application of many downstream processes. For a long time, these compounds are synthesized using ordinary chemical catalysis. However, this technique is lacking of producing an enantiomerically pure compound where the undesirable racemic mixtures have often formed. Enantiomeric purity is a key issue in synthetic chemistry especially for use in food and consumer products. The quest for enantiomerically pure compounds is motivated by the increasing health concerns among consumers since non-purity compounds lead to adverse side effects to the human body. Due to this reason, an application of biocatalysis has started to spread because of its extraordinary capability to produce compounds with highly enantiomeric purity. In the area of biotransformation, non-conventional systems are gaining importance with the aim to achieve high productivity as well as to provide ease of handling process. A continuous-closed-gas-loop bioreactor (CCGLB) is a new design approach of bioreactors and has been applied in this work. The CCGLB possesses a continuous gas phase reaction which can overcome some major problems associated with the conventional system and these include; 1) eliminating mass transfer limitation which resulted in an increase in the biotransformation rate; 2) providing *in-situ* product removal thus, reduce a number of downstream processes and; 3) reducing substrate and/or product inhibition.

A model of reaction proposed in this work is an asymmetric reduction of geraniol into citronellol using the whole cells of *Saccharomyces cerevisiae* (baker's yeast type-II). Citronellol which has a pleasant rose scent has been recognized as safe (GRAS) and commercially used in food, fragrance and pharmaceutical sectors. Baker's yeast type-II was chosen because it has shown a great performance in reduction reaction, its availability in the market is in bulk and also generally inexpensive. Those aspects make it favorable for this particular investigation. An effort in optimizing parameters that might give a strong impact to the biotransformation rate was closely investigated in the experimental work.

1.7 Objectives

The main goal of this research is to investigate the efficiency of CCGLB system in conducting the biotransformation of geraniol into citronellol mediated by baker's yeast. Therefore, in order to achieve this goal, a few integrated objectives have been addressed as follows:

1) To investigate the compatibility and performance of CCGLB for biotransformation.
2) To compare the performance of baker's yeast in catalyzing gas-phase biotransformation to that of the liquid-phase biotransformation.
3) To determine the optimum parameters such as biomedium pH, agitation rate, substrate flow rate and glucose concentration for which highest product formation can be obtained in the CCGLB.
4) To explore the baker's yeast efficiency in catalyzing biotransformation during its growth.

5) To determine the kinetic parameters and simulate the biotransformation process in CCGLB.

1.8 Scope of study

The biotransformation of geraniol into citronellol using the whole cells of *S. cerevisiae* was conducted in liquid and gas phase systems. The difference between these two systems is in the form of geraniol being consumed during the reaction. For the liquid-phase biotransformation, experiments were conducted in shake-flask culture, while the gas-phase biotransformation was conducted in the CCGLB. Taking into account the highest production of citronellol obtained, the reaction was optimized at several parameters such as initial pH and substrate concentration. While in the CCGLB system, the effects of agitation rate, substrate flow rate and amount of glucose required were also investigated.

The study begins with a preliminary work where the kinetics of yeast growth were observed at several fermentation conditions such as initial pH, temperature and glucose concentration. Such work is important in order to maximize the productivity of biomass during *S. cerevisiae* cultivation. In addition, the optimum temperature obtained from this work was further used in later biotransformation experiments. Specific growth rate was determined in order to evaluate the cell growth behavior.

Liquid-phase biotransformation of geraniol was then conducted in shake-flask culture. The potential of growing and resting cells of baker's yeast in catalyzing the reaction were investigated. The purpose of this work is mainly to determine the efficiency of *S. cerevisiae* to transform geraniol using both states of cells. From this work, one might explore the behavior of yeast growth with and without the presence of substrate for biotransformation (geraniol). It also provides information of the

affinity of yeast to grow and simultaneously to perform the biotransformation. In contrast, the biotransformation using resting cells strictly focused on the rate of reaction at several specified parameters.

In the CCGLB system, the same states of cells were applied as in the aforementioned preliminary work. Parameters that give major effects to the rate of reaction such as biomedium pH, agitation rate, substrate flow rate and glucose concentration were closely investigated. These experiments were carried out with the aim to observe the performance of CCGLB and later to compare with the results of the liquid-phase biotransformation.

The last part of the work is on process modeling of the biotransformation with regard to the CCGLB system. This was carried out by preparing material balances of substrate and product in gas and liquid phases. A complete solution of the model is then numerically solved using codes implemented in MATLAB® and later graphically simulated for further understanding of the working system. Validation of the model was undertaken by comparing the simulated results to the experimental data at different substrate flow rates. The model was also used to estimate the overall mass transfer coefficient of geraniol within the system.

1.9 Thesis structure

This thesis contains six chapters. **Chapter 1** introduces the background and the objectives in this study. **Chapter 2** gives a survey of the literature relevant to this study, which includes the baker's yeast mediated biotransformation, biological and non-biological catalysis of geraniol, and describes the previous work and development in the area of biotransformation. **Chapter 3** illustrates the experimental procedures and materials applied in this project. The experimental results are

presented and discussed in **Chapter 4**, which includes the experimental results from yeast growth study, liquid-phase biotransformation, and the results from the study in the CCGLB. **Chapter 5** presents and discusses the modeling of biotransformation of geraniol into citronellol in the CCGLB, and the results predicted by the model. **Chapter 6** draws the conclusions from this work and suggests some recommendations for future studies.

CHAPTER TWO

LITERATURE REVIEW

2.0 Overview

In this chapter, the published knowledge of flavors production by microorganisms is reported. Further review on stereoselective biotransformation mediated by baker's yeast is presented in order to explore the versatility of this microorganism in catalyzing variety of useful compounds. Factors that influence the yeast growth as well as the biotransformation was summarized, which can be helpful for future investigation. Apart from that, a compilation of citronellol production using whole-cell biocatalysis as well as chemical catalysis is also presented. The last part of this chapter discusses on the improvements in the field of biotransformation processes including whole-cell engineering, medium engineering and bioreactor design.

2.1 Production of flavors by microorganisms

Fruits and flowers have their own perception of flavors and odors due to the occurrence of sensory substances in the form of essential oils. Traditionally, man has extracted these essential oils directly from plants for commercial use as food additives and also as components in consumer products such as perfumes and cosmetics. However, the demand of flavor compounds can no longer be met by the supply of plant sources. This is due to the fact that, this method usually produces low concentration of the desired compound. Apart from that, the extraction process is rather complex which implies to high production cost (Naik *et al.*, 1989). Nowadays, majority of artificial flavors were prepared in bulk quantity by chemical catalysis. Alongside with this means, the employment of biological catalysis especially in food

processing and pharmaceutical manufacturing has increased considerably in the past decades because the process is regarded as safe (Straathof *et al.*, 2002).

Terpenes are the major class of natural products found in nature and widely used as starting materials in flavor synthesis. Classification of terpene family is based on the number of isoprene units (C_5) incorporated in the molecular skeleton of the compound. For example, two isoprene units represent monoterpene ($C_{10}H_{16}$) while three isoprene units represent sesquiterpene ($C_{15}H_{24}$). Modified terpenes with the addition of an oxygen atom in their structures are known as terpenoids and being the largest constituents in essential oils. About 23,000 single terpenoids were found in nature and most of them are in the form of monoterpenoids (Demyttenaere, 2001). Almost all terpenoids are acknowledged by US Food and Drug Administration (FDA) as GRAS (generally recognized as safe) for their intended use as flavoring substances (The Terpene Consortium, 2002).

Monoterpenoids can exist as aldehydes, alcohols, ketones or ethers, in acyclic and cyclic structures. Figure 2.1 gives the structure of some representative monoterpenoids.

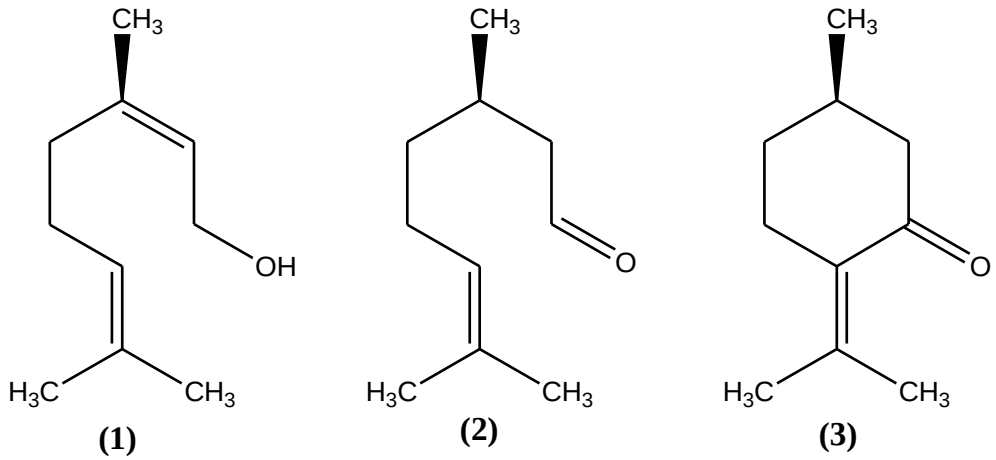


Figure 2.1: Monoterpenoid derivatives structure; **(1)** geraniol (alcohol); **(2)** citronellal (aldehyde); **(3)** pulegone (cyclic ketone).

Prior to the biocatalysis, microorganisms, plant cells and isolated enzymes have shown their capability in catalyzing various types of terpene compounds (Demyttenaere, 2001). According to a survey conducted by de Carvalho and da Fonseca (2006), approximately two-thirds of the published works on the terpenes biotransformation in the last decade had employed microorganisms as the biocatalyst and indeed, bacteria and fungi are the most dominant as indicated in Figure 2.2. Plant cells contributed about 11% while only 7% had used isolated enzymes.

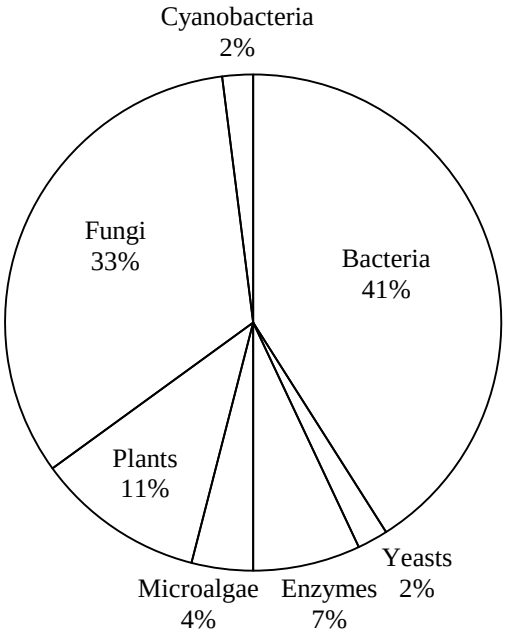


Figure 2.2: Percentage of published papers on various biocatalysts used in biotransformation of terpenes in the last ten years (de Carvalho and da Fonseca, 2006).

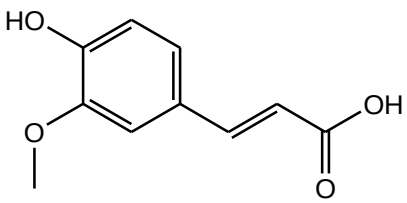
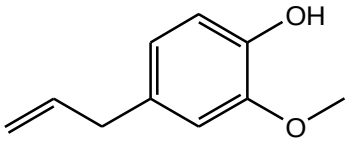
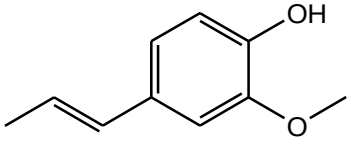
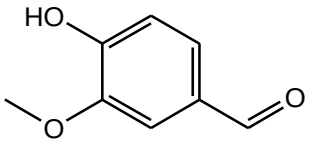
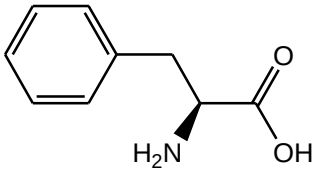
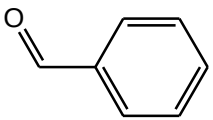
The reason why the microorganisms are of choice is eventually because they are capable to catalyze a broad range of chemical reactions and also much cheaper than that of isolated enzymes. In comparison to the plant cells, the growth of microorganisms is much faster, which makes it feasible for use in biotransformation.

Almost all classes of microorganisms have been utilized as biocatalysts in synthesis of flavors which are from bacteria, fungi, yeast and higher fungi families

(Berger, 1995). *Bacillus*, *Pseudomonas* and *Acetobacter* species are among bacteria employed (Hua *et al.*, 2007), while *Aspergillus*, *Basidiomycetes* and *Penicillium* are of favorable strains from higher fungi (Lomascolo *et al.*, 1999; Pescheck *et al.*, 2009). In the yeast family, *Candida*, *Saccharomyces* and *Rhodutorula* are among the most popular strains used in the synthesis (Miyazawa *et al.*, 1995; Muller *et al.*, 2006). The strain of *S. cerevisiae* has successfully transformed a number of monoterpenes such as monoterpene ketones of menthone, carvone, isopiperitenone and phenylalanine to the corresponding alcohols (Xu *et al*., 2007; Yadav *et al*., 2007), allylic alcohols such as geraniol to citronellol (Gramatica *et al*., 1982), linalool to α-terpineol (King and Dickinson, 2000) and monoterpene aldehyde of citral to citronellal (Muller *et al*., 2006). Other potential microorganisms in the synthesis of flavors are presented in Table 2.1.

The development in terpene synthesis has led to an improvement of the corresponding reaction systems such as the increase in their yield and productivity. Such a work may involve utilizing different types of microorganisms, changing reaction conditions and optimizing the duration of biotransformation (Aniol and Huszcza, 2005). The improvement in this system with an integration of other methods such as genetic engineering, cell immobilization and medium engineering could increase the potential of whole cells in organic synthesis purposes.

Table 2.1: Potential microorganisms for synthesis of flavors

Biocatalyst	Substrate	Product	Flavoring assessment	Reference
Serratia *Amycolatopsis* *Pseudomonas* sp.HR 199	 Ferulic acid  Eugenol  Isoeugenol	 Vanillin	Vanilla	(Xu *et al.*, 2007)
Ischnoderma	 L-Phenylalanine	 Benzaldehyde	Cherry	(Xu *et al.*, 2007)