ON THE WHOLE-CELL CELL *SACCHAROMYCES CEREVISIAE* BIOTRANSFORMATION OF KETOISOPHORONE AT DIFFERENT SUBSTRATE CONCENTRATIONS

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by

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LIST OF SYMBOLS

Symbol	Description	Unit
C	Concentration	g/L
M	Molarity	M
pH	Potential of hydrogen	-
ΔT	Rate of change of temperature	$^{o}C/min$
T	Temperature	^{o}C
T	Time	Hr
L	Volume	L
λ	Wavelength	nm

LIST OF ABBREVIATIONS

(6R)-levodione	2,2,6-trimethylcyclohexane-1,4-dione
(4R,6R)-actinol	(R)-hydroxy-2,2,6-trimethylcycloheanone
(4S)-phorenol	4-hydroxy-2,6,6-trimethylcyclohex-2-enone
ACT	Actinol
ADHs	Alcohol dehydrogenases
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C=C	Carbon-carbon double bonds
C=O	Carbon-oxygen conjugated double bonds
CO_2	Carbon dioxide
DOIP	6R-dihydro-oxoisophorone
ERs	Enoate reductases
FID	Flame ionization detector
FMN	Flavin mono-nucleotide
GC	Gas chromatography
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KIP	2,6,6,-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone)
NADH	Nicotinamide adenine dinucleotide
NAD^+	Reduced form of nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

$NADP^+$	Reduced form of nicotinamide adenine dinucleotide phosphate
OYE	Old yellow enzymes
OIP	4-oxoisophorone
Tris-HCL	Tris-hydrochloric acid
UV-vis spectrometer	Ultraviolet visible spectrometer

BIOTRANSFORMASI KETOISOPHORON DENGAN MENGGUNAKAN KESELURUHAN *SACCHAROMYCES CEREVISIAE* PADA KEPEKATAN SUBSTRAT YANG BERBEZA

ABSTRAK

Jenis ragi Baker II telah digunakan dalam kajian ini sebagai biopemangkin untuk mengkaji penurunan 2, 6, 6-trimethylcyclohex - 2-ene-1,4-dione (ketoisophorone) kepada perantara kiral yang berguna dan meghasilkan produk yang bernilai tinggi iaitu (4R, 6R) -4-hidroksi-2,6,6-trimethylcyclohexanone atau nama ringkasnya (4R, 6R) -actinol. Lima substrat ketoisophorone yang berbeza telah diperkenalkan pada kultur yang berbeza untuk menyiasat kesan regenerasi kofaktor dan kursus masa ketoisophorone untuk menghasilkan perantara yang sepadan dengan 2,6,6-trimethylcyclohexane-1,4-dione [(6R) - levodione] dan 4-hydroxy-2,6,6-trimethylcyclohex-2-ene-1-one [(4S) -phorenol] dan produk utama (R)-hydroxy-2,2,6-trimethylcycloheanone [(4R, 6R) -actinol] di seluruh sel *Saccharomyces cerevisiae*. Ketersediaan dan kestabilan kofaktor telah dikaji dengan menggunakan spekta penyerapan ultraviolet-yang boleh dilihat. dan didapati bahawa pada 2.0 g / L konsentrasi substrat ketersediaan kofaktor adalah yang paling rendah disebabkan pembekuan nutrien glukosa. Tiada kesan kepekatan substrat terhadap perencatan sel dari 0.2 g / L hingga 2.0 g / L. Kromatografi gas digunakan untuk menganalisis substrat, perantara; (6R) -levodione dan (4S) -phorenol dan produk utama (4R, 6R) -actinol. Kepekatan (6R) -levodione mempunyai kepekatan yang lebih tinggi berbanding dengan (4S) -phorenol kerana persaingan koenzim dan kadar pengurangan bon karbon-karbon yang lebih tinggi berbanding dengan kadar tindak balas pengurangan karbon.

ON THE WHOLE-CELL *SACCHAROMYCES CEREVISIAE* BIOTRANSFORMATION OF KETOISOPHORONE AT DIFFERENT SUBSTRATE CONCENTRATIONS

ABSTRACT

Baker's yeast type II has been utilized in this study as a biocatalyst to investigate the reduction of 2, 6, 6-trimethylcyclohex--2-ENE-1,4-Dione (ketoisophorone) into a useful chiral intermediates as well as for its valuable product that is (4R,6R) -4-hydroxy-2,6,6-trimethylcyclohexanone or in short (4R, 6R) -actinol. Five different substrate of ketoisophorone has been introduced at different culture in order to investigate the effect of the cofactor regeneration and the time courses of ketoisophorone to produce corresponding intermediates of 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione] and 4-hydroxy-2,6,6-trimethylcyclohex-2-ene-1-one [(4S)-phorenol] and the main product (R)-hydroxy-2,2,6-trimethylcycloheanone [(4R,6R)-actinol] on the whole- cell *Saccharomyces cerevisiae*. The cofactor availability and stability has been investigated by using ultraviolet-visible and it was found that at 2.0 g/L of substrate concentration the cofactor availability is the lowest as the nutrient-limiting of glucose. There is no effect of substrate concentration towards cell inhibition from 0.2 g/L to 2.0 g/L. Gas chromatography was used to analyse the substrate, intermediates; (6R)-levodione and (4S)-phorenol and main product (4R, 6R)-actinol. The concentration of (6R)-levodione has higher concentration compared to (4S)-phorenol due to the competition of coenzymes and higher rate of carbon-carbon double bond reduction compared to the reaction rate of carbonyl reduction.

CHAPTER ONE

INTRODUCTION

1.1 Research Background

1.1.1 Biotechnology

Biotechnology can be defined as the use of biological processes, system, or organism by manufacture products intended to improve our daily lives. Over century ago, biological processes of microorganism have been used to make good products such as bread and cheese, and to preserve dairy products. Biotechnology has application in four main industrial areas, namely in health care (medical), agriculture and crop production, food processing and environmental. These broad application can be classified and organized based on colour coded classification in promoting public perception and understanding of biotech applications for the cause of science, development, and the current and post human future of humankind. Table 1.1 shows the area of biotechnology activities based on the colour codes.

Red biotechnology implies the uses of biotechnology in medicine. Red biotechnology includes producing vaccines and antibiotics, developing new drugs, molecular diagnostics techniques, regenerative therapies and the development of genetic engineering to cure diseases through genetic manipulation (DaSilva, 2012). White biotechnology related to industrial process that employ microorganism in chemical production. This technology is gene-based technologies that used to generate efficient production strains.

Table 1.1: Colours of biotechnology (DaSilva, 2012).

Colour classification	Area of Biotechnology Activities
Red	Health, Medical, Diagnostics
Yellow	Food Biotechnology, Nutrition Science
Blue	Aquaculture, Coastal and Marine Biotech
Green	Agricultural, Environmental Biotechnology, Biofuels, Biofertilizers, Bioremediation, Geomicrobiology
Brown	Arid Zone and Desert Biotechnology
Dark	Bioterrorism, Biowarfare, Biocrimes, Anticrop warfare
Purple	Patents, Publications, Inventions, IPRs
White	Gene-based Bioindustries
Gold	Bioinformatics, Nanobiotechnology
Grey	Classical Fermentation and Bioprocess Technology

Green biotechnology is commonly focused on agriculture as working field. Green biotechnological approaches and applications include creating new plant varieties of agricultural interest, producing biofertilizers and biopesticides, using in vitro cultivation and cloning plants. The aims of green technology are to get varieties resistant to pests and diseases, developing varieties with improved nutritional properties (e.g., higher content of vitamins) with the use of transgenic plants, developing plant varieties able to act as bio-factories and produce substances of medical, biomedical or industrial interest in quantities easy to be isolated and purified

of transgenesis in plants (DaSilva, 2012). Ulrich and Franskenberg-Dinkel (2015) stated that as for blue, yellow, grey, black and even gold biotechnology leads this other major technologies due to constant technological advancement.

1.1.2 Biocatalysis

Biocatalysis can be defined as the use of enzymes or whole cells as biocatalysts for industrial synthetic chemistry. They has been implemented in the past several decades in many biocatalytic processes to produce a wide variety of products in various industries (Johannes et al., 2006). For example, they have been used for centuries and developed in several fields as genetics, molecular biology, fermentation biotechnology, biotransformation, nanotechnology, material sciences, advanced spectroscopy and others (Illanes et al., 2012).

Forti et al. (2015) reported that biocatalytic processes are increasingly used for replacing conventional chemical process and to make possible the formation of new products. The use of biocatalysis can be justify as the high specificity and selectivity, and the possibility to use "environmental-friendly" conditions (e.g., solvent-free approaches, low working temperatures and pressures, and waste reduction) represent, undoubtedly, the most attractive ones. Besides that, biocatalysis can sometimes allow reduction of the number of steps in a synthetic route (Matsuda et al., 2009; Bastos Borges et al., 2009). Nevertheless, the use of enzymes in organic synthesis has been difficult to adopt in industrial scale and has several disadvantage. The disadvantages of this enzyme technology are high cost, instability and poor performance under reactor conditions, narrow substrate specificity and requirement of complex cofactors (Berenguer-Murcia and Fernandez-Lafuente, 2010). Table 1.2 below indicates the advantages and disadvantages of biocatalysis in comparison with chemical catalysis.

Table 1.2: Advantages and disadvantages of biocatalysis in comparison with chemical catalysis (Faber, 1997).

Advantages	Disadvantages
Generally more efficient (lower concentration of enzyme needed)	Susceptible to substrate or product inhibition
Can be modified to increase selectivity, stability, and activity	Solvent usually water (high boiling point and heat of vaporization)
More selective (types of selectivity: chemo-selectivity, regio-selectivity, diastereo-selectivity, and enantio-selectivity	Enzymes found in nature in only one enantiomeric form
Milder reaction conditions (typically in a pH range of 5–8 and temperature range of 20–40 ^{o}C)	Limiting operating region (enzymes typically denatured at high temperature and pH)
Environment friendly (completely degraded in the environment)	Enzymes can cause allergic reactions

1.1.3 Yeast

Yeast are single-celled microorganism, eukaryotic that classified as fungus kingdom. Yeast are known for their biotechnology applications and mainly used for the production of beverages, cereal-based food, enzymes, fine chemicals, single-cell protein, and flavouring compounds (Ejik and Johannes, 1995; Gatto and Torriani, 2004). Yeasts can act as biocatalyst in the form of either enzyme or whole-cell and are very important sources of biotechnology relevant to enoate reductase (Raimondi et al.,

2010). In addition, yeast can be exploited as source of new OYE genes (Raimondi et al., 2011). Types of yeast comprise *Saccharomyces. rouxii (Zygosaccharomyces rouxii), Saccharomyves. delbrueckii (Saccharomyces unisporus, Torulaspora delbrueckii), Saccharomyces willianus, Zygosaccharomyces bailii* and *Candida. tropicalis*. *Sacchromyces cerevisiae* reproduces asymmetrically by budding off new daughter cells which is genetically identical with their parent cells (Sinclair et al., 1998; Arthur and Watson, 1976). Baker's yeast is commonly made by strains belonging to *S. cerevisiae* and mainly used in biotransformation for producing flavours and fragrance.

1.1.4 Biotransformation

Biotransformation is defined as the use of biological systems (organism /enzymes system) to produce chemical changes on compounds that are not their natural substrates (Pimentel et al., 2011). This mechanism has been developed by microbes to acclimatize to environmental changes and it is useful in a wide range of biotechnological processes (Cresnar and Petric, 2011). The most significant aspect of biotransformation is that it maintains the original carbon skeleton after obtaining the products (Bianchini et al., 2015). There are two types of biotransformation namely enzymatic and nonenzymatic. Enzymatic biotransformation are further divided into microsomal and non-microsomal (Smitha et al., 2017). Biotransformation occurred due to various enzymes present in the body is the enzymatic elimination. Meanwhile microsomal biotransformation is caused by enzymes present within the lipophilic membranes of smooth endoplasmic reticulum (Jones and Fawcett, 1996). Non-microsomal biotransformation involves the enzymes which are present within the mitochondria (Smitha et al., 2017). For instant, alcohol dehydrogenase responsible for

metabolism of ethanol into acetaldehyde and tyrosine hydrolases enzymes, xanthine oxidase converting hypoxanthine into xanthine and others (Smitha et al., 2017).

1.2 Problem Statement

Flavour and fragrance playing an important role for the food and beverages, feed, cosmetic and pharmaceutical industry. There are many flavours compounds available and produces via synthesis or extraction. However, flavours and fragrances belong to many different structural classes that is challenging target for academic and industrial research. Ketoisophorone (KIP) or 2,6,6-trimethylcyclo-hex-2-ene-1,4-dione is one of the flavour and fragrance agent used mostly in food and cosmetic industries as it has unusual and rare odour and taste. The reduction of KIP has its valuable and interesting through biotransformation as it was first analyse decades ago and only recently the reduction mechanism of KIP is about fully understood (Leuenberger et al., 1976b; Yamazaki et al., 1988)

S. cerevisiae is used in this study as a whole cell to catalyse carbon-carbon double bonds and carbonyl compound for biotransformation of KIP. This study primarily focuses on the stationary phase of cell in the phosphate buffer of *S. cerevisiae* at different substrate concentration. Buque-Taboada et al. (2005) stated that existence of carbonyl reductase severally limits the chemoselectivity by producing side-products and binder product yields. Hence, five different substrate concentrations of KIP is injected into sample after inoculation in order to identify the substrate, intermediate and main product of the biotransformation.

During the biotransformation, cofactor (NADH/NADPH) availability and stability need to be observe as the presence of cofactor has been proven to assist the product formation. It keeps a continuous flow of a hydrogen ions to and from the

reduction system, while the amount of enzymes present determine the rate of both intermediate and product formed during the biotransformation. Thus, the prepared sample must be measured from time to time in order to identify the rate of biotransformation of substrate and the availability of the cofactor present.

1.3 Research Objectives

The objectives for this research,

i) To study the effect of substrate concentration towards cell inhibition.

ii) To investigate the time course of biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone) to produce corresponding intermediates of 2,6,6-trimethylcycohexane-1,4-dione [(6R)-levodione] and 4-hydroxy-2,6,6-trimethylcyclohex-2-ene-1-one [(4S)-phorenol] as well as desired product (R)-hydroxy-2,6,6-trimethylcyclohexane [(4R, 6R)-actinol] on a whole-cell *S. cerevisiae.*

iii) To determine the availability of cofactor of stationary phase biotransformation at different substrate concentrations.

1.4 Scope of Study

In this study, the biotransformation of ketoisophorone has been experimentally on whole-cell Baker's yeast type II (*S. cerevisiae*). Baker's yeast was utilized as biocatalyst because of it is remarkably easy to maintain in laboratory and has ability to reduce a variety of substrates into many optically active compounds. The reduction or transformation of ketoisophorone is based on two enzymes naturally produced by *S. cerevisiae* which are enoate reductase and carbonyl reductase. Enoate reductase from *S. cerevisiae* also known as Old Yellow Enzyme (OYE) catalyse the reduction

of carbon-carbon double bonds, while carbonyl reductase or alcohol dehydrogenase (ADHs) is the constituent enzyme in the reduction of aldehyde and ketone to alcohol (Raj et al., 2014). *S. cerevisiae* is competent to transform 2,6,6-trimethylecyclohex-2-ene-1,4-dione (ketoisophorone) to 2,2,6-trimetyhlcyclohexane-1,4-dione or (6R)-levodione or [6R]-levodione as the intermediates in phosphate buffer to (**S**)-4-hydroxy-2,6,6-trimetylcyclohex-2-enone and to final product (R)-4-hydroxy-2,2,6-trimethylcyclohexanone or in short (4R,6R)-actinol simultaneously with the help of ketoisophorone reductase and levodione reductase. The phosphate buffer with the existence of glucose acts as a medium. The medium is kept in the incubator for at least 16 h at 37 ^{o}C with the shaking speed of 150 rpm. The top layer (organic phase) resulted from the mixture of sample and ethyl acetate after being vortexed and centrifuged was then being analyse using gas chromatography to analyse the substrate, intermediate and the product of biotransformation. Sonication was conducted in this experiment to break the cell debris after the cell pellet was suspended in the Tris-HCL buffer. The sample was then being carried out in UV-vis to determine the ability of the cofactor.

CHAPTER TWO

LITERATURE REVIEW

2.1 Choices of Yeast

Bhattacharya et al. (2013) suggested that yeast are known for their biotechnology application and thus interest in searching and characterizing new species has increased over the years. Based on Walker (2009) around 1000 species of yeast have been described, but new species are being characterized on a regular basis and there is considerable untapped yeast biodiversity on earth. Yeast taxonomy can be summarize as indicates in Table 2.1 with *S. cerevisiae* as an example due to the most commercially exploited yeast species (Walker, 2009).

Table 2.1: Taxonomic hierarchy of yeast (Walker, 2009).

Taxonomic category	Example (*Saccharomyces cerevisiae*)
Kingdom	Fungi
Division	Ascomycota
Subdivision	Ascomycotina
Class	Hemiascomycete
Order	Endomycetales
Family	Saccharomycetacae
Subfamily	Saccharomyetoideae
Genus	Saccharomyces
Species	cerevisiae

Various types of yeast has been used and experimented for biotransformation of ketoisophorone namely *Aspergillus niger, Saccharomyces cerevisiae*

Saccharomyces rouxii, *Saccharomyces delbrueckii*, *Saccharomyces willianus*, *Zygosaccharomyces bailii*, *Candida tropicalis*, to catalyze OIP reduction (Fukuoka et al., 2011; Yamazaki at al., 1988). Leuenberger et al. (1976a) and Buque-Taboada et al. (2004) mentioned that using baker's yeast (*S. cerevisiae*) as catalyst gave a comparable yield of 80-85% DOIP and a very attractive enantiomer excess of e.e. ≥ 98%. In addition, the application of *S. cerevisiae* as a whole-cell has shown a substantial productivity, particularly on the product's regio- and stereoselectivity as compared to other organism such as *Thermomonospora curvata* and *Bacillus stearothermophilus* (Yamazaki et al., 1988; Hori et al., 1984; Khor and Uzir, 2011; Nishii et al., 1990). In spite of the fact that baker's yeast also degrades the product to an unwanted by-product known as (4S-6R)-Actinol or S-ACT as depicts in Figure 1, this process has been regarded as the best option for industrial application (Leuenberger, 1985).

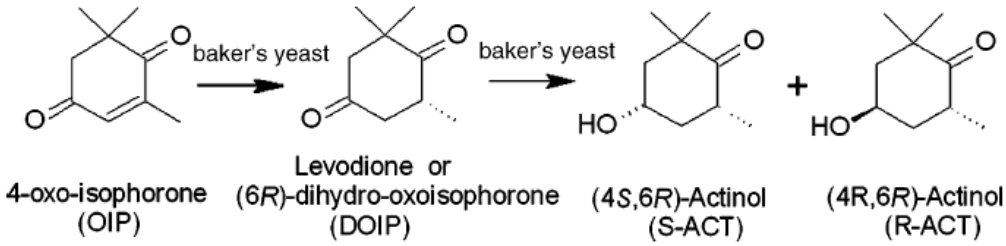

Figure 2.1: Model reaction: OIP reduction to DOIP's subsequent reduction to actinol (Buque-Taboada, 2005).

2.1.1 *Saccharomyces cerevisiae*

S. cerevisiae is a model yeast that commonly known as baker's yeast and brewer's yeast (Ernest, 2002). This yeast species is traditionally used for producing breed, beer, wine and some ethnic fermented foods and beverages mostly in Asia, Africa and South Africa (Walker, 1998). *S. cerevisiae* is commonly applied to whole-cell biocatalysts in biotransformation

(Rehm et al., 1993). The best physiological state of cells must be considered when the whole-cells are employed as biocatalyst.

Whole-cells are often preferable than enzyme as biocatalyst because they are more convenient and stable sources than purified enzymes, with no need for costly purification of coenzyme addition (Forti et al., 2015). The number of enzyme is still quite limited even it is commercially available in market (free or immobilize) and the fact it is considered as powerful tools. Albuquerque et al. (2007) reported that whole-cells biocatalyst are able to work under mild condition, such as room temperature and atmospheric pressure, minimizing problems of isomerization, racemization, epimerization and rearrangement that may occur during traditional chemical catalysis. Hence, biocatalytic asymmetric synthesis attracts considerable attention due to its simple, cheap and benign methodologies that combines green chemistry with high efficiency (Faber & Patel, 2000; Roberts et al., 1995)

Yeasts has widely utilized as biocatalyst because they are easy to handle, broad substrate acceptability and production of enzymes belonging to different classes (Bialecka-Florjanczyk and Kapturowska, 2012). Moreover, baker's yeast is economical source of enzymes because it can simply grow in open jar without sterilization. Rehm et al. (1993) also stated that yeast has high reducing capacity and hence addition of cofactor is not required.

Despite the benefits of yeast, yeast has major drawback when operated at high temperature. Besides that, yeast can inactivate in freezing temperature, grow slowly at cold temperature and steadily at moderate temperature (mild condition) at 24 ^{o}C and without limit at 38 ^{o}C (Moyad, 2007).

Biocatalyst are increasingly synthesis used during synthesis of enantiomerically pure compounds of industrial interest (pharmaceutical, food and crop

protection), due to their regioselectivity and stereoselecttivity (Chaparro-Riggers et al., 2007). *S. cerevisiae* is used as stereospecific bioreduction biocatalyst as it can catalyse compounds with carbonyl groups or carbon-carbon bonds (Khor and Uzir, 2011). In addition, Shimizu et al. (1998), Rodrigues and Moran (2001) and Pereira (1998) mentioned that yeast reductase have been employed to prepare chiral alcohols from prochiral ketone reduction, and *S. cerevisiae* has been extensively used as biocatalyst for that purpose.

There are two important enzymes that responsible for catalytic activity in the reduction of carbonyl group and carbon-carbon double bond (C=C). Carbonyl reductase or known as alcohol dehydrogenase (ADH) that mediates the reduction of aldehyde and ketone to alcohol, while enoate reductase is responsible to catalyse the reduction of the C=C. These two enzyme are stereoselectivity reduced ketoisophorone. Figure 2.2 illustrate the possible routes of ketoisophorone biotransformation on *S. cerevisiae* (Uzir and Najimudin, 2018). The presence of compounds **(3)** and **(4)** as in the figure were not observed during the course of biotransformation on Baker's yeast. The 'X' mark on Figure 2.2 indicates that there are no presence of the compound or the route of ketoisophorone based on the two enzymes.

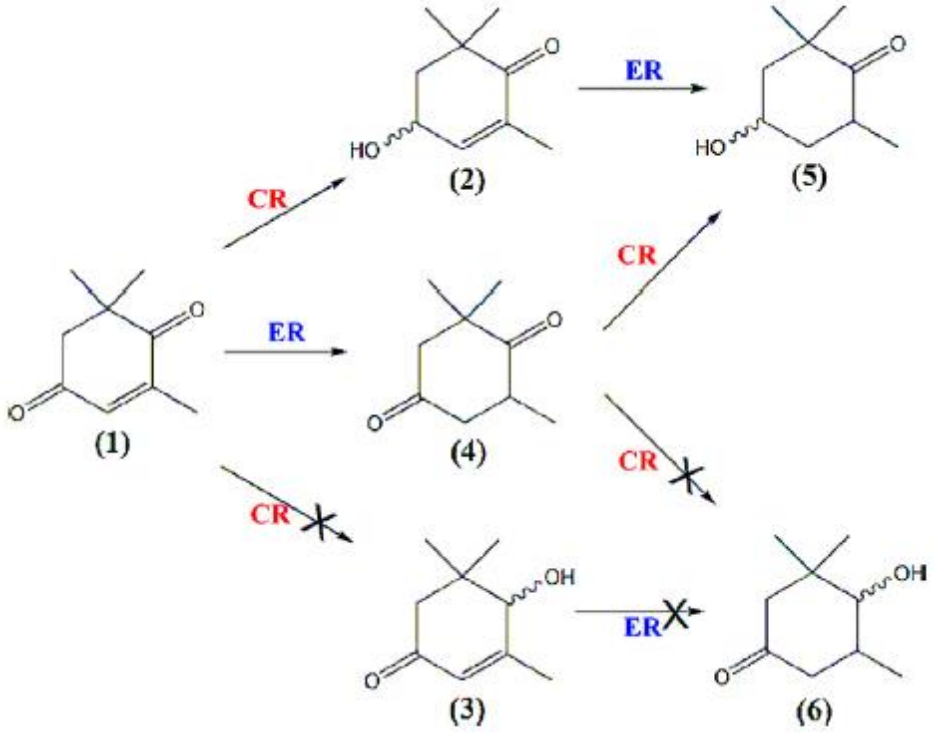

Figure 2.2: Possible routes of ketoisophorone reduction based on two enzymes naturally produced by itself (Uzir and Najimudin, 2018). Note that **ER** represent enoate reductase, **CR** refers to carbonyl reductase, KIP **(1)**, (4S)-phorenol **(2)**, 4-hydroxy-3,5,5-trimethylcyclohex-2-ene-1-one **(3)**,(6R)-levodione **(4)**, (4R, 6R)-actinol **(5)**, and 4-hydroxy-3,5,5-trimethylcyclohexanone **(6)**.

2.2 Enzyme Mediating the Reaction

2.2.1 Enoate reductases (ERs)

In 1993, enoate reductases (ERs) from *S. cerevisiae*, *carlsbergensis,* also widely notable as 'Old Yellow Enzyme' (OYE), was the first isolated flavouring containing enzyme (Warburg and Christian, 1933). Reductases or dehydrogenases can be utilized for the stereocontrolled reduction of C=C bonds and also can catalyse the chemo- and stereoselective hydrogenation of electron poor alkenes (Raimondi et al., 2011). Enoate reductase can be employed in preparation of enantiomerically pure aldehydes, ketones,

and ester as (ERs) selectivity reduce carbon-carbon double bonds in α,β-unsaturated carbonyl compounds (Chaparro-Riggers et al., 2007). Hence, this bioreduction can repercussion in important chiral synthons towards synthesis of fragrances, chemicals and pharmaceuticals (Toogood et al., 2010).

Stuermer et al. (2007) reported that OYEs has the biotechnology potential on the ability to generate up to two diverse stereocenters by the stereoselective trans-hydrogenation of the C=C, which is highly demanded in asymmetric synthesis but hardly to perform with conventional methods. Other than α,β-unsaturated carbonyl compound, conjugated enals, enones, imides, nitroalkenes and yiones are substrate that OYEs can reduce (William and Bruce, 2002; Struermer et al. 2007).

In addition, OYEs are flavin mono-nucleotide (FMN) containing NADPH-dependent oxidoreductases (Quertinmont and Lutz, 2016). For example, in OYE1p enzymes, the reduced cofactor FMN mediates a net anti-hydrogenation of the olefinic bond, acting as a transitory reservoir electrons that are transferred from NAD(P)H to the substrate (Kohli and Massey, 1998). Bi Ping Pong mechanism is applied in this reaction and both molecules use the same binding. β-NADPH is most favour in reduction of OYEs but OYEs also accepting α-NAPDH and NADH as cofactors because OYEs are loosely specific (Raimondi et al., 2011).

Whole-cell biocatalyst is partially one of a way to overcome the issue of regeneration of reducing cofactor OYE enzymes due to the presence of competing carbonyl reductases (CRs). Buque-Taboada et al. (2005) mentioned that existence of (CRs) severally limits the chemoselectivity by producing side-products and binder product yields. Moreover, OYEs often possess identical stereoselectivity and generate the same enantiomer, and this resulting in extreme limit to their application (Jäckel et al., 2008).

2.2.2 Carbonyl reductase (CRs)

Carbonyl reductase (CRs) also known as alcohol dehydrogenase (ADH) are enzymes accountable to catalyze the reversible reduction of aldehydes or ketones to corresponding primary or secondary alcohols (Loderer et al., 2018). For the synthesis various chiral compounds, ADHs have been employed in biocatalysis by asymmetric reduction (Matsuda et al., 2009). ADHs were applied as purified enzyme, enzyme immobilizes or whole cell biocatalyst (Loderer et al., 2018).

Baker's yeast is often used for the reduction of aldehydes and ketones as a whole-cell biocatalyst. For instant, *S. cerevisiae* is used in fermentation to produce ethanol. The energy metabolism of *S. cerevisiae* involved two major pathways namely glycolysis and aerobic respiration. During aerobic respiration, ADHs catalyze the interconversion of acetaldehyde and ethanol (De Smidt et al., 2008).

2.3 Cofactor

Cofactor can be defined as a non-protein chemical that assists with a biological chemical reaction and commonly required for enzymes or enzymes complex or increases the rate of catalysis. Besides that, cofactors acts as stoichiometric in biotransformation reactions and undergo chemical reactions with substrates (Liu and Wang, 2007). Biotransformation of KIP or OIP involved two important cofactors that are NADPH-dependent and NADH-linked oxidoreductase in baker's yeast (Buque-Taboada et al., 2005). Figure 2.3 depicts the cofactor that coupled the ketoisophorone. (Uzir and Najimudin, 2018) mentioned that compounds X_{Red} and X_{Ox} represented in the figure are the reduced and oxidized formed of X respectively, that occur during oxidation.

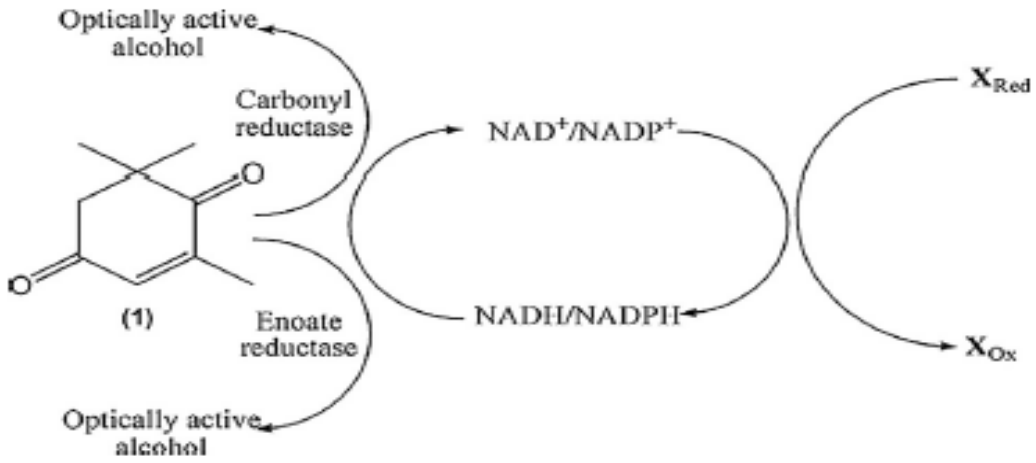

Figure 2.3: The use of cofactor as hydrogen ion transfers to both reductases-mediated reduction. Compounds X_{Red} and X_{Ox} represent the reduced and oxidized X respectively, where X is species that undergoes oxidation (Uzir and Najimudin, 2018)

Cofactor plays an important role in all biotransformation involving redox reaction as it transfer hydrogen ion (H^+) from an oxidized system to that of the reduced system (Uzir and Najimudin, 2018; Chin et al., 2009). NADPH and /or NADH are initially available in cells, yeast or bacteria for the reduction reaction of substrate. However, there is only limited amount of cofactors inside the yeast and co-substrate such as glucose are necessary for recycling the cofactors inside the metabolic pathway. In addition, the generation of cofactor is dependent on the most basic cellular metabolism of glucose breakdown or glycolysis (Chin et al., 2009). Thus, an adequate amount of glucose should be dispensed in order to achieve an optimum biotransformation rate (Arifin et al., 2011). The main source of cofactor regeneration is shown in Figure 2.4 (Uzir and Najimudin, 2018) and it is the simplified version of glycolysis steps combined with TCA (tricarboxylic acid) or the Kerb's cycle.

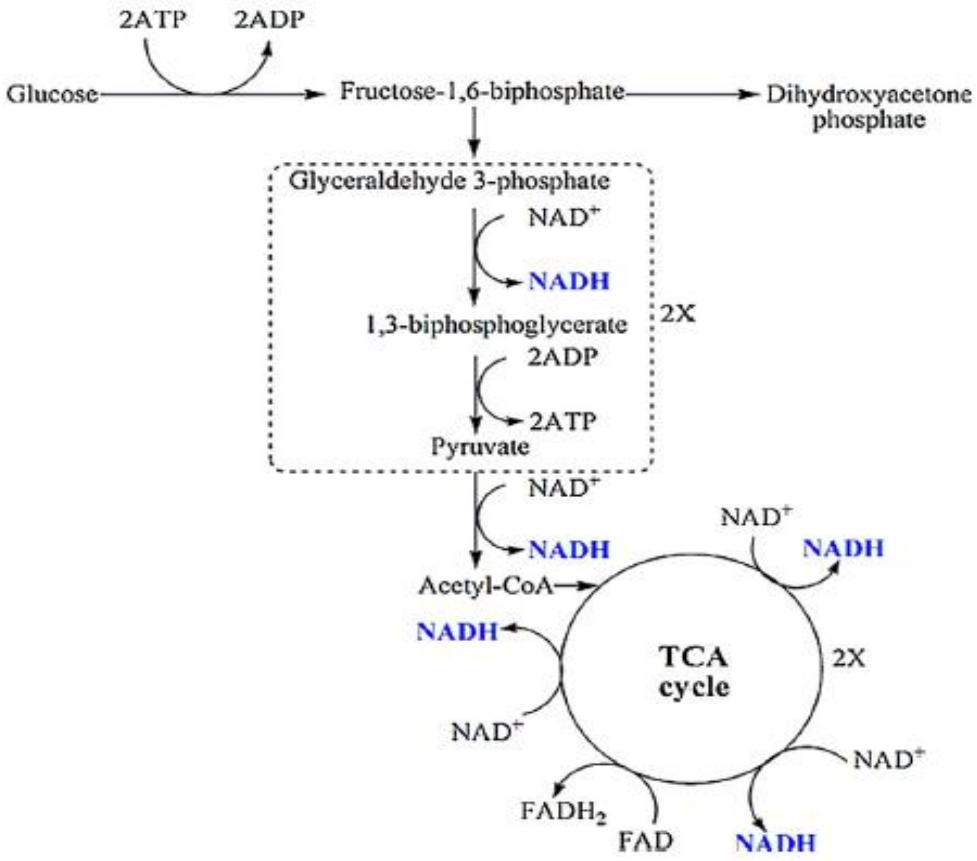

Figure 2.4: A simplified version of the glycolysis combined with TCA cycle showing the theoretical formation of cofactor (NADH) in yeast/ bacteria (Uzir and Najimudin, 2018)

2.4 Biotransformation of Ketoisophorone.

Ketoisophorone (KIP) is an industrially important cyclic endione as it is a key intermediate in the synthesis of carotenoids and flavouring agents (Ernest, 2002). 6R-dihydro-oxoisophorone (DOIP), also known as (6R)-levodione, is a key intermediate in the synthesis of some carotenoids and flavours, and synthesized via the enantioselective reduction of 4-oxoisophorone by *S. cerevisiae* (baker's yeast), where in situ removal of the product is employed by an external crystallization step (Buque-Taboada et al., 2004)

In biotransformation or bioreduction of ketoisophorone, used enoate reductase (OYEs) and alcohol dehydrogenases (ADHs) to catalysed the reaction. Enoate reductases (Ers) also known as Old Yellow Enzyme (OYE) selectively reduced carbon- carbon double bond in α,β-unsaturated carbonyl compound.(Chaparro-Riggers et al., 2007) While alcohol dehydrogenase are enzymes responsible for the reversible oxidation of alcohols to aldehydes with the associated reduction of NAD^+ or $NADP^+$. Biotransformation of KIP is observed on GLC spectrum where the conversion yield of the product was determine on the basis of the peak ratio of substrate and product (Hegazy et al., 2009). The possible route of KIP biotransformation has been mentioned earlier.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

Baker's yeast type-II (*S. cerevisiae)* in dried form is used in this study as one of the microorganism that act as biocatalyst. The yeast supplied was purchased from Sigma-Aldrich, USA.

3.1.2 Chemicals

All of the chemicals used in this study were purchased commercially.

Table 3.1: List of chemicals.

Chemical	Supplier
Potassium hydrogen phosphate (K_2HPO_4)	Quality Reagent Chemical (QRëC)
Potassium dihydrogen phosphate (KH_2PO_4)	Fisher Scientific
D-(+)-glucose (>99.5%)	Sigma-Aldrich
2,6,6-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone)	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Tri-Hydrochloride	Calbiochem
Ethyl acetate	Merc KGaA

3.2 Methodology

3.2.1 Preparation of phosphate buffer solution

Phosphate buffer was used as an aqueous medium for cell suspension for a stationary phase biotransformation. Potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were used to prepare phosphate buffer solution at pH 7 at a concentration of 0.1 M. In order to prepare 0.1 M buffer solution, 10.7 g of K_2HPO_4 and 5.24g of KH_2PO_4 were added and dissolved in 1000mL of medium bottle with 1 L of deionized water. Then, 5g/L of glucose was added to provide additional source of carbon. 250 mL of the prepared medium was measured and poured into five of 500mL of baffled shake-flask individually. The flasks was sterilized at 121 ^oC for one day in the autoclave.

3.2.2 Preparation of Tris-HCL buffer solution.

50 mM of Tris-hydrochloric acid (Tris-HCL) buffer solution was prepared by adding 3.94 g of Tri-HCL and 3.57 g of bovine serum albumin (BSA) and dissolved with 500mL deionized in 500mL of medium bottle. The medium bottle was shaken until the solution was well mixed.

3.2.3 Stationary phase biotransformation at different substrate concentration.

The experiment was carried out in 250 mL phosphate buffer in the 500 mL baffled shake-flask that had been autoclaved earlier. 1.25 g of dried Baker's yeast type II (*S. cerevisiae*) was measured and added to the 250 mL phosphate buffer resulting into $5g_{dew}/L$ of cell concentration. The procedure was carried out aseptically in a laminar flow cabinet to ensure the medium is free from contamination. Then, the baffled shaker-flask was placed on an incubator-shaker (IKA, KS4000i Control,

Korea) at 37 ^{o}C with the shaking speed of 150 rpm for 30 minutes for reaction and stabilization. 0.2 g/L of substrate (ketoisophorone) was introduced into the cell suspension 30 min after the inoculation by using a micro pipette. Samples were withdrawn every hour in order to determine the remaining substrate, the amount of products and the cofactor availability during the biotransformation. The same procedures were conducted for different substrate concentration of ketoisophorone at 0.5 g/L, 0.8 g/L, 1.0 g/L and 2.0 g/L.

3.2.4 Analytical Method

3.2.4.1 Quantifications of Substrates and Products.

Samples were withdrawn every hour for at least two times. 0.5 mL of sample was withdrawn from the biotransformation medium and transferred into 1.5 mL Eppendorf tube. 0.5 mL of ethyl acetate was then added as solvent into the sample for organic separation. The mixture was vigorously vortexed using vortex mixer for at least 5 min to produce homogenous solution. The sample was then centrifuge at 4000 rpm for 10 min in micro centrifuge (Eppendorf 5702 R, Germany) to separate the aqueous and the organic phase. The sample was divided into three layer once the separation was complete. Only the top layer (organic phase) was removed and kept in a sample vial and wrapped with parafilm for gas chromatographic analysis.

3.2.4.2 Cofactor (NADH/NADPH) analysis

Another 1 mL of sample was withdrawn from the reaction medium and transferred into 1.5 mL Eppendorf tube, then subjected to centrifugation at 4000 rpm for 10 min. After centrifugation, all of the resulted supernatant was removed and the cell pellet remained at the base of the tube was added with 1mL of Tris-HCL buffer

solution. The mixture was vortexed for 5 min until the cell pellet was completely suspended in the buffer. In order to break open cell membrane the suspended cell was then undergone sonication process using Misonix Sonicator 3000 (Cole-Palmer, USA). The suspended cell was transferred into small beaker and immersed in the beaker containing ice. Ice cubes were used to prevent the sample heat up too much during sonicating process and to reduce the temperature to about 5 ^{o}C. The sample was sonicated for 10 cycles (5s on 5s off) at the sonication amplitude of 8μm and later the sample was transferred back to the same Eppendorf tube and then undergone centrifuged at 4000 rpm for 10 min to remove the cell debris. After centrifuged, the supernatant of the sample was carefully transferred into a quartz cuvette and quickly checked for absorbance using a UV-vis spectrophotometer (Cary 60, Agilent Technologies, USA) with the absorbance wavelength of NADH/NADPH was set at 340 nm. Samples with absorbance value less than 1 can be defined directly but absorbance value of the samples that greater than 1, need to be diluted with Tri-HCL buffer solution. Dilution using Tri-HCL buffer solution is used in order to obtain absorbance value lower than 1.

3.2.4.3 Gas chromatographic (GC) analysis.

The procedure was continued in subsection 3.2.4.1 where the organic samples were kept in glass vials for chromatographic analysis. Standards were performed prior to samples analysis using 7820A Gas Chromatography, (Agilent Technologies, USA) which equipped with a flame ionization detector (FID) and capillary column (0.25 μm, 0.25 mm, 30m, MEGA –DEX DMT Beta, Italy). The standards and samples were prepared using 10 μL syringe and only 1μL of sample was inserted into a gas chromatography. The initial oven temperature was set at 120 ^{o}C and held for 2 min.

The temperature was then ramped at the rate of 2 ^{o}C/min until it reached at final temperature of 210 ^{o}C. The concentration of substrate, intermediate and products along the reaction time can be determined after approximately 47 min. The GC needs to be cooled down to 120 ^{o}C for almost a few minutes before the injection of the next sample. The presence of substrate, intermediate and products can be determined based on area under the peak curve in the GC spectrum and it being differentiate based on the standards.

3.3 Experimental Activities

The overall experimental activities carried out in this study are presented in Figure 3.1

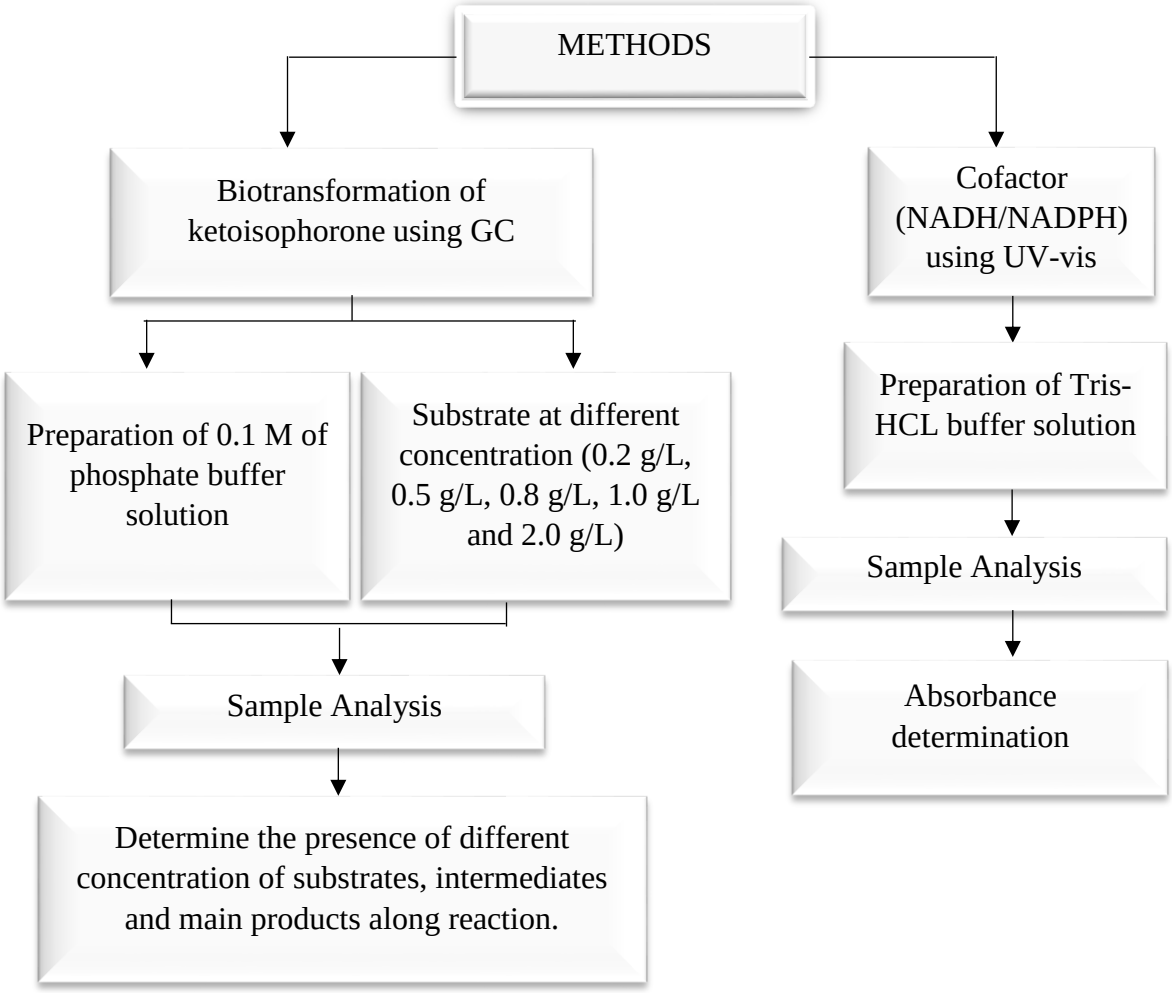

Figure 3.1: Experimental Activities