BIOTRANSFORMATION OF *CIS*/*TRANS* 3,7-DIMETHYL-2,6-OCTADIEN-1-OL MEDIATED BY *SACCHAROMYCES CEREVISIAE*

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UNIVERSITI SAINS MALAYSIA

2011

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by

KHOR GUAT KHENG

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

AUG 2011

ACKNOWLEDGEMENT

Firstly, I would like to extend my deepest gratitude to my supervisor, Dr. Mohamad Hekarl Uzir for his excellent guidance, encouragement and support during this study. His willingness to spend innumerable time sharing his profound knowledge and effort, has assisted my completion of this project. His constant support, encouragement, never ending enthusiasm and confidence in me has been a source of motivation for me.

I am thankful to my co-supervisor, Prof. Mohd Roslee Othman, who had put in great effort and time to give some advices as well. My appreciation is also dedicated to the technicians and the staffs in School of Chemical Engineering for providing their duly assistance in the operation of various equipment in the laboratories and support. The financial support in terms of Fellowship Scheme for a period of two years awarded by Universiti Sains Malaysia (USM) is also vastly acknowledged.

Heartfelt thanks to all my friends who make every moment enjoyable and meaningful throughout my study. The delightful times we spent together will always be treasured. I must concede that there was lot more who helped me directly or indirectly in the completion of this work, whose name I could not mention. Last but not least, my heartfelt gratitude to my family for their endless love and support.

Thank you.

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LIST OF ABBREVIATIONS

2-KLGA	2-keto-*L*-gulonic acid
3-D	three dimensional
ADH	alcohol dehydrogenase
ANOVA	analysis of variance
ATP	adenosine triphosphate
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic
DXP	1-deoxy-*D*-xylulose 5-phosphate
e.e.	enantiomeric excess
ER	enote reductase
FCM	free cell method
FDA	Food and Drug Administration (U.S.)
FID	flame ionization detector
FPP	farnesyl pyrophosphate
g	gram
GC	gas chromatography
GPP	geranyl pyrophosphate
GRAS	generally regarded as safe
h	hour
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
ICM	immobilized cell method
ID	internal diameter

IPP	isopentenyl pyrophosphate
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
L	litre
L-AA	*L*-ascorbic acid
m	mili
M	molarity
MCC	3-methylcrotonyl-coenzyme A carboxylase
MEP	2-C-methyl-*D*-erythritol 4-phosphate
MHO	6-methyl-5-hepten-2-one
M-M	Michaelis-Menten
MVA	mevalonic acid
n	nano
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of NAD^+
$NAD(P)^+$	nicotinamide adenine dinucleotide phosphate
NAD(P)H	reduced form of $NAD(P)^+$
O.D.	optical density
rpm	revolutions per minute
RSM	response surface methodology
TCA	tricarboxylic acid
µ	micro
w/v	weight per volume

LIST OF SYMBOLS

Symbols

A or B or C	coded factors
E_o	total enzyme
E	free enzyme
ES	enzyme-substrate complex
SE	enzyme-inhibitor complex
SES	enzyme-substrate-inhibitor complex
K_i	dissociation constant SE complex
K_m	Michaelis-Menten constant
K_p	reaction constant
S	substrate concentration
t	time
X_1	cell concentration at time point t_1
X_2	cell concentration at time point t_2

Greek symbols

μ	specific growth rate of cells
v	biotransformation rate
v_o	initial rate of biotransformation
v_{max}	maximum biotransformation rate

BIOTRANSFORMASI *CIS/TRANS* 3,7-DIMETIL-2,6-OKTADIEN-1-OL OLEH *SACCHAROMYCES CEREVISIAE*

ABSTRAK

Biotransformasi geraniol and nerol menggunakan *Saccharomyces cerevisiae* telah dijalankan. Linalool dan geraniol dihasilkan sebagai produk utama dengan menggunakan sistem sel tumbuh, tetapi hanya pengisomeran nerol kepada geraniol berlaku apabila sel rehat digunakan. Reduktif biotransformasi geraniol adalah lebih berkesan menggunakan sistem sel rehat. Kombinasi rekabentuk factoran penuh dengan kaedah respons permukaan (RSM) digunakan untuk menilai kesan ketumpatan sel, kepekatan substrat dan masa biotransformasi atas penghasilan produk. Produktiviti meningkat seimbang dengan ketumpatan sel. Namun, kerencatan sel tumbuh diperhatikan apabila substrat dalam kepekatan tinggi digunakan. Keadaan yang optimum untuk biotransformasi geraniol adalah 1.36 g/L kepekatan substrat dan 60 g/L ketumpatan sel dalam masa 38 jam, dengan penghasilan 2.21 g/L citronellol dan 1.03 g/L nerol. Kepekatan optimum untuk linalool dan α-terpineol ialah 0.568 g/L dan 0.209 g/L masing-masing, dan dicapai dengan menggunakan 1.98 g/L kepekatan substrat nerol dan 35.8 g/L ketumpatan sel dalam masa 8.5 hari. Dengan anggapan penglibatan dua tapak aktif (*ES* yang produktif dan *SE* yang tidak produktif), kajian kinetik menunjukkan bahawa kerencatan kompetitif terlibat dalam proses biotransformasi tersebut. Nilai pemalar Michaelis-Menten, K_m, dan kadar maksimum biotransformasi, v_{max}, diperolehi. K_i ialah pemalar pemisahan untuk kompleks *SE* yang tidak produktif. Kenaikan kepekatan substrat menyebabkan kenaikan pembahagian kompleks *SE* dan *SES* (ternar) dalam enzim, justeru mengakibatkan kerencatan walaupun tapak kerencatan mempunyai tarikan yang lemah untuk substrat ($K_i > K_m$).

BIOTRANSFORMATION OF *CIS/TRANS* 3,7-DIMETHYL-2,6-OCTADIEN-1-OL MEDIATED BY *SACCHAROMYCES CEREVISIAE*

ABSTRACT

Biotransformation of geraniol and nerol mediated by *Saccharomyces cerevisiae* was performed. Linalool and geraniol were mainly obtained from nerol using growing cells but only isomerisation to geraniol occurred using resting cells. Reductive biotransfomation of geraniol to citronellol was apparently more effective with resting cells system. Combination of full factorial design with response surface methodology (RSM) was applied in order to evaluate the effects of cell density, substrate concentration and biotransformation time on product concentrations. The productivity increased proportionally with cell density. However, inhibition was observed at higher substrate concentrations. The optimum conditions for biotransformation of geraniol were found to be 1.36 g/L substrate concentration and 60 g/L cell density at 38 h, with 2.21 g/L citronellol and 1.03 g/L nerol formed. Linalool and α-terpineol achieved their optimums at concentrations of 0.568 g/L and 0.209 g/L respectively, using 1.98 g/L of substrate nerol and cell density of 35.8 g/L after 8.5 days. Kinetic studies assumed a two-site model (productive *ES* and non-productive *SE*) and revealed that a non-competitive substrate inhibition was involved in the biotransformation processes. The corresponding values of Michaelis-Menten constant, K_m, and maximum biotransformation rate, v_{max} were obtained. K_i represents the dissociation constant for non-productive *SE* complex. Increase in substrate concentration leads to the increase in the proportion of *SE* and *SES* (ternary) complexes in the enzyme, causing inhibition even though the inhibitory sites has poor affinity for the substrate ($K_i > K_m$).

CHAPTER 1

INTRODUCTION

1.1 Flavors and Fragrances

The ever-growing market and potentiality in food and beverages industries have triggered the development and a strong demand for flavors and fragrances. Successful production of new items with assured quality, commercialization and enhancement on the characteristics of current products has always been a target for these companies when facing a tough market competition in the world of food and beverages. In order to fulfill the market of more energetic demands from exigent consumers, flavor can be among the most valuable ingredient as it plays a crucial role in determining the satisfactoriness for a specified food product and influences consumers' further consumption of food.

Flavors can be defined as a complex combination of attributes such as taste, aroma, texture and pungency (Bomben et al., 1973). Volatile organic compounds (VOC) of food and beverages, generally called aroma compounds, are divided into classes such as aldehydes, alcohols, ketones, esters, lactones, ethers and terpenes. They are present at very low concentrations, typically at mg/L or g/L levels in natural sources (Ribeiro Jr. et al., 2004) and are the main contributor to the flavors. Analysis on different kinds of fruits indicates more than 6000 compounds are constituents of their aroma (Bai et al., 2007) and every single aroma compound has different contributions on the final aroma of a fruit due to their dissimilar molecular characteristics and concentrations (Raisi et al., 2008). In addition, purification of natural materials, especially essential oils, led to the identification of aroma-active

materials such as benzaldehyde, cinnamaldehyde and vanillin (Rowe, 2005). The increased knowledge of organic chemistry has made a revolution to the flavor and fragrance industry, when these aroma compounds could now be synthetically and biologically produced.

1.2 Biotechnological Route

More than one hundred years ago, biotechnology was employed to do chemical transformations on non-natural man-made organic compounds. At present, the application of biotechnological route encompasses several areas of interests, especially pharmaceutical, food, beverage and fuel, which leads to development of new products with improved economics. A substantial increase has been observed in the application of biocatalysis and biotransformation to produce fine chemicals.

Numerous bacteria, yeasts and fungi are used to catalyze chemical reactions from renewable feedstocks and ever since, have reaped in much attention owing to its higher reaction rate, higher product purity, less energy consumption and fewer negative impacts on the environment. Enhancement in molecular biology and genetic engineering have also steered the improvement and understanding of biochemical pathways which make possible the production of flavor molecules in a natural way. Microbial cells and their components are used in bioconversions and *de novo* syntheses in order to produce individual flavor compounds such as alcohols, aldehydes, ketones, acids, esters, aromatic compounds, terpenes, lactones, pyrazines etc. This knowledge is now applied to designing commercially viable processes, including scale-up, process optimization, and improved product recovery (Labuda, 2009).

Biocatalysis and biotransformation have always been active participants in the field of biotechnology and the fact that the number of available enzymes exploitable in performing selective biotransformation into a myriad of desired products continues to expand, bioconversion processes are advancing at an increasing rate. The utilization of biocatalysts in bioprocesses has continued to emerge and constantly being recognized as attractive alternatives for the synthesis of various important and commercially useful compounds as auxiliaries to the traditional chemical approaches over the past years. Consequently, infiltration of biotechnological production processes to the chemical industry will extend into agriculture and pharmaceuticals sectors. Production of chemicals using engineered microorganisms and enzymes could generate global revenue of $1.8 trillion (USD), with one-fifth of the chemical industry is expected to be dependent on synthetic biology by 2015 (Singh, 2011).

1.3 Problem Statement

Aroma compounds are key impact substances in the food and fragrance industries. The increased customer demand for natural flavors and fragrances has led to intensified attempts to increase the rate of production of these enantiomerically pure substances; both the percentage yield as well as its enantiomeric excesses (*e.e.*). The use of flavors in particular, does not only target the food and cosmetic industries. Its application has also been introduced in the cancer treatment scheme (Carnesecchi et al., 2001). It is well-known that the consumption of fruits and vegetables could reduce and at the same time prevent the occurrence of cancer (Steinmetz et al., 1994), and this has therefore triggered a new scope of research where the formation of

active compounds mainly found in fruits such as terpenes and terpenoids are mimicked based on their natural existence.

The major sources of natural flavorings in general are plants and some of these are also chemically synthesized. However, the preference of consumers towards products defined as “natural” has triggered the increasing demand for flavors isolated from natural sources, in which the starting materials are natural (plants and animal sources). Nevertheless, several shortcomings of the production are stalwartly elucidated through the large fluctuations of quality, price and supply of the natural flavors extracted from plants, attributed to the variability in the composition and yield of the final products from different geographical sources, in addition to the dependence of sources on the weather, risk of plant diseases and seasonal variation in supply etc (Berger et al., 2000).

Recent developments on biotechnology have enabled the production of natural flavorings to be carried out economically and more efficiently. Microbial whole cells have shown great potential for biotransformation owing the ease with which the microorganisms can be cultivated and used in the bioreactors (Kashi et al., 2008). However, existing successful chemical processes and several biocatalyst-associated problems, such as lack of stability and limited operating range, have often thwarted efforts to substitute them with biotransformation processes. Most of the aroma compounds are secondary metabolites that cells do not require for growth. They are present at low concentrations during the logarithmic growth phase, but appear in large quantities during the stationary phase. Very often, flavor compounds partition to lipid structures due to their hydrophobicity, targeting cellular membranes for product accumulation during microbial processes. In other words, these compounds and/or their precursors inflict cytotoxicity effects on the cells (Schrader,

2007). Inhibitory effects often cause impediments for higher yield production, resulting in high costs for downstream processing comprising isolation and purification steps.

The aim of this research is to study the potentiality of *Saccharomyces cerevisiae* to mediate the biotransformation of monoterpenoids geraniol and nerol, respectively. *S. cerevisiae* or baker's yeast is a species of budding yeast. It is perhaps the most useful yeast owing to its use since ancient times in baking and brewing. Besides being widely used in the fermentation and brewing industries, *S. cerevisiae* has found its application in the production of useful chemical compounds (Attfield, 1997) and has been proven effective with relatively high *e.e.* (Ward and Young, 1990).

While bioconversion of terpenes has been extensively explored using both prokaryotic and eukaryotic microorganisms (de Carvalho and da Fonseca, 2006), less interest has been allocated for the biotransformation of acyclic monoterpenes (e.g., geraniol and nerol) utilizing baker's yeast. Then again, for many instances, the performance of *S. cerevisiae* is often outshone by other organisms. The presence of different enzymes causing overlapping activities of the cells plus many of the reductases involved have yet to be fully characterized also contributed to the little attention given.

Nevertheless, with its distinct characteristics such as process robustness, ability to grow anaerobically, high tolerance to low pH, high sugar/ethanol concentrations that lower the risk of contamination (Nevoigt, 2008), and involvement in well-established large-scale fermentation, baker's yeast offers significant advantages as a versatile biocatalyst. Other attractiveness includes being inexpensive, readily available and non-pathogenic which earns its status to be categorized as

GRAS (Generally Regarded As Safe) organism (Ostergaard et al., 2000). Besides, the capability of *S. cerevisiae* in carbon–carbon double bonds reductive biotransformation has been well defined and made it possible to perform biotransformation of terpenes and their derivatives.

1.4 Objectives

The objectives set in for this particular research can be classified into three key areas, as listed below:

1. To study the biotransformation of *cis* and *trans* 3,7-dimethyl-2,6-octadiene-1-ol (geraniol and nerol) respectively, using baker's yeast type-II (*Saccharomyces cerevisiae*).
2. To investigate the effect of substrate concentration, cell density, glucose concentration and time towards the biotransformation processes.
3. To optimize the biotransformation processes by using Response Surface Methodology (RSM) and to propose a kinetic model that describe the biotransfomation processes.

1.5 Scope of Research

In this research, biotransformation experiments were conducted in batch mode with monoterpenoids geraniol and nerol as the main precursors. The capability of microorganisms in converting these substrates into more valuable aroma compounds offers a remarkable alternative source of natural flavors (Demyttenaere, 2001). Baker's yeast type-II in its dried form was used as the biocatalyst throughout

the work. Evaluation of the sustainability and stability of the baker's yeast towards inhibitory effects was carried out based on cell density, substrate/product concentration and biotransformation time. Identification and concentration of products were later verified using gas chromatography techniques. Subsequently, optimum values for the parameters involved in the biotransformation processes were determined using statistical approach. Lastly, a suitable model was developed for kinetic inhibitory studies.

1.6 Organization of Thesis

This thesis contains five chapters encompasses Introduction, Literature review, Materials and methods, Result and discussions, Conclusion and recommendations for future research. Chapter 1 introduces the background and importance of flavors and fragrances and their production via biotechnological route, followed by objectives of this study. Chapter 2 highlights the review on literatures relevant to this study, which includes several successful works on biotransformation of terpenoids and the significant usages of baker's yeast (*Saccharomyces cerevisiae*) in this particular area of interest. The materials and experimental procedures required in this study is placed under Chapter 3, while Chapter 4 presents and discusses the experimental results and optimization via statistical approach, wrapped up with the development of model for kinetic studies. Last chapter draws the conclusion for this research work accompanied with several recommendations for future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Terpenes

According to Encyclopedia Britannica, terpenes are any of a class of hydrocarbons occurring widely in higher plants and animals, and empirically constructed from isoprene. These natural organic substances are ubiquitous in all classes of living things and constitute the largest and most widespread family of natural products. To date, over 55,000 terpenes have been isolated, doubling the amount each decade (Breitmaier, 2006). Terpenes exist in the form of hydrocarbons, alcohols, ethers, aldehydes, ketones, carboxylic acids, esters. They play an important role as the major biosynthetic building blocks within nearly every living organism.

Terpenoids, also known as isoprenoids, belong to a subclass of the prenyllipids (terpenes, prenylquinones, and sterols) and represent the oldest group of small molecular products synthesized by plants (Leray, 2009). Terpenoids are relatively inexpensive, readily available and renewable natural precursors (Demyttenaere and De Kimpe, 2001). These compounds comprise one, or more, basic isoprene units, assembled and modified in thousands of ways. In other words, terpenoids are the resulting compounds after modifications on terpenes, such as by rearrangement of the carbon skeleton wherein methyl groups are moved or removed and also oxidation (addition of oxygen atoms). Most of them are multi-cyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons.

Just like terpenes, classification of the terpenoids can be established according to the number of isoprene units incorporated in the basic molecular skeleton used, as shown in Table 2.1. The basic molecular formulas of terpenes are multiples of that, $(C_5H_8)_n$ where n is the number of linked isoprene units. The isoprene units attach to one another by linking the head of one unit to the tail of another to form linear chains or they are arranged to form rings. These observations are known as the isoprene rule or the C5 rule.

Table 2.1: Classification of terpenoids (Leray, 2009)

Terpenoids	Number of Isoprene Units	Number of Carbon Atoms
Hemiterpenoids	1	5
Monoterpenoids	2	10
Sesquiterpenoids	3	15
Diterpenoids	4	20
Sesterterpenoids	5	25
Triterpenoids	6	30
Tetraterpenoids	8	40
Polyterpenoids	Large number of isoprene units	5n (n>8)

Terpenes and terpenoids are generally associated with characteristic fragrances as they impart a wide variety of pleasant and floral scents and are used extensively for their aromatic qualities. In particular, monoterpenes and sesquiterpenes are the primary constituents of essential oils from many types of plants, while the other terpenes are constituents of balsams, resins, waxes, and rubber (Leray, 2009). Generally, all terpenoids are synthesized through the condensation of C5 isomeric precursors dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which can be derived either from mevalonate (MVA) or non-mevalonate (MEP) pathways from pyruvate of higher plants, as shown in Figure 2.1.

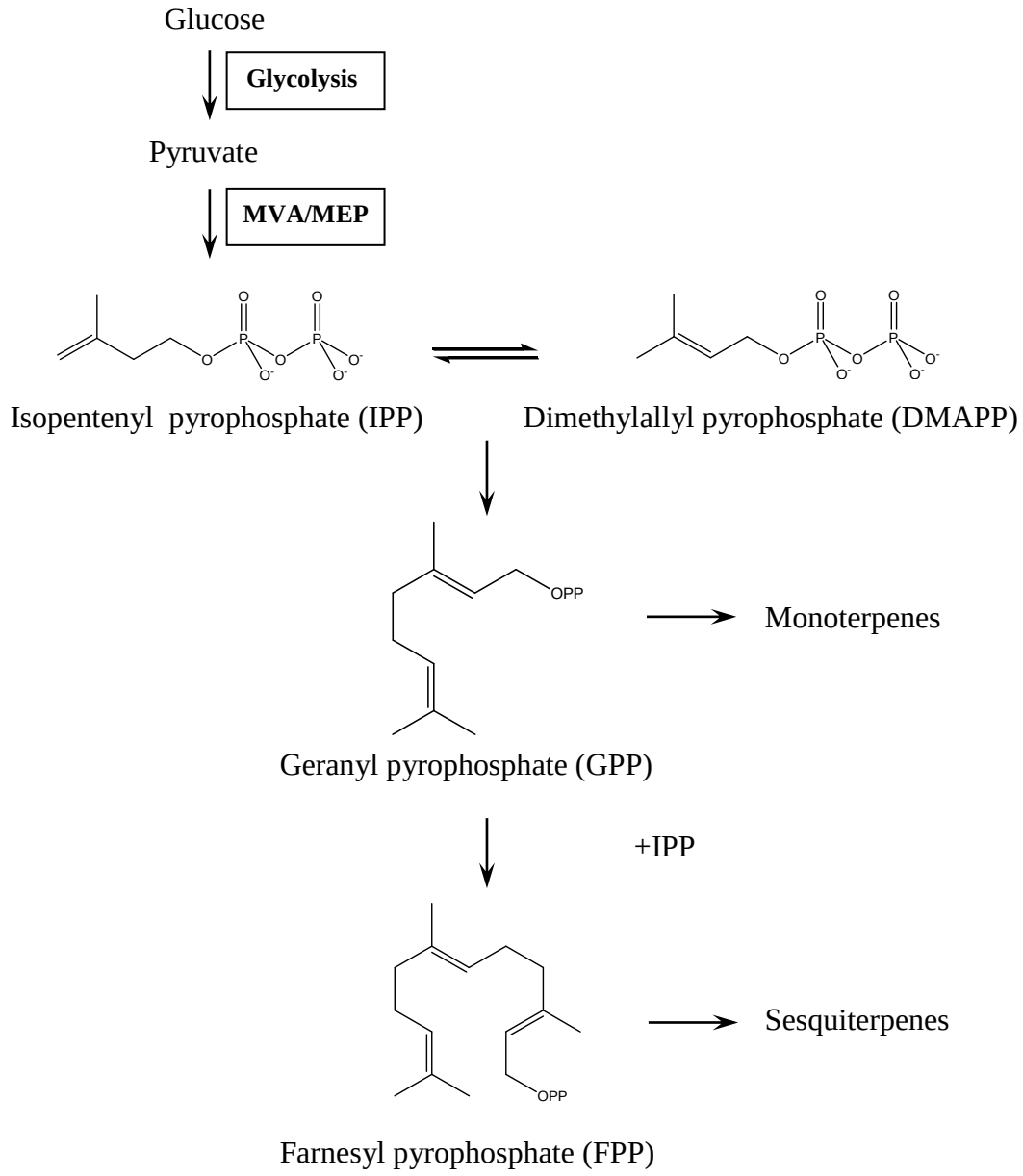

Figure 2.1: Biosynthetic pathways of terpenes (Carrau et al., 2005)

The important products generated by the action of enzymes known as prenyltransfereases include geranyl, farnesyl, geranylgeranyl pyrophosphates, squalene and phytoene, leading to the diverse formation of terpenoids (Humphrey and Beale, 2006). While geranyl pyrophosphate (GPP) plays the role as a precursor for monoterpenes as well as of a number of secondary plant metabolites, farnesyl

pyrophosphate (FPP), the product of FPP synthase, is the precursor to a wide variety of sesquiterpenes.

The mevalonic acid pathway (MVA) takes place in the cytosol. Organisms including higher eukaryotes and many bacteria produce terpenoids through MVA or HMG-CoA reductase pathway, the pathway that also produces cholesterol. Isopentenyl pyrophosphate (IPP) is formed from acetyl-CoA via mevalonic acid, which is then be isomerized to DMAPP by IPP isomerase. The mevalonic acid independent pathway is an alternative metabolic pathway discovered in the late 1980s that leads to the formation of IPP and DMAPP. Initially, pyruvate and glyceraldehyde 3-phosphate are condensed to 1-deoxy-D-xylulose 5-phosphate (DXP) by DOXP synthase. In the second step, DXP is rearranged and reduced by DOXP reductase yielding 2-C-methyl-D-erythritol 4-phosphate (MEP). MEP is subsequently converted into 4-diphosphocytidyl-2-C-methylerythritol, and a multiple steps ensuing will form IPP and DMAPP.

In an investigation work of mono- and sesquiterpene biosynthesis by baker's yeast (Carrau et al., 2005), monoterpenes α-terpineol and linalool were found to be the main products afforded by all the *S. cerevisiae* wine yeasts tested. Judging by the effect of nitrogen and redox conditions (microaerobic and anaerobic) on the terpenes production, it was suggested that leucine catabolism pathway (MCC pathway), known to occur in mitochondrion in eukaryotic cells, could have been accounted for the formation of the monoterpenoids, while the sesquiterpenoids followed the cytosolic sterol metabolic pathway already described in yeast (Carrau et al., 2005). In another work, both wild-type and farnesyl diphosphate synthase (FPPS)-defective *S. cerevisiae* mutant strains were also discovered in having the ability to produce monoterpene-derived compounds (Oswald et al., 2007).

2.1.1 Monoterpenoids

Monoterpenes hydrocarbons dominate in most essential hydrocarbons. They are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures. Monoterpenes consist of two isoprene units that belong to the C_{10} representatives of the terpenoid family of natural products, and found their usefulness in the perfumery and flavor industries. Monoterpenoids (monoterpene oxygen-containing derivatives) diverge from higher isoprenoid biosynthesis at the level of GPP. The vast majority of the several hundred naturally occurring monoterpenes are cyclic, and they represent a relatively small number of skeletal themes multiplied by a very large range of simple derivatives, positional isomers and stereochemical variants (Croteau, 1987).

It is well-known that geraniol and nerol are both geometric isomers (*trans* and *cis* isomers of 3,7-dimethyl-2,6-octadiene-1-ol, respectively) and belong to the most fundamental acyclic monoterpenoid alcohols. Their esters and aldehydes are also common. Figure 2.2 shows the chemical structures for both geraniol and nerol. Besides their use in food and flavor industry, these alcohols are also used for the synthesis of vitamins A and E (Mercier and Chabardes, 1994).

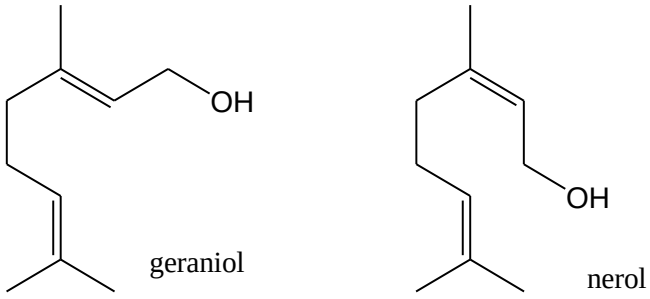

Figure 2.2: Chemical structures of geraniol and nerol (Surburg and Panten, 2006)

Geraniol

Geraniol is found widely as a chief constituent in essential oils including ilang-ilang oil, palmarosa oil, geranium oil, orange flower oil, lemongrass oil, hops oil, and lavender oil. A common usage of geraniol is found in perfumery and flavoring industries, where it is used for flavors such as peach, raspberry, red apple, orange, lemon etc. Geraniol is also a natural antioxidant. It has been shown that geraniol inhibited the proliferation of MCF-7 human breast cancer cells and rodent mammary tumor development (Duncan et al., 2004). On top of that, geraniol suppressed the growth and polyamine biosynthesis in human colon cancer in which it caused a 70% inhibition of cell growth and a 50% decrease of ornithine decarboxylase activity, a key enzyme of polyamine biosynthesis, which is enhanced in cancer growth (Carnesecchi et al., 2001). Burke and co-workers in their studies about the effects of isoprenoids on cancer of the pancreas reported that farnesol, geraniol, and perillyl alcohol suppressed pancreatic tumor growth without significantly affecting blood cholesterol levels (Burke et al., 1997).

Nerol

Nerol occurs in small quantities in many essential oils such as lemongrass, where it is always accompanied by geraniol. Nerol was originally isolated from neroli oil, and was later discovered in the oils of petitgrain, rose, Mexican lignaloes and *Helichrysum angustifolium*. Nerol possesses an agreeable, rose-like odor and reveals close similarity with geraniol in terms of its chemical behavior. Nerol undergoes the same reactions as geraniol, but cyclizes more readily in the presence of acids as illustrated in Figure 2.3 and is commonly used in perfumery and for bouquetting citrus flavors (Surburg and Panten, 2006).

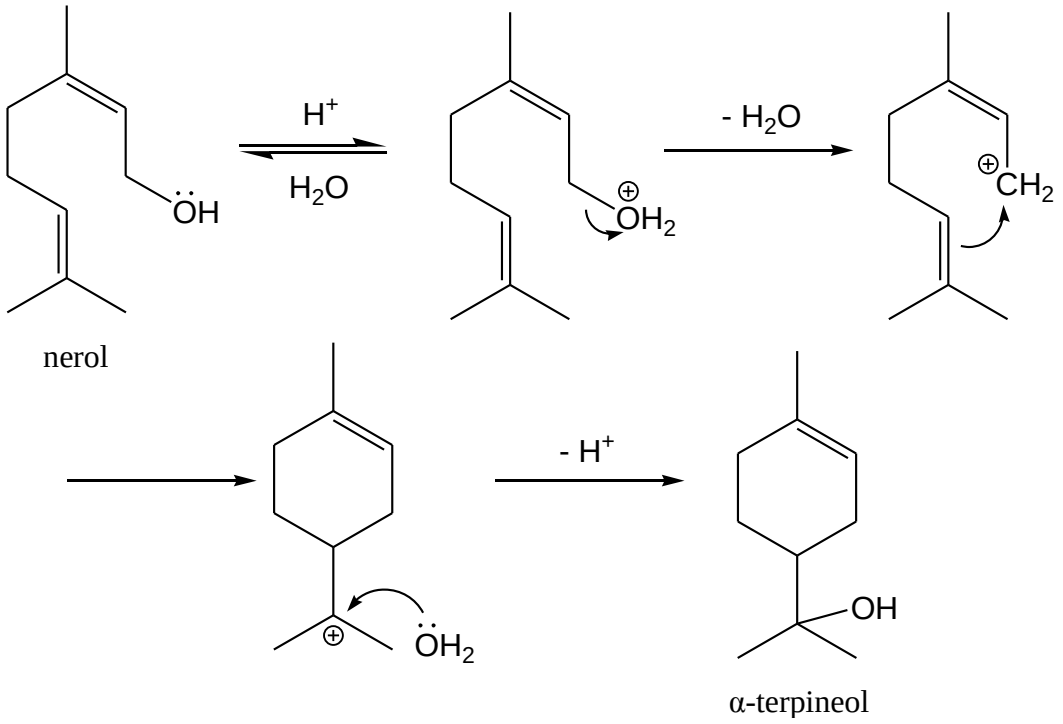

Figure 2.3: Cyclization of nerol in the presence of acid (Fogiel, 1978)

Citronellol

Citronellol occurs as a mixture of its two enantiomers; the pure (+) or (–) form is seldom found. Citronellol in the (–)-form is common in geranium and rose oils, both of which may contain up to 50% citronellols (Surburg and Panten, 2006) and has a finer rose odor than the (+)-enantiomer with a sweet, peach-like flavor. The United States FDA considers both substances GRAS (Generally Recognized as Safe for food use) (Weatherston, 2005). Figure 2.4 shows the chemical structures for both enantiomers of citronellol.

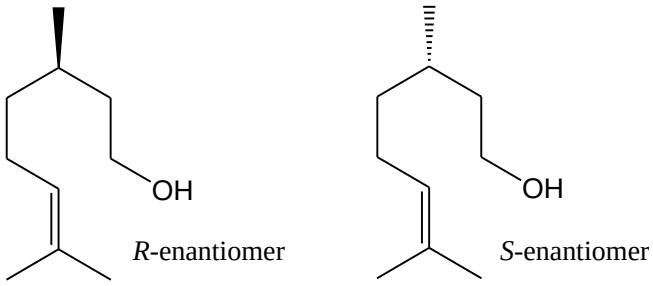

Figure 2.4: Chemical structures of *R*,*S*-citronellol (Surburg and Panten, 2006)

Citronellol undergoes the typical reactions of primary alcohols. Compared with geraniol, which contains one extra double bond, citronellol is relatively stable and can be converted to citronellal by dehydrogenation or oxidation (Surburg and Panten, 2006). Besides, citronellol has a long history as one of the most widely used fragrance materials in cosmetics, flavorings and fragrances particularly for rose notes and for floral compositions in general. As a flavor material, citronellol is added for bouquetting purposes to citrus compositions (Surburg and Panten, 2006). Citronellol also acts as a pesticide active ingredient that is used on food crops and ornamentals to attract mites, a major agricultural pest. Citronellol is usually used in tiny amounts and shows minimal to no toxicity in laboratory studies, hence, no harm is expected to humans or the environment if label instructions are followed (Weatherston, 2005).

Linalool

Linalool is a colorless liquid that can be found mainly in rosewood, freesia, lavender, coriander, basil, citrus leaves and linaloe. It is an allylic isomer of geraniol and nerol (Sell, 2003). Linalool occurs as one of its enantiomers in many essential oils. For example, (+)-linalool is a chief constituent of coriander. On the other hand, (–)-linalool can be found in Ho oils from *Cinnamomum camphora* (Surburg and Panten, 2006). Figure 2.5 illustrates the chemical structures of (±)-linalool.

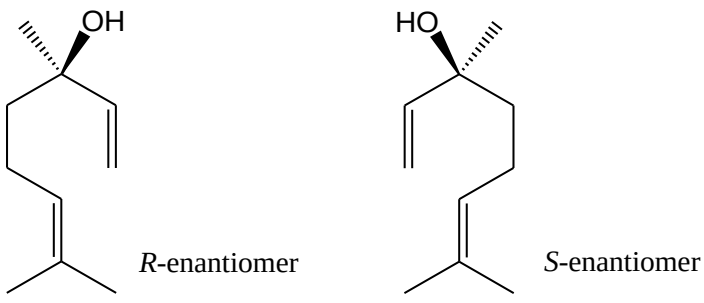

Figure 2.5: Chemical structures of *R,S*-linalool (Surburg and Panten, 2006)

Due to its fresh, floral odor, linalool is considered an important fragrance material for manufacturing scented products and cleaning agents. Besides, it has been shown that linalool and its corresponding acetate play a major role as potentially anti-inflammatory agents (Peana et al., 2002). Tests performed also shown that linalool provide pharmacological effects of antinociceptive/antihyperalgesic (reduction of pain sensitivity) (Peana and Moretti, 2009). Linalool possesses antioxidant properties which is vital for the prevention of lipid peroxidation. It can also be used in therapeutic approaches to limit the damage caused by oxidation of unsaturated fatty acids (Peana and Moretti, 2009). Linalool can also be treated as insecticidal compound, showing effectiveness against fleas, mites, houseflies etc. and hence useful for household pest control, fumigation of food handling and storage facilities (Olkowski et al., 1995).

α-Terpineol

Terpineol or α-terpineol, is one of the most prevalent monocyclic monoterpenoid alcohols in nature. (*R*)-(+)-α-terpineol has a floral, typically lilac odor, while (*S*)-(−)-α-terpineol that occurs in conifer and lavandin oils, carries a pine smell characteristic (Sell, 2003). Although α-terpineol is found in many essential oils, only small quantities are commercially produced. Its typical commercial application includes in the production of soaps, cosmetics and flavors which is mainly used as various flavor compositions such as berry, lemon, lime, nutmeg, orange, ginger etc (Tan and Day, 1998). On top of that, α-terpineol displayed antimicrobial activity against *Trichophyton mentagrophytes* (Park et al., 2009), *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Jirovetz et al., 2005). The structures of both enantiomers are illustrated in Figure 2.6.

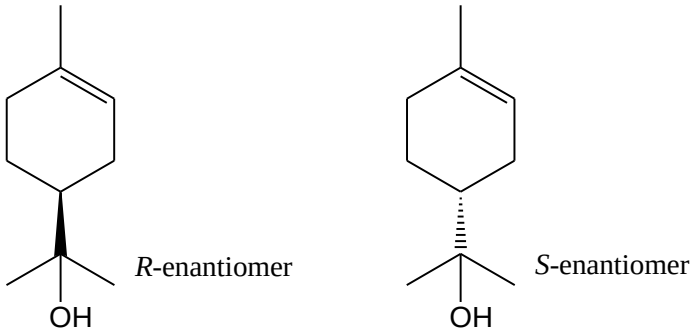

Figure 2.6: Chemical structures of *R*,*S*-α-terpineol (Surburg and Panten, 2006)

It is well-known that herbs and spices exhibit antimicrobial activity and the principle behind it is often attributed to the essential oil present in these spices. Many studies have been carried out and found that citronellol, geraniol and nerol shows antimicrobial properties (Arldoğan et al., 2002; Shin, 2003; Duarte et al., 2007). These substances may be useful in clinical situations, where infectious diseases caused by a large variety of pathogenic bacteria in humans can be controlled and treated. In addition to that, these monoterpenoids are found to be one of the active ingredients that produce effective natural plant-based insect repellents (Taylor and Schreck, 1985; Barnard and Xue, 2004; Gillij et al., 2008), which find their potentialities to prevent and control the outbreak of insect-borne diseases.

2.1.2 Production of Terpenoids

Traditionally, terpenoids can be acquired via physical separation from plant or animal sources, which include essential oils, extracts and distillates or from animal sources. These extraction methods are usually vacuum- or steam- distillation, expression, concentration and extraction with organic solvents (Berger et al., 2000; Margetts, 2005).

Nevertheless, active components within these sources are often present in small quantities or in bound form or are only found in exotic plants, making isolation difficult and the flavor products expensive (Janssens et al., 1992). Also, the stumbling blocks such as heat, chemical solvents, and problems associated with product contamination and degradation (Raisi et al., 2008) found in these techniques have rendered them less effective when aromatic compounds obtained from the raw materials tend to be distorted from the original character of the odor. In addition, large-scale productions are often inhibited by seasonal variation, and variability in the composition and the yield of the final product is rather difficult to control. Consequently, the quality and price of the given essential oil frequently fluctuates over the year (Berger et al., 2000).

Currently, advances in chemical processes have enabled these flavour compounds to be produced synthetically in satisfactory yields and high production rates. However, one of the drawbacks of chemical processes is the formation of undesirable racemic mixtures that could only be avoided through the use of chiral building blocks suitable for the total synthesis of the target molecule. However, these building blocks could hardly be obtained from readily available compounds or natural products (Watanabe et al., 2005). In contrast, biotechnological production provides a feasible alternative for the production of flavour compounds. The US and European legislations have acknowledged that enzymatic or microbial processes as capable of producing enantiospecific compounds labelled as 'natural', which has been advantageous for the production of terpenoids (Demyttenaere, 2001). Biotechnological production of terpenoids can be either via *de novo* syntheses (fermentation) using simple substrates like sugar and alcohol or through biotransformation of precursor compounds to flavour end-products.

2.2 Biotransformation

Biotransformation is a chemical modification process on an organic compound using microorganisms as biocatalyst. Usually the catalyst is an enzyme, or whole-cell microorganism containing an enzyme or several enzymes. Conversion from one material into another using whole living organisms is usually called "bioconversion" (Pandey et al., 2007). Biotransformation produced a wide range of products as advances in this technology have led to the increase in development of scientifically novel and commercially successful processes to manufacture specialty chemicals, especially flavors and fragrances. These processes often use novel enzyme systems and have acquired multidisciplinary research in their development (Cheetham, 1993).

Figure 2.7 shows the rapid increase on the total number of biotransformation processes that have been started on an industrial scale over the past years. Currently, biotransformation products are being produced at a scale from below 100 up to 10000 tons per annum and the number of biotransformation used at the industrial scale is doubling every decade, indicating the enormous biotechnological potential of the use of cells/enzymes as biocatalysts (Rehm, 2008).

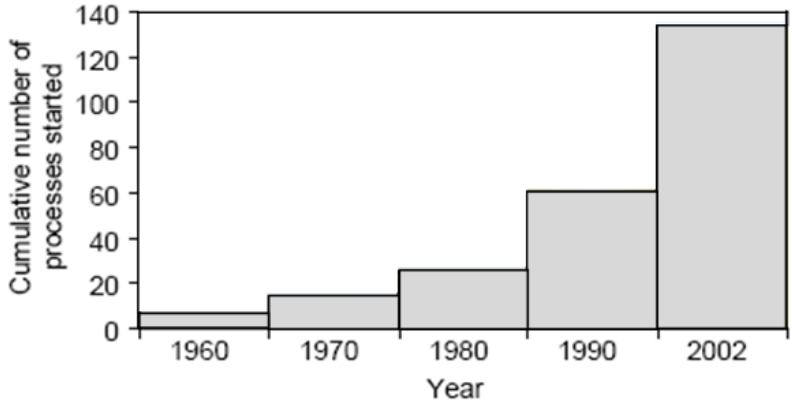

Figure 2.7: Cumulative number of biotransformation processes on an industrial scale (Straathof et al., 2002)

Generally, the flavor products obtained by microbial routes are optically pure, extra-cellular, and suitable for commercial exploitation because of easy downstream processing and high yields (Agrawal, 2004). Some of the high-value biotransformation products are chiral compounds serving as intermediates or precursors for the synthesis of pharmaceuticals. For example, many enzymes isolated from pseudomonads as well as some whole *Pseudomonas* cells are currently used as biocatalysts in industrial biotransformations (Rehm, 2008)

2.2.1 Biocatalysts versus Chemocatalysts

Biocatalysts refer to enzymes, cell organelles or whole-cells, in which the latter can be growing or non-growing, and viable or non-viable. They can be free or immobilized and of microbial, plant or animal origin. Microbial cells are genetically engineered both for the purpose of increasing the enzyme production and for use as biocatalysts (Averill et al., 1991). Due to high metabolic rates which promote the rapid microbial growth of microorganisms, attributed to their high surface to volume ratio, microbial cells are often considered as preferred choice for biotransformation, which in turn translates to a far more efficient and economical process. Furthermore, microbial cells are easier to grow in culture and possess effective cell wall structure that gives its high mechanical strength (Roberts et al., 1995).

The most important requirements for a catalyst in technical processes are selectivity, activity and stability. Most of chiral natural flavors occur in enantiomerically enriched form. Because different enantiomers or regioisomers provide different sensorial properties, their specific synthesis is much useful and favorable (Brenna et al., 2003). Biocatalysts are often significantly superior to

classical chemocatalysts due to their capability of catalyzing a large number of stereo- and regioselective chemical manipulations (Serra et al., 2005). The use of enzymes and several wild-type microorganisms in particular can prevent undesirable secondary reactions and side products (Carballeira et al., 2009a). Another important advantage of biocatalysts is that they can reduce or eliminate the use or generation of hazardous substances (Carballeira et al., 2009a) and are completely biodegradable (Serra et al., 2005), thus creating a more environmentally-friendly approach.

However, there are still some major drawbacks on the acceptance of biocatalysis on the whole over the last 20 years (Bommarius and Riebel, 2004). Limited number and availability of enzymes plus long development times of new biocatalysts owing to an insufficient knowledge base of biocatalysis and biotechnology remained a problem and a challenge. Some of the advantages and disadvantages of biocatalysts over chemical catalysts are listed in Table 2.2.

Table 2.2: Advantages and disadvantages of biocatalysts (Buchholz et al., 2005)

Advantages	Disadvantages
• Low temperatures required • Active at moderate pH • Stereo- and regioselective • Low energy consumption • Less by-products • Non-toxic when correctly used • Can be reused (immobilized) • Can be degraded biologically	• Unstable at high temperatures • Unstable at extreme pH values • Unstable in aggressive solvents • Inhibited by some metal ions • Hydrolyzed by peptidases • Some enzymes are still very costly • Require expensive co-substrates

Despite the shortcomings of biocatalysts, recombinant DNA technology and development of molecular biology have led to the discovery of many novel enzymes of improved catalytic performances (Riva, 2001). Alongside with process

development towards higher stability and volumetric productivity, the strengthening of biocatalytic approach in synthetic routes has eventually become more significant and dramatically advanced in recent years (Bommarius and Riebel, 2004).

Many literatures have reported the successful use of biocatalysts in several chemical processes (Zaks, 2001; Thomas et al., 2002) and a prominent example can be observed in the production of vitamin C. Vitamin C or *L*-ascorbic acid (*L*-AA) is an indispensable nutrient for human to carry out several physiological functions (Bremus et al., 2006). For the past 70 years, *L*-AA is being produced via Reichstein process, which involves a series of chemically based unit operations and a single biocatalytic step. However, there has been increasing pressures to develop a sustainable alternative process as the catalyst used involves environmentally hazardous chemicals (Hancock and Viola, 2002). Besides, process efficiency and economic factor give rise to huge interest to find other substitutes as this process is still the most energy consuming (Bremus et al., 2006). As a result, microbiological biotransformations using reasonable raw materials have been given due attention.

Several strategies have been followed in shifting the present *L*-AA synthesis methods from chemical to biocatalytic routes, by using different bacterial strains (Hancock and Viola, 2002). However, most of the known approaches do not involve direct *L*-AA synthesis, instead produce 2-keto-*L*-gulonic acid (2-KLGA) as a key intermediate that can be converted to *L*-AA via conventional chemical processing technology (Bremus et al., 2006). Therefore, the strategy for the improvement of 2-KLGA production is to generate recombinant strains using genetic engineering methods. Figure 2.8 shows the comparison of the *L*-ascorbic acid synthetic processes. The traditional Reichstein Process takes in all five chemical steps with one biocatalytic step, produced an overall yield of only 55% (Bommarius and Riebel,

2004), while the one-step fermentation (glucose fermentation) and two-step fermentation (sorbitol fermentation) processes to 2-KGLA by way of subsequent chemical steps into *L*-AA, provide better alternatives with fewer processing steps.

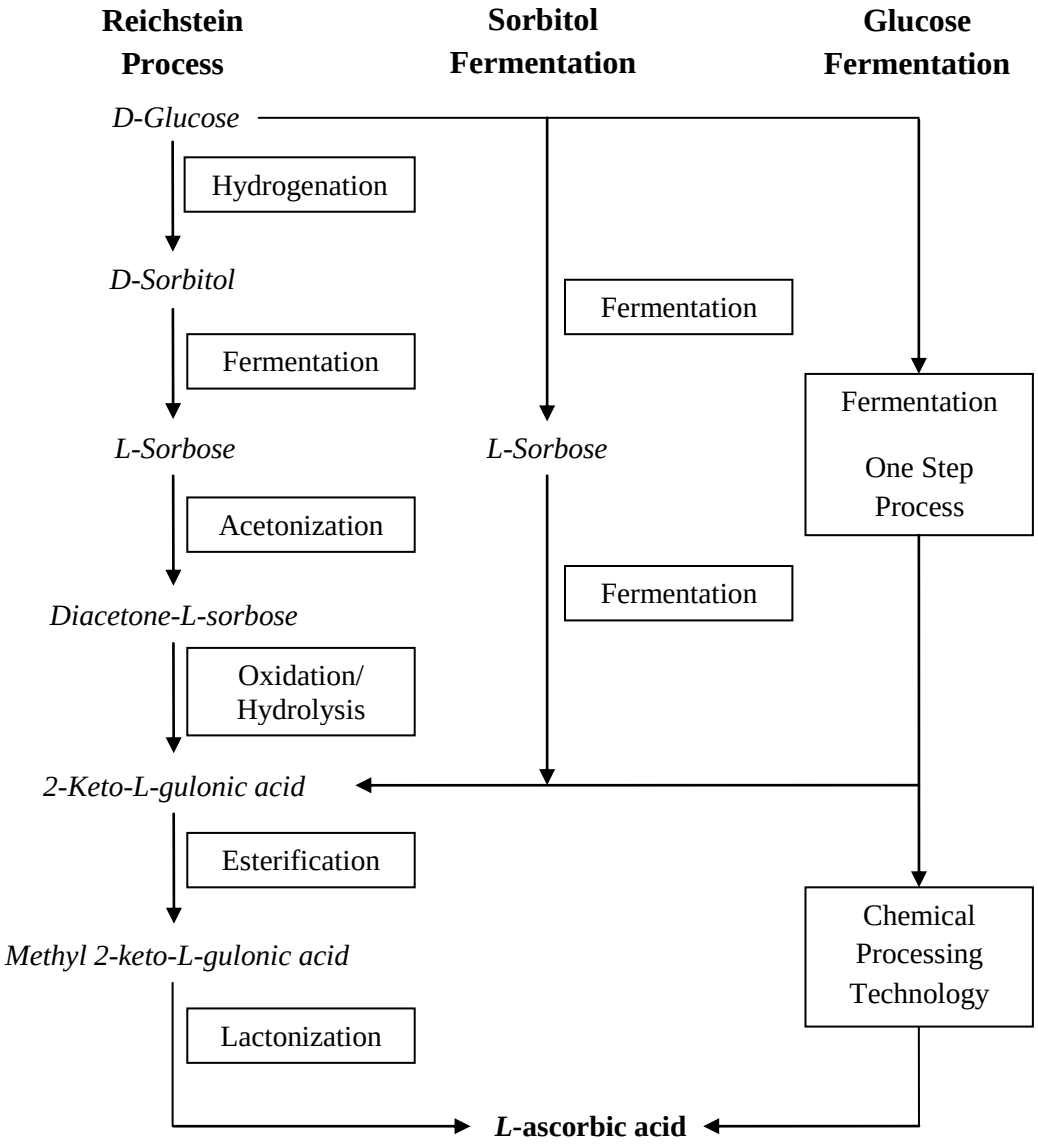

Figure 2.8: Comparison of processes for the synthesis of *L*-ascorbic acid (Bommarius and Riebel, 2004)

2.2.2 Whole-Cell versus Isolated Enzymes

Generally, biocatalysts can be divided into two main subgroups: whole-cells or their isolated enzymes. Currently, at least 134 biotransformation processes are

operating industrially with a mix of whole-cell and isolated enzyme catalysts (Straathof et al., 2002), and according to a poll conducted, 22 out of 38 large scale asymmetric syntheses involved enzymatic or whole-cell biotransformations (Yazbeck et al., 2004).

Enzyme-catalyzed reactions have been widely used in laboratory or industrial scale, whereas, whole-cell catalyzed reactions have been commonly restricted to special cases (Carballeira et al., 2009b). In choosing the best biotransformation system, factors to be taken into consideration in deciding whether an enzyme or whole-cells should be used include the availability, the number of steps involved, yield, cofactor requirement, byproduct formation, the required product purity, price, etc (Averill et al., 1991). Whole-cell biocatalysts can offer several advantages over the use of isolated enzymes in biocatalysis. They are a convenient and stable source of enzymes that are often synthesized by cells in response to the presence to the substrate (D'Arrigo et al., 2000). *S. cerevisiae* and *Penicillium* sp. are some of the inexpensive whole-cells that are usually available from fermentation processes.

Whole-cell can be obtained in alternative forms of growing cultures, resting cultures, spore cultures or immobilized cultures (Roberts et al., 1995). Growing cell cultures represent the simplest biotransformation procedures whereby in a batch-grown culture, substrate will be added to the medium, followed by incubation that will be carried out until all of the added substrate disappears and/or the bioconversion ceases. At fixed intervals, a fresh sample of the growth medium will be drawn out to monitor the extent of substrate disappearance and product formation. Resting cells are non-growing live cells obtained by removing growing cells at a time in the growth phase when the potential of the cells to take on the desired biotransformation is optimal. The cells are then resuspended in a weak compatible