# OPTIMISING THE PRODUCTION OF CHIRAL ALCOHOL VIA *ASPERGILLUS NIGER* CATALYSED BIOTRANSFORMATION OF 1-(4-BROMO-PHENYL)-ETHANONE

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**UNIVERSITI SAINS MALAYSIA**

**2011**

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**By**

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**Thesis submitted in fulfilment of the**

**requirements for the degree of Master of Science**

**NOVEMBER 2011**

**ACKNOWLEDGEMENTS**

**Bismillahirrahmanirrahim**

This special part of thesis gives me an opportunity to express my gratitude to all beloved persons around me who made this thesis possible and become realism. First and foremost, I would like to say Alhamdulillah and thank Allah for the strength and all His guidance for me to finish this project. To the infinite perseverance, enthusiasm and patient guidance of my dearest supervisor Dr. Mohamad Hekarl Uzir, I would like to express my deepest appreciation and gratitude for your encouragement, guidance and unconditional support from the initial to the final level enabled me to develop an understanding of the subject. Working with such a talented scientist, a friendly and funny supervisor as you was the most precious experience for me and I would like to say that you are the best mentor I ever had. It is an honour to me to thank my co-supervisor, Assoc. Prof. Dr. Azlina Harun @ Kamaruddin for her precious advice, encouragement and assistant during my study.

My best regards to University Sains Malaysia for the financial support for my study by rewarding a Fellowship Scheme under the Research University Status and USM-PGRS funding to support this research. I am heartily thankful to my beloved husband, Wan Izzudin Shaharudin, who has been the most influential in my life, made available his support in a number of ways and has inspired me to this point in my study. I would like to extend my gratitude to my beloved friends Azyati, Syida, Aimie, Fadzil, Azam, Azan, Faisal, Kak Aziah, Kornisah, Fikri, Fattah, Afrizal, Kak Jus and Kak Syura for their companion and care throughout my time in USM. Thank you so much for your motivation and unparalleled help. You are incredible people and I had an amazing time with you, guys. Special thanks to all USM staffs

especially Mr. Faiza, Mr. Syamsul, Mr. Osmarizal, Mr. Raqib, Mrs. Yusnadia, and Mrs. Latifah for their valuable help during completion of my research.

Last but not list, I would like to show my deepest gratitude to my beloved parents Hj. Abas bin Jusoh and Hajjah Hindun bt Muda for their endless love, support and blessing to pursue resume and successfully accomplish my studies. Mom and dad this thesis is made for you. To my beloved brothers, sisters, nieces and nephews, thank you for your love and care. To those who indirectly contributed in this research, your kindness given shall not be forgotten. Thank you so much.

Fatimatul Zaharah Abas

November 2011

**TABLE OF CONTENTS** **Page**

**LIST OF TABLES** **Page**

**LIST OF FIGURES** **Page**

**LIST OF SCHEMES** **Page**

## LIST OF ABBREVIATIONS

| Abbreviation | Description |
|---|---|
| ATP | Adenosine Triphosphate |
| ARB | Angiotensin Receptor Blocker |
| ACE | Angiotensin Converting Enzyme |
| ATCC | American Type Culture Collection |
| *A. niger* | *Aspergillus niger* |
| ANOVA | Anallysis of Variance |
| CCD | Central Composite Design |
| DOE | Design of Experiment |
| DNS | Dinitrosalicyclic acid |
| % e.e | Percentage of enantiomeric excess |
| GC | Gas Chromatography |
| GRAS | Generally Recognized as Safe |
| HPLC | High Performance Liquid Chromatography |
| $H_2SO_4$ | Sulphuric acid |
| h | hour |
| $KNaC_4H_4O_6 \cdot 4H_2O$ | Potassium sodium tartrate |
| mM | milimolar |
| mmol | milimol |
| NaCl | Sodium chloride |
| $Na_2SO_4$ | Sodium Sulphate |
| NAD(P)H | Nicotinamide Adenine Dinocleotide Phosphate |
| $NADP^+$ | Oxidized form of NADPH |
| NADH | Nicotinamide Adenine Dinucleotide |
| $NAD^+$ | Oxidized form of NADH |

| | |
|---|---|
| rpm | Rotation per minute |
| RAS | Renin Angiotensin System |
| i-propanol | isopropanol |
| UV-VIS | Ultraviolet-Visible |
| $CO_2$ | Carbon dioxide |
| CV | Coefficient Variation |
| Pred $R^2$ | Predicted $R^2$ |
| RMSD | Root Mean Squared Deviation |
| g | gram |
| ml | mililiter |
| CVD | Cardiovascular diseases |
| AT1 | Angiotensin II type 1 receptors |
| AT2 | Angiotensin type 2 receptor |
| FDA | Food and Drugs Administration |
| L | Liter |
| sp. | Species |
| VALUE | Valsartan Antihypertensive Long-term Use Evaluation |
| DNA | Deoxyribonucleic acid |
| ADH | Alcohol dehydrogenase |
| v/v | Ratio volume per volume |
| $\varepsilon$ | Random error |
| $R^2$ | Determination coefficient |
| $R^2$adj | Adjusted determination coefficient |
| $R^2$pred | Predicted determination coefficient |
| ES | Enzyme-substrate complex |
| E | Enzyme |
| $\beta_o$ | Constant coefficient |

| | |
|---|---|
| $\beta_i$ | Coefficient for the linear effect |
| $\beta_{ii}$ | Coefficient for the Square effect |
| $\beta_{ij}$ | Coefficient for the interaction effect |
| $X_i$ | Input variables |
| $X_i^2$ | Square effect |
| $X_{ij}$ | Interaction effect |
| KOH | Potassium hydroxide |
| HCl | Hydrochloric acid |
| N | Molar |
| BPT | Biphenyltetrazole |
| Bp | Boiling point |
| Mp | Melting point |
| Mw | Molecular weight |
| Σ | Total |
| n | Number of cell |
| $pO_2$ | Dissolve oxygen |

## LIST OF SYMBOLS

| Symbol | Description | Unit |
|---|---|---|
| $\mu$ | Specific growth rate | $h^{-1}$ |
| $S$ | Substrate concentration | mM |
| $\tau_d$ | Time doubling | h |
| $K_m$ | Michaelis-menten constant | g/L |
| $K_s$ | Substrate inhibition constant | g/L |
| $V_{max}$ | Maximum reaction rate | mM/h |
| $X$ | Cell mass concentration | g/L |
| $t$ | time | h |
| $S^*$ | Substrate Inhibitor concentration | mM |
| $X_0$ | Initial cell mass concentration | g/L |
| $q_s$ | Substrate utilization rate | $h^{-1}$ |
| $q_p$ | Product formation rate | $h^{-1}$ |
| $Y_{x/s}$ | Biomass yield coefficient | g/g |
| $Y_{p/x}$ | Growth-associate product yield | g/g |
| $Y_{p/s}$ | Product yield coefficient | g/g |
| $m_s$ | Maintenance coefficient | g/g.h |
| $V$ | Initial reaction rate | mM/h |
| $V_{max}^{app}$ | Apparent maximum reaction rate | mM/h |
| $K_m^{app}$ | Apparent Michaelis-menten constant | g/L |
| $S_0$ | Initial substrate concentration | g/L |
| T | Temperature | °C |
| $\mu X$ | Volumetric growth rate | g/L.h |
| $K_p$ | Product constant | g/L |

**PENGOPTIMUMAN PENGHASILAN ALKOHOL KIRAL MELALUI BIOTRANSFORMASI 1-(4-BROMO-FENIL)-ETANON BERMANGKINKAN KULAT *ASPERGILLUS NIGER***

## ABSTRAK

Alkohol kiral merupakan bahan permulaan yang sangat berguna untuk penghasilan pelbagai sebatian biologi yang aktif. Dalam kajian ini, penurunan keton bermangkinkan biologi melalui proses penurunan tidak simetri menggunakan *Aspergillus niger* sebagai pemangkin biologi telah dijalankan bagi menghasilkan *(R)*-1-(4-bromo-fenil)-etanol. Daripada hasil kajian ini, didapati fasa eksponen adalah masa yang terbaik bagi proses biotransformasi jika dibandingkan dengan fasa pegun kerana kadar penghasilan pada fasa eksponen (0.063 mM/j) lebih tinggi daripada fasa pegun (0.028 mM/j). Tambahan pula, kajian pengoptimuman biotransformasi yang dilakukan melalui sistem kelalang goncang menunjukkan bahawa keadaan optimum yang didapati ialah pada jam yang ke 48 masa pengeraman, pH 6.68, suhu 31.85$^{o}$C dengan kepekatan substrat yang rendah iaitu 6.78 mM. Lebihan enantiomer dan penukaran produk adalah sangat tinggi pada keadaan optimum ini iaitu hampir 100 %. Penambahan amaun kepekatan substrat dalam proses biotransformasi ini telah menyebabkan berlakunya rencatan substrat. Tambahan pula dengan menggunakan 2.5 L sistem bioreaktor, ianya boleh memaksimumkan peratusan lebihan enantiomer, penukaran produk dan juga kadar penghasilan produk. Parameter-parameter yang dikaji dalam sistem bioreaktor ialah kelajuan pengadukan pada kadar 50 rpm ke 150 rpm dan juga masa pengeraman biotransformasi dari 0 hingga ke 56 jam. Daripada keputusan kajian yang didapati, kelajuan pengadukan yang maksimum ialah pada

tahap 150 rpm dengan masa pengeraman optimumnya pada jam ke 33.34 proses biotransformasi yang mana menghasilkan 99.94 % lebihan enantiomer dan 88.82 % penukaran produk. Tambahan pula, sebanyak 0.097 mM/j kadar penghasilan *(R)*-1-(4-bromo-fenil)-etanol telah dicapai pada keadaan optimum ini. Oleh yang demikian, daripada keputusan kajian yang diperolehi, ianya secara jelas menunjukkan bahawa, terdapat perbezaan yang ketara dalam kadar penghasilan dan juga penukaran produk *(R)*-1-(4-bromo-fenil)-etanol dengan menggunakan sistem bioreaktor. Masa pengoptimuman juga boleh dipendekkan jika dibandingkan dengan sistem kelalang goncang. Keputusan kajian yang diperolehi daripada sistem bioreaktor boleh digunakan untuk kajian-kajian lain pada masa akan datang seperti kajian menggunakan pelbagai jenis substrat, pengaliran udara yang berbeza dan pelbagai jenis pemangkin biologi. Selain itu, bagi kajian kinetik penapaian, kadar spesifik pertumbuhan, ($\mu$) *A. niger* ialah 0.29/j dengan 0.164/j kadar pembentukan produk, ($q_p$) dan 0.191/j kadar penggunaan substratum, ($q_s$). Berdasarkan analisis MATLAB® untuk kajian kinetik biotransformasi yang dilakukan, nilai $V_{max}$, $K_m$ dan $K_s$ yang diperolehi masing-masing ialah 2.23 mM/j, 76.69 mM and 0.21 mM.

**OPTIMISING THE PRODUCTION OF CHIRAL ALCOHOL VIA *ASPERGILLUS NIGER* CATALYSED BIOTRANSFORMATION OF 1-(4-BROMO-PHENYL)-ETHANONE**

## ABSTRACT

Chiral alcohols are useful starting materials for the synthesis of various biologically active compounds. In this study, the biocatalytic reduction of ketone via asymmetric reduction process using *Aspergillus niger* as a whole-cell biocatalyst was carried out in order to produce (*R*)-1-(4-bromo-phenyl)-ethanol. From the result obtained in this sudy, it shows that the exponential phase was the best period for biotransformation to take place compared to the stationary phase, since the production rate (0.063 mM/h) at exponential phase was higher than the production rate (0.028 mM/h) at stationary phase. In addition, the optimization study for biotransformation via shake-flask system shows that the optimum condition was found to be at $48^{th}$ hr incubation time under pH 6.68 and at $31.85^{o}$C with the lowest amount of substrate concentration which is 6.78 mM. The highest % enantiomeric excess and product conversion which almost 100 % were achieved at this optimum condition. The increasing amount of substrate concentration used in this biotransformation process leads to substrate inhibition. In addition by using the bioreactor system, it can maximize the % of enantiomeric excess, production conversion, and rate of product formation. The parameters varied on bioreactor system were agitation speed in the ranges of 50 rpm to 150 rpm and the incubation time of biotransformation from 0 to $56^{th}$ h. From result obtained, the maximum agitation speed was found to be at 150 rpm with optimum incubation time at $33.34^{th}$

hr of biotransformation with 99.94 % e.e and 88.22 % product conversion. In addition, almost 0.097 mM/h of the production rate was achieved at this optimum condition. Therefore, from the result obtained it clearly showed that there was quite a different range of production rates as well as product conversion of *(R)*-1-(4-bromo-phenyl)-ethanol when using the bioreactor system. The optimum incubation time also can be shortened compared to the shake-flask system. The result obtained from the bioreactor system can be used for further study in verifying the different types of substrate used, different aeration and different types of biocatalyst. Besides that, for the fermentation kinetic study, the specific growth rate, ($\mu$) of *A. niger* is 0.29 $h^{-1}$ with 0.164 $h^{-1}$ of product formation rate, ($q_p$) and 0.191 $h^{-1}$ of substrate utilization rate, ($q_s$). In addition, according to MATLAB® analysis for the biotransformation kinetic study, the values of $V_{max}$, $K_m$ and $K_s$ were found to be at 2.23 mM/h, 76.69 mM and 0.21 mM, respectively.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Research Overview

### 1.1.1 Significance of Chirality

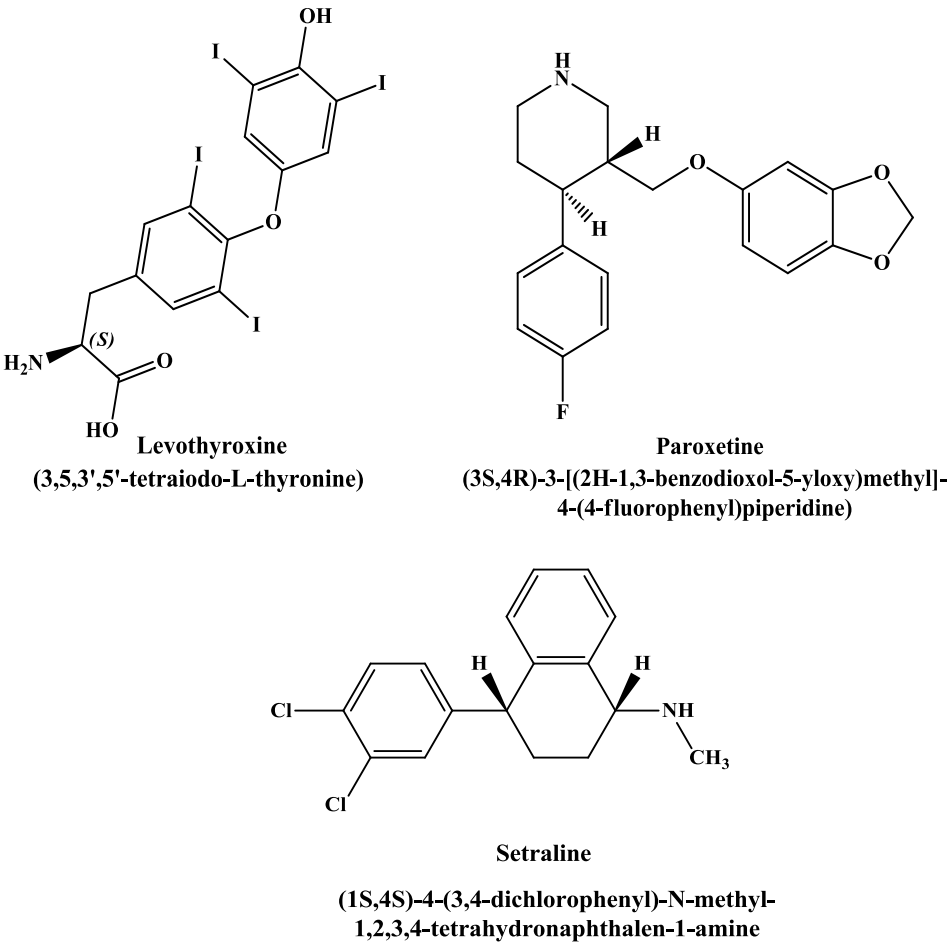
Molecular chirality is a fundamental phenomenon that plays an important role in biological processes. The increasing popularity of chirality in pharmaceutical activity has stimulated an increasing demand for economical and high productive methods for commercial synthesis of pure enantiomers. The demand for these optically pure therapeutic agents is becoming more stringent due to its more-specific characteristics compared to any racemic mixtures (Long *et. al.,* 2002; Hassan and Mohamed, 2004). In addition, the demand for fast-growing drug industries for enantiomerically pure compounds and specialty chemicals are the driver for many companies to pursue biocatalytic technology. Enantiomerically pure alcohols are valuable chiral building blocks for many such industrials where the compounds act as key intermediates in the production of pharmaceuticals, fine chemicals and natural products. Recently, much attention has been focused on the production of chiral alcohols since the chiral drugs are major contributors to the global pharmaceutical market (Long *et. al.*, 2005; Pękala *et. al.*, 2007).

In addition, the issue of chirality has emerged as a major theme in drug design, discovery and development, since stereoisomer distinction is a significant component in many pharmacological events. The advances in chiral technology and the ability to produce enantiomerically pure compounds have an important impact on drug design, research and development as well as on the strategies and policies of the pharmaceutical industry (Agranat and Caner, 1999). In the early twentieth

century,the relevance of chirality to the pharmaceutical industry was established by the fact that one enantiomer of hyoscyamine possessed greater pharmacological activity than the other. Today, most of the new drugs and those under development consist of a single optically active isomer (Wei *et. al*., 2009). In addition, regulatory agencies throughout the world are currently reviewing the importance of chirality since it has becoming an issue for agrochemical, pharmaceutical and other industries such as flavor and fragrance industry (Challener, 2002; Leffingwell, 2003). Besides, chiral therapeutics or chiral drugs already made up over one-third of pharmaceutical drugs currently sold worldwide. This is a growing industry with global chiral drug sales for 2002 increasing by 12 % to $160 billion of a total drug market of $410 billion as reported by Technology Catalysts International (Chirality, 2004).

The earliest recognition of chirality in drugs was closely linked to the discovery of molecular chirality. As well, drug chirality is now a major theme in the design, discovery, development, launching and marketing of new drugs chiral molecules are constituents of a large proportion of therapeutic agents (Caner *et. al*., 2004). This discovery was accomplished mainly in France during the first half of the $19^{th}$ century. By the beginning of the $20^{th}$ century, investigation of the role of chirality in drug action had begun and continued to occupy pharmacologist and chemist during the remainder of century. As a result enantioselectivity in the effects or character of chiral drugs was found in many cases, especially in a large variety of pharmacological effects and chemical structures (Caner *et. al.*, 2004 and Welch, 2004). Besides, vast majority of synthetic chiral drugs introduced by 1987, where 88 % were racemic and by the late 1980s roughly a quarter of the drugs on the market were chiral and racemic (Francotte and Lindner, 2006).

In addition, chirality is a key factor in the safety and efficiency of many drug produced. Meanwhile, chiral alcohols are useful as a starting material for the synthesis of various biologically active compounds and thus, the production of single enantiomers of drug intermediates has become increasingly important in the agrochemical as well as pharmaceutical industries (Patel, 2002; Kurbanoglu *et. al.*, 2007). Many familiar drugs were introduced to the market as single enantiomers such as Levothyroxine, Paroxetine, Setraline, and to mention a few which as illustrated in Figure 1.1.




**Levothyroxine**
**(3,5,3',5'-tetraiodo-L-thyronine)**

**Paroxetine**
**(3S,4R)-3-[(2H-1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine)**

**Setraline**
**(1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine**

Figure 1.1: Available familiar drugs that market as single enantiomers.

In fact, single enantiomers can be produced by chemical or chemo-enzymatic synthesis. Biocatalysis often offers advantages over chemical synthesis since

enzyme-catalyzed reactions are often highly enantioselective and regioselective (Patel, 2002). Furthermore, great attention has been paid to enantioselective syntheses of enantiomerically pure compounds or chiral synthons that are increasingly in demand for the development of modern drugs and agrochemicals (Baldassarre *et. al.,* 2000). In addition, chiral alcohols with additional functional groups are promising building blocks for the synthesis of enantiomeric pure pharmaceuticals and other chemicals (Shimizu *et. al.,* 1998). Chiral alcohol such as (*R*)-1-(4-bromo-phenyl)-ethanol is one of the important precursors or key intermediate that normally used in pharmaceutical for drug synthesis.

### 1.1.2 Biotransformation of Chiral Alcohol

Biotransformation has been accepted as a method for generating optically pure compounds and for developing efficient routes to target compounds. Biotransformation also provides an alternative to the chemical synthetic methodology that is sometimes competitive, and thus represents a section of the tools available to the synthetic chemist (Loughlin, 2000). As well, according to Xiao and co-workers, biotransformation using growing cells is still a preferable method for the synthesis of most of the cofactor-dependent products industrially (Xiao *et. al.,* 2005). The biotransformation is bound to condition which can significantly affect the enantioselectivity and productivity of the chiral alcohol which some of them includes; the pH of the media, temperature of the media, incubation time, and substrate concentration. The advantages offered by biotransformation over chemical catalysis are summarized in Table 1.1.

Table 1.1: Comparison between biotransformation and chemical catalysis

| Biotransformation | Chemical catalysis |
|---|---|
| Product specificity | Product with racemic mixtures |
| Environmental friendly | Used heavy metal catalysts |
| Operated at mild condition | Operated at high temperature and pressure |
| Acknowledged as 'natural' | Acknowledged as artificial/synthetic |

In addition, biotransformation has been applied in various industries due to the two main reason which includes; i) the availability of microorganism such as bacteria, in order to produce large amounts of biomass and a great variety of different enzymes in a short time and also; ii) biotransformation offers a product with outstanding properties of chemo, regio and stereospecificity. Chemospecificity refers to a restricted single chemical reaction when several functional groups present at the compound's structure, and thus, will avoid the side reaction. Meanwhile regiospecificity indicates that the reaction of substrate molecules at the same time with enzyme while stereospecificity can be described as the enzyme preference to attack with one of enantiomers from two entities of *R*-or *S*- configuration resulted in a single enantiomer compound, thereby avoiding difficulties of racemic mixtures (Leuenberger, 1990; Leresche and Meyer, 2006; Arifin, 2010).

Among the current methodologies that have been applied to obtain chiral alcohols include the biocatalytic reductions of the corresponding ketones, as well as the kinetic resolution with lipases of the racemic alcohols via esterification (Goldberg *et. al.,* 2007). In addition, the reaction of acetophenone to chiral phenyl-ethanol has been widely studied as a model of reaction for ketone bioreduction. Normally the synthesis of optically active alcohols was performed by asymmetric reduction of ketone through biocatalytic methods (Brzezińska-Rodak *et. al.,* 2006;

Chen *et. al.*, 2008). The enantioselective reduction of ketones in organic synthesis plays a major role in the production of chiral intermediates which can be modified later for the synthesis of fine chemicals (Valadez-Blanco and Livingston, 2009). Asymmetric reduction of substituent of acetophenone to a chiral phenyl-ethanol using the whole-cell biocatalyst is one of the most successful and popular applications of biotransformation. This is due to their small sizes, bacteria are by far have the largest surface-to-volume ratio in the living world, which allow them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface. This metabolic flexibility requires that these microorganisms are also able to produce hundreds of different enzymes for all sorts of reactions. Figure 1.2 shows the example of microbial strains used as biocatalyst for the conversion of acrylonitrile to acrylamide.

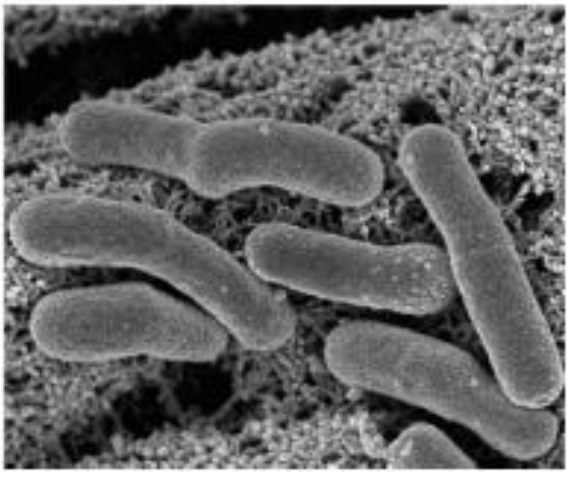


Figure 1.2: The photograph on the bacterium belongs to the genus *Rhodococcus*, a polymorphic organism able to grow into very long filamentous (Leresche and Meyer, 2006).

## 1.2 Problem Statement

In this new millennium, the pharmaceutical industry faces new opportunities created by the completion of the Human Genome Project and the increased emphasis on genomics and proteomics in drug discovery. Technological advances have focused on the drug discovery effort towards the search for drugs directed at molecular targets or pathways believed to have a causal role in the disease (Koh *et. al*., 2003). The chiral alcohols are important intermediate for numerous drug in pharmaceutical, flavours and fragrance and also play an important role in many biological reactions inside human body. Both isolated enzyme and whole-cells have been used on laboratory scale and industrially as biocatalyst (Rozzell, 1999; Baldassarre *et. al.,* 2000; Milagre *et. al*., 2008). Furthermore, for the ideal biotransformation process, the efficient and stable biocatalyst need to be chosen in order to get the highest % enantiomeric excess (% e.e) and product conversion, to minimize the side reaction and also to ensure the biocatalyst stable under the optimal reaction conditions (Burton, 2001).

There are various whole-cell microorganisms that can be used as a biocatalyst for the production of chiral alcohol such as *Escherichia coli*, *Saccharomyces cerevisiae, Aspergillus niger*, *Pseudomonas fluorescens* and to mention a few but the *S. cerevisiae* (baker's yeast) has widely been chosen by many researchers since its availability, simplicity to use as well as gives the highest yield as well as % of enantiomeric excess compared to other microorganism as mention in Table 1.2 (Lorraine *et. al*., 1996; Gervais *et. al*., 2003; Guo *et. al*., 2005; Brzezinska-Rodak *et. al*., 2006; Lin *et. al*., 2008). Meanwhile, the *Aspergillus niger* is rarely being used as biocatalyst for biotransformation of chiral alcohol. Only a few of studies such as

done by Demyttenaere *et al*., (2001); Snajdrova *et. al*., (2004); Kurbanoglu *et. al*., (2007) and Zilbeyaz et. al., (2008) used *A. niger* as biocatalyst. Therefore, in this present project the wild-type of *A. niger* ATCC 6275 has been chosen as a biocatalyst in order to know how efficient this microorganism affects on the production of (*R*)-1-(4-Bromo-phenyl)-ethanol.

Table 1.2: Whole-cell biocatalyst for synthesis of optically active alpha-hydroxy acids (Guo *et. al.*, 2005)

| Microorganism | Conversion (%) | e.e (%) | Configuration |
|---|---|---|---|
| *Stenotrophomonas maltophilia* CECT 112 | 90 | 49 | *S* |
| *Corynebacterium flaccumfaciens* CECT 5039 | 94 | 71 | *S* |
| *Camdida boidinii* CECT 10139 | 72 | 94 | *R* |
| *Pseudomonas fluorescens* AS1867 | 70 | 97 | *R* |
| *Saccharomyces ellipsoideus* JUC Y | 82 | >99 | *R* |
| *Saccharomyces cerevisiae* CECT 1929 | 88 | >99 | *R* |
| *Saccharomyces cerevisiae* CECT 1477 | 93 | >99 | *R* |
| *Saccharomyces cerevisiae* JUC WXJ-2 | 98 | >99 | *R* |

In fact, normally biotransformation was carried out at exponential phase without knowing the effect at different phase such as during stationary phase on the % e.e as well as product conversion. The fermentation process usually stops and produces lower cell biomass at stationary phase but the biotransformation process might be occurred and produces the chiral alcohol during that phase. Therefore, the biotransformation process needs to be investigated during stationary phase too and the efficiency of *A. niger* also could be investigated during both phases. Besides, the effect of incubation time for the shake-flask and bioreactor system should be

scrutinized in order to ensure which system shows the best incubation time as well as produces the highest % e.e, product conversion and production rate.

In addition, most of the biotransformation of chiral alcohols was not being optimized and in order to get the optimum condition, they only verified the effect of each parameter such as incubation time, pH, temperature, agitation speed and to mention a few. However, the relationship between each parameter involved which includes; incubation time, pH, temperature, agitation speed, as well as substrate concentration and the profile for every parameter at optimum condition has not being verified by the most researchers. The optimization and the relationship between each parameter can be verified by using response surface methodology (RSM) in order to get the highest % e.e as well as product conversion at optimum condition. Moreover, biotransformation system can be characterized biochemically in order to provide the information which then can be used in mathematically modelling and also scale-up of the bioreactors (Burton, 2001). The growth kinetics of *A. niger* and modelling for the biotransformation of (*R*)-1-(4-Bromo-phenyl)-ethanol need to be investigated too, since there might be the substrate inhibition occurred in the biotransformation process. This is due to the kinetics and modelling for the substrate inhibition that occurred previously in the study done by Zilbeyaz and Kurbanoglu, (2008) has not been carried out. In fact, the substrate concentration in the bio-medium is of paramount importance in the performance of biotransformation since in many cases, it will inhibit the biocatalyst activity as well as affecting the enantioselectivity of the product (Valadez-Blaco and Livingston, 2008). The kinetic and modelling actually is important to find the value of $V_{max}$, $K_m$, and $K_s$.

## 1.3 Research Objective

In order to achieve the overall aims, several specific objectives were defined:

i) To study the efficiency of *A. niger* as a biocatalyst at different growth phase in the reduction of ketone to chiral alcohol for the synthesis of chiral drug.

ii) To determine the optimum pH, temperature and substrate concentration in order to obtain the maximum % enantiomeric excess as well as production of chiral alcohol via shake-flask system using response surface methodology (RSM).

iii) To identify the optimum incubation time and agitation speed using 2.5 L bioreactor system and to compare the formation rate between shake-flask study and bioreactor system.

iv) To obtain the kinetic growth of *A. niger* and the model for the production of (*R*)-1-(4-bromo-phenyl)-ethanol using POLYMATH® and MATLAB®.

## 1.4 Organization of the Thesis

This thesis consists of six chapters in which every chapter describes the sequence of the research and represents valuable as well as tangible information about the research study.

Chapter 1 briefs the term of chirality, the history and the application of the chirality in pharmaceutical field. It also concisely described the market trends throughout the world and the techniques available in order to obtain the chiral alcohol. The problem statements, research objectives and the organization of thesis were also highlighted in this chapter.

Chapter 2 represents the details of every subtopic that are related to the biotransformation of the chiral alcohol. It also covers the advantages of using whole-cell microorganism as biocatalyst for the biotransformation of ketone to chiral alcohol. The general schematic process of reduction was also described here.

Chapter 3 emphasizes on the materials and method used, which also describes the procedures for the production of chiral alcohol using *A. niger* as a biocatalyst via shake-flask method. This chapter also explains the analytical method, kinetic and modeling, and optimization studies for every biotransformation parameters. The overall experiment flow chart was also presented here.

Chapter 4 refers to the experimental result and details discussion of the fermentation growth of *A. niger* and also the results of biotransformation of (*R*)-1-(4-bromo-phenyl)-ethanol for every parameters studied.

Chapter 5 discusses about the kinetics and modeling of *A. niger* growth and biotransformation of (*R*)-1-(4-bromo-phenyl)-ethanol by using POLYMATH® and MATLAB® analysis.

Chapter 6 summarizes and concludes the overall finding based on the results obtained in the previous chapter. The recommendations for future studies are also given here.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Chirality

### 2.1.1 The Term of Chirality

Chirality is well known as the key factor in the efficiency of many drugs and it also plays an important part in human life. In fact, it is an essential property of the bio-molecular building blocks of life (Young *et. al.,* 2006). The concept of "chirality" has been known in chemistry since the 1870's although it would be nearly a hundred years before chemists began using the term. In simple words, chirality is "handedness," that is, the existence of left/right opposition. For example, the left hand and right hand are mirror images and therefore "chiral". The term chiral is derived from the Greek word *kheir* meaning "hand" and it is used to express an object that is non-superposition on its mirror image (Leffingwell, 2003). In addition, any carbon atom that is bonded to four different functional groups is termed as a chiral or an asymmetric carbon. Molecules containing one or more of these carbon centers are considered as chiral molecules. Chiral centers can exist in two forms called enantiomers (*R* and *S*). Figure 2.1 shows a few examples of chiral molecules.

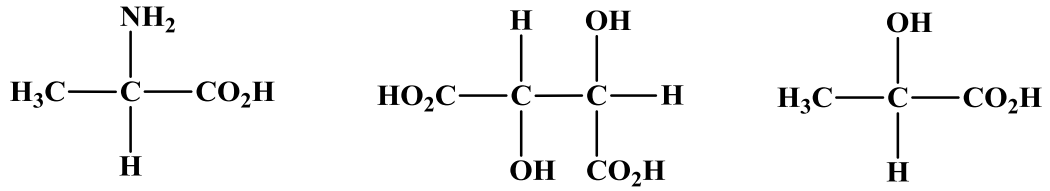



Figure 2.1: Examples of chiral molecules.

### 2.1.2 Application of Chirality in Industries

The importance of chirality in life sciences and the production of new materials has been growing rapidly, and is highly appreciated by the pharmaceutical industry for decades. This makes the manufacturing of chiral molecules one of the highlighted topics in modern fine chemical industry (Akutagawa, 1995). As technologies for producing and measuring enantiopure materials keep improving, the production of enantiopure pharmaceuticals has become commonplace, with many of the top selling drugs in the world are now being sold in enantiopure forms. Consequently, the subject of chirality in pharmaceutical industries is a topic of considerable interest and importance (Welch, 2004; Nguyen *et. al.,* 2006; Mohan *et. al.,* 2011). Apart from that, chirality continues to attract interest in the chemical, biological and pharmaceutical sciences. Nowadays, chiral synthesis is becoming increasingly important for research and development in other various applied industries. It is even more important to synthesize these enantiopure products in an environmentally friendly process in order to build a sustainable society (Nakamura *et. al.,* 2002; Hasan *et. al.,* 2004).

## 2.2 Single Enantiomer

### 2.2.1 Single Enantiomer of Chiral Molecule

Chiral molecules such as amino acids, sugar, proteins and nucleic acids are important components in all living organisms. An interesting feature of these chiral bio-molecules is that, they naturally exist in only one of the two possible enantiomeric forms (*R* or *S*). However, only a single enantiomer of a chiral molecule is usually desired, as is the case when the target molecule is a chiral drug that will be used by both human and animals. Drug molecules can be likened to tiny keys that fit

into locks in the body and elicit a particular biological response. Since the ‘locks’ in living organisms are chiral, and exist in only one of the two possible enantiomeric forms, only one enantiomer of the ‘key’ molecule should be used (Welch, 2004; Cox, 2005; Mohan *et. al.,* 2011; Nagori *et. al.,* 2011).

Indeed, many of the major pharmaceutical manufacturers are seeking more efficient and cleaner routes in order to produce single enantiomeric compounds, either in the form of drug intermediates or active pharmaceutical ingredients. Among the 100 top-selling drugs in today’s market, 50% are single enantiomer drugs. The production of single enantiomers of drug intermediates is increasing and many biologically active substances exist as single enantiomers in living organisms (Misl'anová and Hutta, 2003; Patel, 2006). Enantiomers can have different biological activities which determine the efficacy of the compound. The use of enantiomerically pure drugs in chemotherapy is becoming mandatory, not only to realize the enhanced specificity of the drug’s action but also to prevent any possible toxicity and undesirable load on the patient’s metabolism by the other enantiomer (Sonawane *et. al.,* 1991; Islam *et. al.,* 1997; Creagh *et. al.,* 2004; Webster, 2006; Sweet, 2009; Peeplwala *et. al.,* 2010).

In addition, chiral compounds are typically optically active where they often have one or more chiral centers. It has been reported that more than one-half of marketed drugs are chiral (Misl'anová and Hutta, 2003; Jurcek *et. al.,* 2008). In the meantime, the methodologies for preparing optically active compounds have been available for over a 100 years which includes; crystallization, liquid chromatography and kinetic resolution, to mention a few. Furthermore, a wide variety of techniques

for chiral separations have been developed in the past 15 years (Rekoske, 2001). Normally, the enantiomers can be separated by conventional methods, such as crystallization, chromatography on silica or other stationary phases. Chromatography is a process that often resulted in high enantiomeric excess which it is suitable on an analytical scale, but its scale-up can be difficult. In this present project, the High Performance Liquid Chromatography (HPLC) technique has been chosen in order to separate the enantiomers.

### 2.2.2 Development of Single Enantiomer in Industries

According to Patel (2002), single enantiomers can be produced through a chemical or chemo-enzymatic synthesis. However, biocatalysis offers better advantages over chemical synthesis as enzyme-catalyzed reactions are often highly enantioselective and regioselective. Thus, the synthesis of optically active alcohols is normally performed by asymmetric reduction of ketone through biocatalytic methods (Chen *et. al.,* 2008). Moreover, according to Wei *et. al.* (2009), the enantiomers of a chiral drug are usually different in their physiological activities whereby one enantiomer is only effective to cure a disease, while the other one may be less effective, ineffective or even toxic if consumed. Hence, chiral drugs need to be carefully separated into single enantiomers during their development and production processes. Thus, enantio-separation is very important for the pharmaceutical industry and it has attracted many researchers' attention to develop effective enantio-separation techniques (Wei *et. al.,* 2009). Indeed, single enantiomer drugs continue to take an increasing share in the market with worldwide sales of these drugs surging by 21 % between 1996 and 1997 up to almost $90 billion (Gervais, 2003). Sandra (2006) reported that based on estimates from Technology Catalysts International

(Falls Church, VA) and IMS Health, single enantiomer therapeutics had sales of $225 billion in 2005, representing 37 % of the total final formulation pharmaceutical market of $602 billion. Figure 2.2 shows the examples of single enantiomers currently available in the market (Hutt and Valentová, 2003).

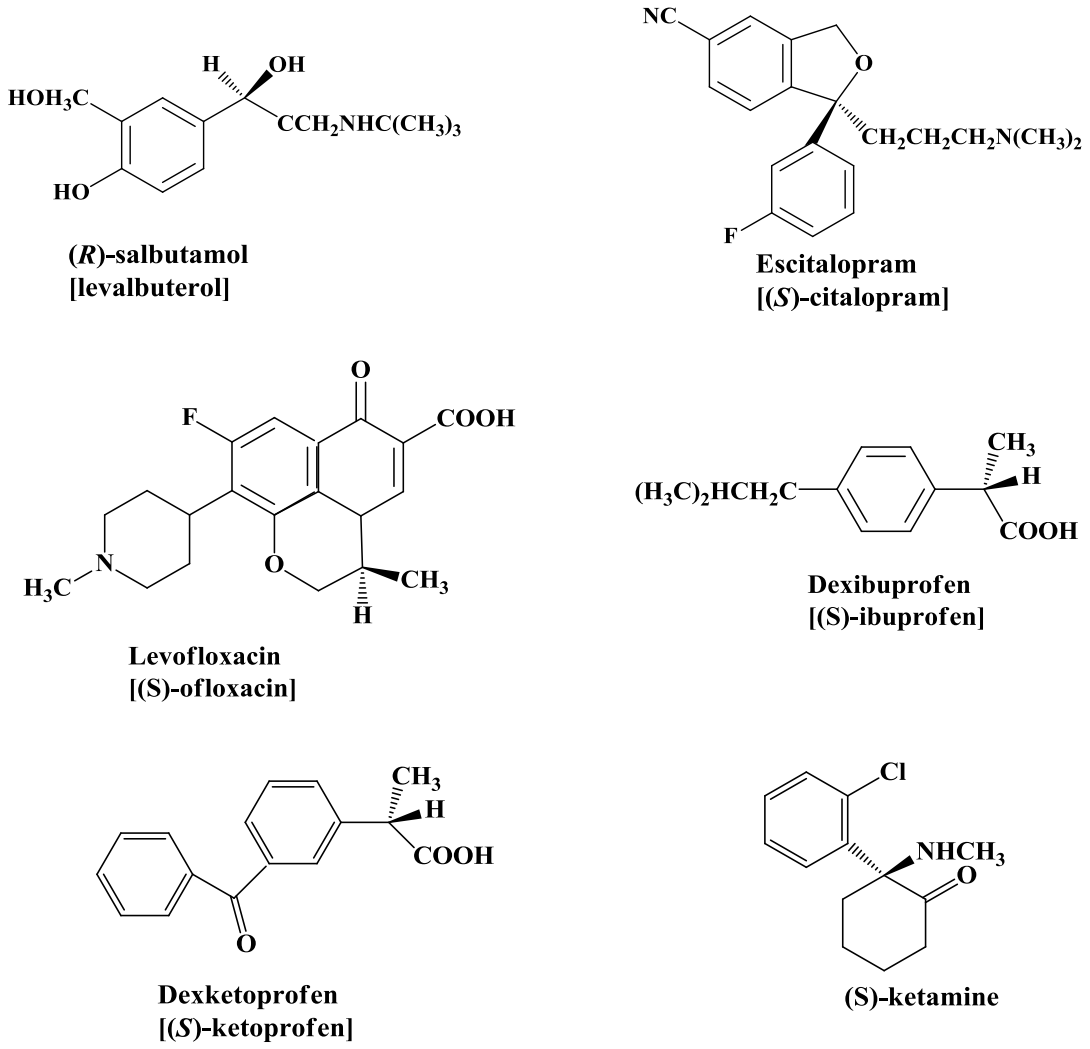



Figure 2.2: Marketed single enantiomers which have undergone the chiral switch (Hutt and Valentová, 2003).

In addition, the compound annual growth rate for single enantiomer products over the past five years is 11 %, which is on par with the pharmaceutical market as a whole. The chiral switch process has resulted in a number of agents being re-

marketed as single enantiomer products. Chiral switch can be defined as the development of a single enantiomer from a previously marketed racemate. Furthermore, single enantiomer drugs will grow at a similar rate as the overall pharmaceutical market. Higher growth in key therapeutic categories will be offset by the large and maturing markets in antibiotics and cardiovascular drugs. Parts of the higher growth will be from sales increase in biologics, but the use of small molecules in certain key classes such as cancer, arthritis, central nervous system and antiviral will give significant impacts as well (Sandra, 2006).

## 2.3 Chiral Alcohol as Chiral Drug

### 2.3.1 Market View of Chiral Alcohol

Among the various chiral compounds, chiral alcohols represent a highly versatile and attractive group of chiral building blocks for the synthesis of various drugs (chiral synthons) and drug intermediates (Soni and Banerjee 2005). Chiral alcohols are the most important kinds of chiral building blocks for many pharmaceuticals, due to their structural property (Zilbeyaz *et. al.,* 2009). The enantiomers administered as drugs often have different actions, toxicities and pharmacokinetic properties (what body does to the drug) due to the macromolecular substances such as enzymes and receptors have inherent chiral selectivity in biological systems (Yang *et. al.,* 2008).

Chiral technology has driven major developments in the pharmaceutical industry, which involves more than just chiral drugs. The emphasis is mainly focused on producing low-volume with high-value products. Chiral drugs continue to be a significant force in the global pharmaceutical market. According to Stinson (2000),

worldwide sales of single-isomer drug accounted for 30 % of the $335 billion total drug sales worldwide in 1998, edging up to 32 % of the $360 billion market in 1999. In addition, according to Smith, (2000), single enantiomer compounds are the major component of the largest selling brands, in both absolute number of drugs and dollar sales volume. Furthermore, Challener (2002) reported that the total sales of chiral drugs had reached $147 billion in 2001 which is an increasing of 3.6 % over the total drug sales in 2000. Besides, government agencies, such as the US Food and Drugs Administration (FDA) have recommended that all new asymmetric drugs being marketed should be a single enantiomer (Agranat and Caner, 1999; Gervais, 2003). Table 2.1 shows the global chiral drug sales from the year 1998 to 2000.

Table 2.1: Global chiral drug sales from year 1998 to 2000 (Smith *et. al.*, 2000).

| | GLOBAL SALES ($) | | |
|---|---|---|---|
| $ Millions | 1998 | 1999 | 2000 |
| Cardiovascular | 21,906 | 24,805 | 26,012 |
| Antibiotics/antifungal | 19,756 | 20,907 | 23,265 |
| Hormones/endocrinology | 12,297 | 13,760 | 17,345 |
| Cancer | 8,006 | 9,420 | 13,360 |
| Central nervous system | 7,027 | 8,592 | 13,720 |
| Haematology | 6,730 | 8,580 | 11,445 |
| Antiviral | 6,131 | 7,540 | 13,446 |
| Respiratory | 4,305 | 5,087 | 8,795 |
| Gastrointestinal | 1,718 | 2,998 | 5,355 |
| Ophthalmic | 1,482 | 1,794 | 2,070 |
| Dermatological | 1,124 | 1,270 | 1,540 |
| Analgesics | 842 | 1,045 | 1,135 |
| Vaccines | 568 | 676 | 1,100 |
| Other | 7,947 | 8,527 | 7,425 |
| TOTAL | 99,839 | 115,001 | 146,013 |

These sales value hit over $100 billion worldwide and increases steadily from one year to another, indicating an increasing demand for optically pure therapeutic agents as the result of its target specific characteristic than racemic mixtures. This

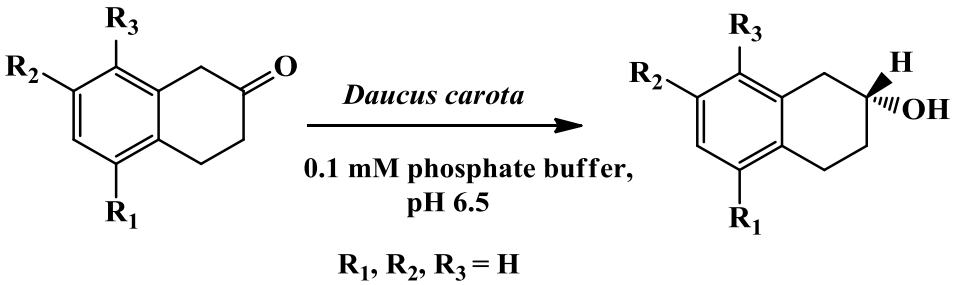
awareness has led to heightened efforts to obtain optically pure compounds (Smith, 2000; Stinson, 2000).

### 2.3.2 The Importance of Chiral Alcohol

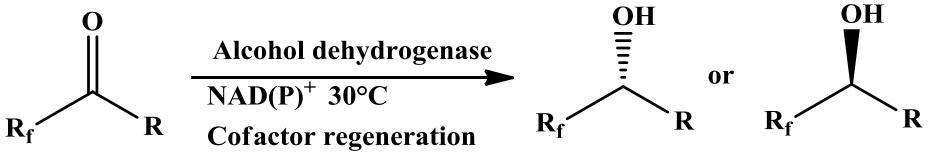
Chiral building blocks could be naturally occurring substances or simple and readily available chiral compounds that could be directly utilized in the synthesis of more complex target molecules (Shen, 2009). In fact, the demand for chiral alcohols has boosted since the need for optically active drugs has increased in the pharmaceutical and agrochemical fields in recent years. Due to this situation, chiral alcohols are becoming increasingly vital as a bioactive compound; being utilized as ligands for various metals in a number of asymmetric reactions (Moon Kim and Jin Kyoon, 1999; Kurbanoglu *et. al.,* 2007). Likewise, it is very important in the pharmaceutical industry since enantiomers of drug compounds may possess different pharmacological and toxicological properties (Zhou *et. al.*, 2009).

Currently, several methodologies can be applied in the process of synthesizing chiral alcohols such as the asymmetric reduction of prochiral ketones, the enantioselective oxidation of a single isomer from its racemate as well as the enantioselective reduction of a carbonyl group using enzymes such as alcohol dehydrogenase or whole-cell microorganism as a biocatalyst (Hasegawa *et. al.*, 1996; Kurbanoglu *et. al.,* 2007). According to Williams *et. al.,* (2001), these methodologies are important in order to measure chiral purity, chiral stability of a drug substance and its stability in metabolism. Chiral alcohols can be efficiently synthesized via asymmetric reduction of the corresponding prochiral carbonyl compounds using

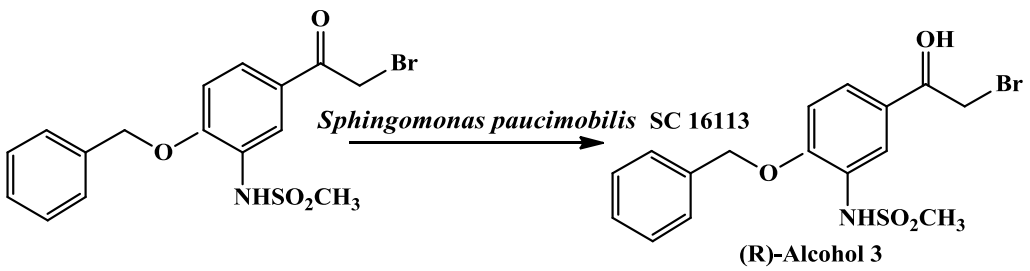
biocatalysts such as oxido-reductase or whole cells (Yang *et al.,* 2008). Scheme 2.1. shows a few examples of chiral alcohol syntheses previously studied by researchers.




i) Reduction of cyclic ketones (tetralones) using *Daucus carota* (Yadav *et. al.,* 2007).




ii) Reduction of perfluorinated ketones with alcohol dehydrogenases (enzyme) (Rosen *et. al.,* 2006).

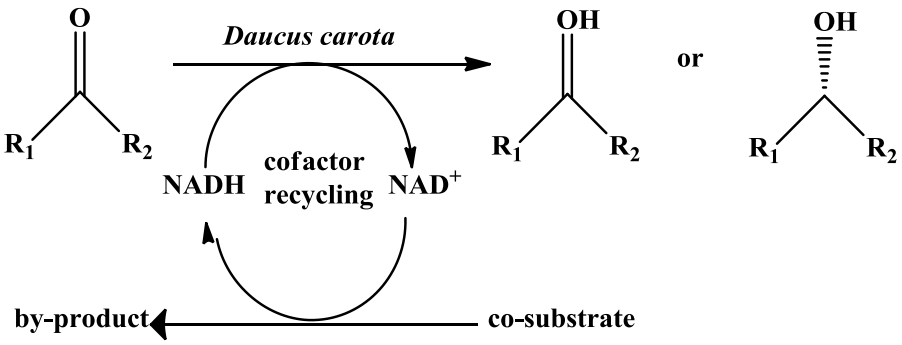



iii) Reduction of 4-benzyloxy-3-methanesulfonylamino-2' bromo-acetophenone 2 to (*R*)-alcohol (Patel, 2001).

Scheme 2.1 : Examples of chiral alcohol syntheses via reduction of various ketones (Yadav *et. al*, (2007); Rosen *et. al*, (2006); Patel, (2001)).

### 2.3.3 Asymmetric Synthesis in Chiral Alcohol

Asymmetric synthesis, also known as chiral synthesis is an organic synthesis which introduces one or more new and desired elements of chirality. Gladiali, (2007) reported that the birth of asymmetric synthesis already exist in 1890, when Emil Fischer recognized that the addition of hydrogen cyanide to L-arabinose had resulted in some 75 % yield of one of the two possible diastreomeric cyanohydrins. In addition, according to Akutagawa (1995), under certain conditions, asymmetric synthesis allows the production of enantiomerically pure compounds that have a wide range of applications in industry. Chemists have fully recognized the importance and practicality of asymmetric synthesis in various fields such as in synthetic organic chemistry, medicinal chemistry, natural products chemistry and pharmaceutical industries. The rise in its importance is due to the development of newer and more efficient methods during the last decade (Ojima *et. al.,* 1998). Scheme 2.2 shows a generic example of an asymmetric synthesis process.




Scheme 2.2: Example of asymmetric reduction of ketone to alcohol using *Daucus carota* as bioctalyst (Kaluzna *et. al.,* 2005).

From Scheme 2.2, it shows that the asymmetric reduction process using *Daucus carota* can regenerate the cofactor itself in order to produce the chiral alcohol continuously. The product produced will be either *R* or *S*. The asymmetric

reduction of ketone is one of the most important fundamental and practical reactions for producing non-racemic chiral alcohols without racemization in order to synthesize industrially important chemicals (Yadav *et. al.,* 2007). The catalysts for the asymmetric reduction of ketone can be classified into two categories: chemical and biological. Both methods have their own peculiarities and further development in selecting the appropriate catalysts for specific purposes is necessary to promote green chemistry (Nakamura *et. al.,* 2003). There are various types of useful ketone used as substrate for the asymmetric reduction process such as an aromatic hydroxyl ketone **(1),** α-alkyl substituted 1,3-diketones **(2),** and α- and β-alkyl substituted β-ketoesters **(3)**. The structures of each ketone mentioned are presented in Figure 2.3.

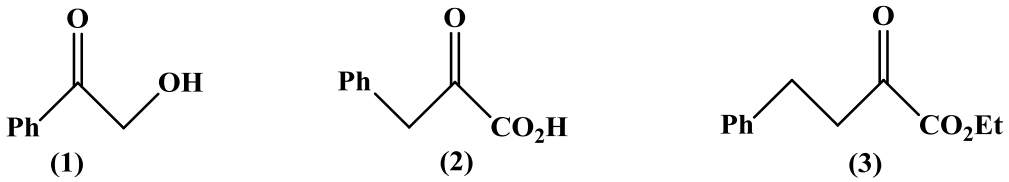



Figure 2.3**:** Several of ketone used as a substrate in asymmetric reduction process *Ph=phenyl, (Kaluzna *et. al.,* 2005).

Furthermore, the asymmetric reduction of ketones to alcohols introduces chirality into a target molecule, which can be used as a valuable intermediate in the preparation of chiral drugs (Brzezińska-Rodak *et. al.,* 2006). Asymmetric synthesis has emerged as one of the most rapidly developing research areas in organic chemistry. Among various strategies involved in the asymmetric synthesis include; the extensive studies of the use of chiral auxiliaries as well as chiral ligands. A number of new auxiliaries and ligands have been successfully utilized for efficient asymmetric synthesis (Moon Kim and Jin Kyoon, 1999). Furthermore, the modification of ligand has played a key role in developing new catalyst precursors for asymmetric synthesis (Zhang *et. al.,* 2009).

## 2.4 Biocatalysis

### 2.4.1 Biocatalysis in Industries

Despite tremendous scientific and technical advances, several chiral syntheses still remain difficult and expensive. Due to this situation, asymmetric biocatalysis employing whole microorganisms has often emerged as a viable alternative (Chartrain *et. al.,* 2001). Biotechnology offers many possibilities for new medical therapies and the manufacturing of new pharmaceuticals, including the synthesis of small molecule pharmaceuticals via biocatalysis is one of the challenging works (Woodley, 2008). A significant breakthrough in the field of biocatalysis came in the late 1950s, when the first three-dimensional enzyme structure was resolved with the help of X-ray analysis (Yuryev and Liese, 2010). Nowadays, the industrial biocatalysis is apparently experiencing a noteworthy growth and gaining momentum especially in the process of synthesizing pure compounds for pharmaceutical intermediates. This phenomenon is hardly surprising considering the enhanced integration of biological catalysts into a variety of industrial processes ranging from manufacturing commodity chemicals to synthesizing highly complex pharmaceutical intermediates and drug substances (Zaks, 2001; Panke and Wubbolts, 2005).

The role of biocatalyst in an asymmetric synthesis continues to become more vital, from a miniaturized micro process to a large-scale macro process. Applying biocatalysis to industrial processes has been shown to be a very advantageous alternative to the conventional chemical methods. Recently, it has also been widely used to prepare enantiomerically pure pharmaceuticals and other compounds of interest. Moreover, biocatalytic approaches using microbial cells and enzymes have become attractive choices for the synthesis of chiral compounds, thanks to the

developments in the discovery, expansion and production of stable biocatalysts. All these developments have brought about new designs for biocatalytic processes in product purification as well as recovery. This is also due to the advances in genomics, screening and evolution technologies, leading to increase availability of new and robust biocatalysts suited for industrial-scale application (Panke and Wubbolts, 2005; de Gonzalo *et al.*,2007; Wohlgemuth, 2010).

Indeed, biocatalysts are responsible for the chemistry of life which includes controlling chemical transformations in primary metabolism and assisting the generation of natural product diversity in secondary metabolism of plants and microbes (Walsh, 2001). Nowadays, various chiral alcohols can be produced via biocatalysis using two methods, that include; a kinetic resolution of the racemic starting material and secondly, a direct synthesis from a prochiral compound. In many cases, highly enantio-selective reduction can be achieved when biocatalysts are used. Both isolated enzymes and whole cells can be utilized as biocatalysts (Pekala *et. al.*, 2007). The application of biocatalyst for preparing molecular asymmetry has led to the higher enantioselectivity, product yield as well as efficiency and therefore, it is currently experiencing a worldwide renaissance (Wohlgemuth, 2010).

In fact, an important element in developing biocatalyst is the identification of the required enzyme activity, in terms of the desired chemical reaction's substrates and products and thereafter, identifying the source of such enzyme activity. Once a suitable source has been found, an efficient production system for the enzyme would be required. In a typical conventional biocatalysis process, the cell would be grown in a fermentation system, then harvested and used as whole-cells or the enzymes