ON 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE

BIOTRANSFORMATION BEYOND THE STATIONARY PHASE OF BAKER'S

YEAST TYPE III

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UNIVERSITI SAINS MALAYSIA

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by

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Mohd Ridzwan bin Remlee

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LIST OF SYMBOLS

Symbol		Unit
A_{340}	Absorbance at 340 nm	-
A	Area under graph	m^2
C_{dcw}	Concentration of dry cell weight	g/L
C_K	Concentration of ketoisophorone	g/L
C_L	Concentration of levodione	g/L
C_P	Concentration of (4S)-phorenol	g/L
T	Time	h

LIST OF ABBREVIATIONS

ACT	Actinol
ATP	Adenosine Triphosphate
CBRs	Carbonyl reductases
CoA	Co-enzyme A
DOIP	6R-dihydro-oxoisophorone (Levodione)
ER	Enoate reductase
FAD	Flavin adenine dinucleotide
GAP	Glyceraldehyde-3-phosphate
GC	Gas chromatography
KIP	Ketoisophorone
LB	Luria-Bertani
NAD+	Nicotinamide adenine dinucleotide
NADH	β-1,4-nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	β- 1,4-nicotinamide adenine dinucleotide phosphate
OYE	Old yellow enzyme
rpm	Rotation per minute
UV	Ultraviolet

BIOTRANSFORMASI 2,6,6-TRIMETILSIKLOHEK-2-ENE-1,4-DIONE MELANGKAUI FASA PEGUN YIS 'BAKERS' JENIS III

ABSTRAK

Pengurangan ketoisoforon oleh seluruh sel, jenis yeast Baker jenis II telah diteliti baru-baru ini. Salah satu produk utama pengurangan biotransformasi ialah 2,6,6-trimetilkiklohex-2-ene-1,4-dione (ketoisoforon) adalah (4R, 6R) -4-hydroxy-2,6,6-trimethylcyclohexanone atau juga dinamakan sebagai (4R, 6R) -aktinol menggunakan '*Saccharomyces cerevisiae'* pada masa kini dengan glukosa sebagai sumber karbon. Biotransformasi ketoisoforon (KIP) membentuk levodione juga dinamakan sebagai 6R-dihydro-oxoisoforone (DOIP), yang merupakan perantaraan sebelum pengurangan selanjutnya menjadi aktinol (ACT). Eksperimen ini hanya tertumpu pada fasa kematian penapaian *S. cerevisiae* di mana substrat, ketoisoforon (KIP) diperkenalkan dan tempoh masa 22h fasa kematian yang bermula dari jam 38 hingga 60 penapaian sel yis. Ketersediaan dan kestabilan kofaktor nicotinamide adenine dinucleotide (NADH) atau nicotinamide adenine dinucleotide fosfat dengan kehadiran glukosa melalui proses kitaran Kreb adalah penting dalam biotransformasi ketoisophorone semasa fasa tersebut ditentukan oleh bacaan penyerapan menggunakan spektrofotometer UV-Vis. Kerja-kerja ini juga menyiasat jumlah substrat dan perantaraan semasa 22 jam tindak balas untuk menentukan aktiviti kedua-dua jenis enzim dalam sel-sel yis yang bertanggungjawab untuk biotransformasi ini iaitu karbonyl reduktase (CBR) dan enoate reduktase (ER) untuk menghasilkan kiral alkohol (4R, 6R) -aktinol. Hasil dari eksperimen menunjukkan bahawa dalam fasa kematian menghasilkan intermediate dan juga produk dalam tindakbalas biotransformasi .

ON 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE BIOTRANSFORMATION BEYOND THE STATIONARY PHASE OF BAKER'S YEAST TYPE III

ABSTRACT

The reduction of ketoisophorone by the whole cell, Baker's yeast cell type II has been researched recently. One of the major product of the biotransformation reduction of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone) is (4R,6R)-4-hydroxy-2,6,6-trimethylcyclohexanone or also named as (4R,6R)-actinol using *Saccharomyces cerevisiae* in the present of glucose as the carbon source. The biotransformation of ketoisophorone (KIP) forms levodione also named as 6R-dihydro-oxoisophorone (DOIP), which is intermediate before further reduction into actinol (ACT). This experiment only focussed on the death phase of S. cerevisiae fermentation where the substrate, ketoisophorone(KIP) was introduced and the time duration of 22h of death phase which is started from 38^{th} hour until 60^{th} of fermentation of yeast cells. The availability and stability of cofactor, nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) by the presence of glucose through Kreb's cycle process was essential in the biotransformation of ketoisophorone during that phase was determined by the reading of absorbance using UV-Vis spectrophotometer. This work was also investigate the amount of substrate and intermediate present during 22 h of reaction in order to determine the activity of the two types of ezymes within the yeast cells which is responsible for this biotransformation which is carbonyl reductase (CBR) and enoate reductase (ER) to produce a chiral alcohol of (4R,6R)-actinol. The result showed that the death phase produced the intermediate as well as product which is actinol with the aid of cofactor availability during the reaction of biotransformation.

CHAPTER ONE

INTRODUCTION

1.1 Biotransformation of ketoisophorone

1.1.1 Carotenoids

Carotenoid is defines as any of a group of non-nitrogenous yellow, orange, or red pigments (also called as biochromes) that are easily obtained from fruits. There are two major types of carotenoid which are the hydrocarbon class, or carotenes, and the oxygenated (alcoholic) class, or xanthophylls (Gloria et al., 2016). These types of carotenoid are synthesized by bacteria, fungi, lower algae, and green plants that makes carotenoids are most conspicuous in the petals, pollen, and fruit such as carrots, sweet potatoes, tomatoes, and citrus fruits. They can also be easily seen in the autumn foliage of deciduous trees and shrubs (Gloria et al., 2016). Carotenoids serve as accessory pigments in photosynthesis, trapping solar energy and passing it to chlorophyll, the primary photosynthetic pigment in the leaves of green plants. All animals and protozoans also consume carotenoids, which they obtain by ingestion. Vitamin A, for example, is one of the substances that animals obtain from the ingestion of carotene besides play a major role in the biological coloration of animals (Gloria et al., 2016).

1.1.2 Actinol

The biocatalytic production of fine chemicals used for the synthesis of naturally occurring carotenoids, vitamins, pigments, flavors and fragrances, is becoming the preferred route for commercial-scale production. Biocatalytic processes, occurring at mild conditions using renewable resources, are usually slow but with competitive yields and desirable enantioselectivities. Actinol (ACT), a key intermediate in the synthesis of optically active xanthophylls, xanthoxin and zeaxanthins (Mayer, 1979, Leuenberger et

al., 1976) as well as aroma constituent of tobacco and saffron (Sode et al., 1987) can be produced by the reduction of ketoisophorone (KIP) with high yield and enantioselectivity.

This conversion has been carried out via hydrogenation of the substrate (KIP) over alumina-supported Pt and Pd catalysts (Arx et al., 1999) or over a Pd/Al_2O_3 catalyst (Pillai and Sahle-Demessie, 2003). The yield obtained is relatively high but the enantioselectivity towards DOIP was still low. The usage of baker's yeast (*S. cerevisiae*) as catalyst had a higher yield of 80–85% DOIP and a very attractive enantiomeric excess which is more than 98% (Arx et al., 1999). Enantiomeric excess is a measurement of purity used for chiral substances. S. cerevisiae facilitates the bioconversion of KIP to production of a doubly chiral compound, ACT with DOIP as the intermediate (Kataoka et al., 2004a).

1.1.3 Yeast

Yeasts are eukaryotic organisms that are classified as fungi. Yeasts are used as starter cultures in cheeses and bread, as well as fermented products such as wine, but they can also initiate spoilage in foods, such as yoghurt, and mayonnaise (Perricone et al., 2017). Types of yeast includes *S. rouxii (Z. rouxii), S. delbrueckii (S. unisporus, T. delbrueckii), S. willianus, Z. bailii* and *C. tropicalis. S. cerevisiae* reproduces asymmetrically by budding off new daughter cells which is genetically identical with their parent cells (Arthur and Watson, 1976, Sinclair et al., 1998). Usually, Bakers' yeast is made by strains belonging to *S. cerevisiae* which is commonly used for the microbial production of actinol (Fukuoka et al., 2002).

1.1.4 Biotransformation

Biotransformation is defined as the series of chemical alterations of a compound such as drug due to the enzymatic activity occurring within the body (Miller-Keane, 2003).

In other word, the compound changes from the origin compound to the product compound or through intermediate compound to become product compound depend on the high or low enzymatic activity. From the other references, biotransformation is defined as the conversion of molecules within an organism, from one form to another often associated with change (increase, decrease, or little change) in pharmacologic activity; refers especially to drugs and other xenobiotics (Miller-Keane, 2003).

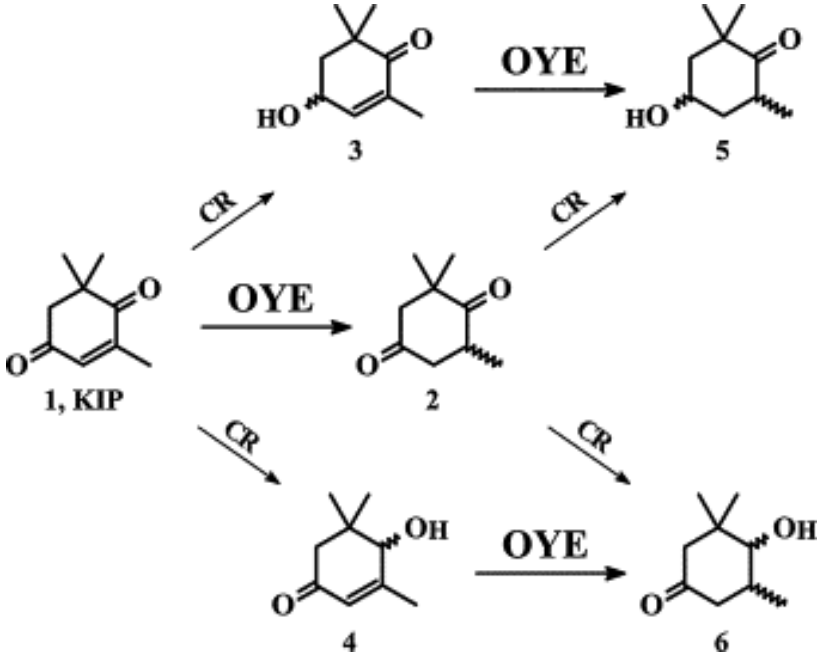

Figure1.1: Reduction of ketoisophorone(KIP) in yeast cells 1 – 2,6,6-trimethylcyclohex-2-ene-1,4-dione [ketoisophorone]; 2 – 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione]; 3 – 4-hydroxy-2,6,6-trimethylcyclohex-2-en-1-one [(4S)-phorenol]; 4 – 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one [hydroxyisophorone];5 – 4-hydroxy-2,6,6-trimethylcyclohexaone [(4R, 6R)-actinol]; 6 – 4-hydroxy-3,5,5-trimethylcyclohexanone. OYE – old-yellow enzyme or called as enoate reductase; CR – carbonyl reductase. (Fukuoka et al., 2002)

1.1.5 Differences between NADH and NADPH

NADH and NADPH are coenzymes that play significant role in various bodily functions. These coenzymes help in the metabolism of alcohol or fatty acid synthesis. These enzymes are also present in plant cells. The coenzyme forms when hydride ion (H^-) is added to NAD^+ (nicotinamide adenine dinucleotide), it leads to the formation of NADH.

On the other hand, addition of hydride ion to $NADP^+$ (nicotinamide adenine dinucleotide phosphate), produces NADPH. In short, NADH and NADPH are reduced forms of NADH and NADPH, respectively by gaining electrons.

Table 1.1 Comparison between NADH and NADPH (BiologyWise, 2018)

Category	NADH	NADPH
Function	NADH is primarily involved in catabolic reactions, where reaction that breaks down molecules to release energy. For instance, synthesis of lipids, carbohydrates, proteins and nucleic acids are broken down into smaller molecules are all catabolic reactions and release biological energy in the form of heat. NADH involves in these reactions that help in energy production.	NADPH plays an important role in anabolic reactions, where the energy is consumed in order to build up or synthesize larger molecules. For example, the synthesis of fatty acids and cholesterol are all anabolic reactions and with condition presence of NADPH.
Type of agents	NADH acts as an oxidizing agent in catabolic reactions, thus it oxidizes and loses an electron. For instance, breakdown of glucose, which requires NADH.	NADPH acts as a reducing agent in anabolic reactions, thus it reduces and gains electrons. NADPH is commonly involved in reductive metabolism.
Process	NADH is utilized in cellular respiration, a process in which body cells obtain energy from nutrients. Plants primarily use NADPH during photosynthesis to make glucose and oxygen.	NADPH is formed from NAPD molecule during the light-dependent reactions of photosynthesis. In the light independent stage of photosynthesis, the NADPH produced helps to convert the absorbed carbon dioxide (CO_2) into carbohydrates (sugar). So, NADPH is crucial in carbohydrate synthesis occurring in plants.
Structure	As far as their structure is concerned, the NADH molecule is a dinucleotide, composed of two nucleotides joined through their phosphate groups. This nucleotide is made up of 5-carbon sugar (deoxyribose) combined to a nitrogen base (adenine) and a phosphate group as shown in Figure 1.2.	The NADPH molecule is also made up of two nucleotides, but contains an extra phosphate group attached to the ribose of adenosine. This additional phosphate group allows NADPH to attach with a different set of enzymes.

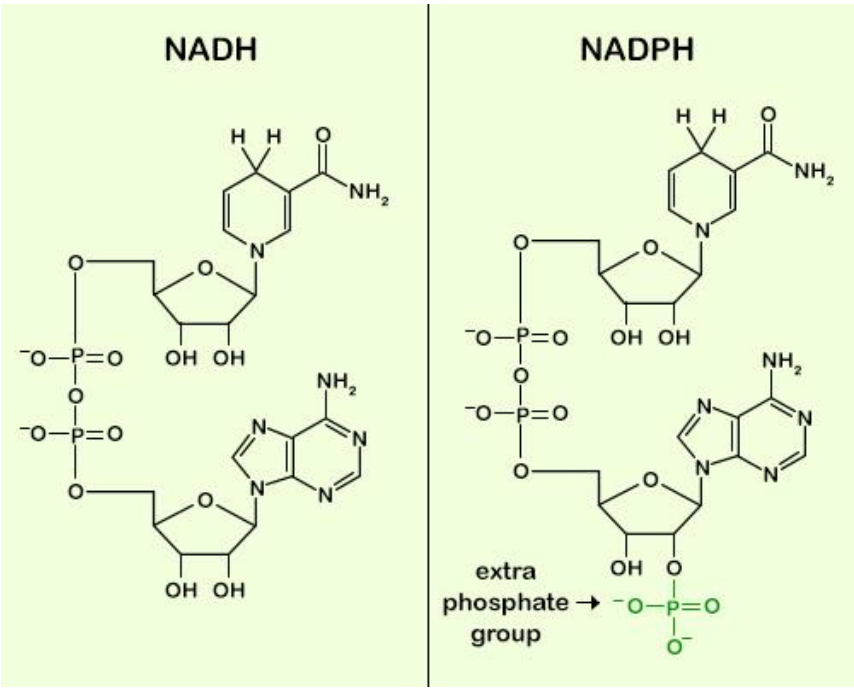

Figure 1.2 Structure compound of NADH and NADPH (BiologyWise, 2018)

1.2 Problem Statement

The Bakers' yeast (*S. cerevisiae*) is used for the biotransformation of ketoisophorone (KIP) to produce alcohol. Some research works on biotransformation of ketoisophorone in a growing whole-cell *S. cerevisiae* was reported (Uzir M.H. and Najimudin N., 2018). The problem is the accumulation of intermediate (6R)-levodione (4) and traces of (4S)-phorenol (2) depend on the activity of enzyme enoate reductase(ER) and carbonyl reductase (CBRs) in the exponential phase. Therefore, this research primarily focuses on the cell activity during the death phase of *S. cerevisiae* in order to determine the intermediates and product produced from the biotransformation of ketoisophorone. Hence, the KIP should be introduced at the beginning of death phase of *S. cerevisiae* to determine the rate of substrate consumed throughout the death phase. The concentration of substrate and the intermediate must be measured during phase involved

to identify the rate of biotransformation of substrate. The availability of cofactor produced from the cells need to be measured to ensure the product will be formed.

1.3 Research Objective

i) To determine the time or duration when yeast culture started the death phase by plotting the growth profile of *S. cerevisiae*.

ii) To determine cofactor availability and products formed beyond stationary phase of *S. cerevisiae* cells.

iii) To determine the concentration of substrate, intermediate and product formed in the death phase.

1.4 Organization of thesis

This thesis consists of five main chapters and each chapter contributes to the sequence of this study. Chapter 1 is introduces biotransformation of ketoisophorone, problem statement, research objective and organization of thesis. Chapter 2 is the discussion about the literature review of this study. Chapter 3 covers the experiment materials and the details of methodology. Explanation on the description of equipment and materials used, experimental procedure and description on types of test analyses. Chapter 4 is about the discussion on the result obtained from the experiment. Chapter 5 provides conclusion about findings of the experiment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Bakers' yeast (*Saccharomyces cerevisiae*)

Saccharomyces cerevisiae is usually known as Bakers' yeast. It is a simple single-celled eukaryote that is often used in scientific research. *S. cerevisiae* is an attractive model organism because of the fact that its genome has been sequenced. Genetic manipulation in yeast is easy and cheap compared to similar experiments in more complex animals such as mice and zebrafish (yourgenome, 2017), and it is very easy to maintain in the lab. The reasons of using Bakers' yeast is this type of yeast is similar to many yeast in sequence and function to those found in other organisms, studies performed in yeast can help us to determine how a particular gene or protein functions in higher eukaryotes (including humans) (Database, 2018).

2.2 Glucose as energy source

Kreb's cycle, also called as the citric acid or tricarboxylic acid cycle (TCA), is a cyclic pathway of enzymatic reactions which oxidizes the compounds derived from molecules such as glucose, fatty acids and amino acids in the matrix of mitochondria (Figure 2.1). The Kreb's cycle involves eight steps which produce in different steps CO_2 and NADH and $FADH_2$, reduced coenzymes which transfer electrons to the respiratory chain. At the same time, the respiratory chain generates ATP for the use in cellular processes. The chemical intermediates of Kreb's cycle can penetrate to the cytoplasm where they can be used as precursors for biosynthetic reactions. Moreover, some of the Kreb's cycle enzymes have isoenzymes in cytoplasm which can process citrate to 2-oxoglutarate, i.e. via aconitase and isocitrate dehydrogenase (Figure 2.1).

Besides, cytoplasmic intermediates can enter the mitochondria and be further processed in the Kreb's cycle (Salminen et. al., 2014; Zhao and Yang, 2016). Firstly, the cycle is provided with new substrates via acetyl-CoA, which can be derived from glycolysis, β-oxidation or amino acid degradation (Figure 2.1). Furthermore, glutamate dehydrogenase can produces 2-oxoglutarate via the deamination of glutamate (Hudson and Daniel, 1993; Li et al., 2012). In fact, Kreb's cycle aids with oxidative phosphorylation is the major pathway in cellular energy production (Salminen et al., 2014).

The NADH/NADPH are the cofactors needed for the biotransformation of ketoisophorone which are produced in the Kreb's Cycle. The Kreb's cycle is part of glycolysis process which converts glucose into energy. Glycolysis is reduced to three reactions. The first reaction describes the conversion from glucose to glyceraldehyde-3-phosphate (GAP) which consumes two ATP molecules and produces two GAP molecules; the second reaction contains the GAP dehydrogenase reaction that produces NADH in the cytosol and 1,3-diphosphoglycerate (1,3-DPG); the third reaction lumps together the remaining glycolysis reactions which produces two ATP molecules per GAP; which are four per glucose molecule and one molecule of pyruvate; which are two per glucose molecule. From Figure 2.1, ACO1 and IDH1 are cytoplasmic enzymes, the rests are in mitochondria. Pyruvate can moves out, be used for another metabolic pathway, or be converted to lactate with the simultaneous oxidation of NADH as shown in Figure 2.1. The pyruvate dehydrogenase reaction with the production of one NADH molecule in mitochondria. Krebs cycle produces four molecules of NADH (Nazaret and Mazat, 2008; Pollak et al., 2007; Singh et al., 2008).

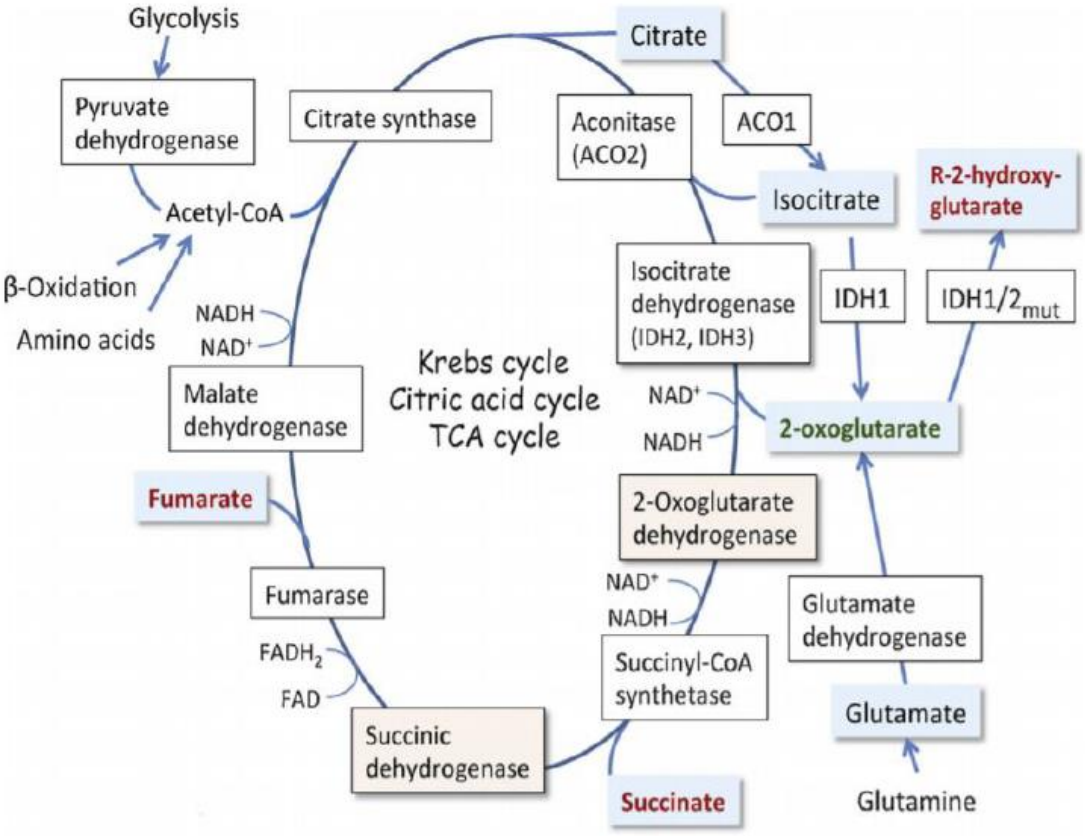

Figure 2.1 Kreb's cycle (Salminen et al, 2014)

2.3 Biotransformation of ketoisophorone

Ketoisophorone (4-oxoisophorone or 2,6,6-trimethyl-2-cyclohexene-1,4-dione) is an industrially important cyclic endione as it is a key intermediate in the synthesis of carotenoids and flavouring agents (Ernst, 2002). Catalytic hydrogenation and enzymatic reduction of ketoisophorone lead to many optically active hydroxycyclohexanone derivatives, such as (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexanone (actinol), that in turn is a useful chiral building block for naturally occurring xanthoxin (Burden and Taylor, 1970) and zeaxanthin (Leuenberger et al., 1976). (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 *Saccharomyces cerevisiae* (Bakers' yeast), where in situ removal of the product is employed by an external crystallization step (Buque-Taboada et al., 2004).

Microbial production of actinol from 2,2,6-trimethylcyclohexanedione has been demonstrated previously by Nishi et al. (1989). Additionally, a two-step production of actinol (ACT) from the commercially available ketoisophorone (KIP) was reported by using old yellow enzyme (OYE) and levodione reductase for enzymatic asymmetric reduction (Kataoka et al., 2002, Wada et al., 2003). In this work, they report for the first time that cultured plant cells can catalyze the stereo- and regioselective reduction of C=C and C=O bonds at C-1 and C-4 position of ketoisophorone.

2.4 Enzyme involved in the reaction.

2.4.1 Enoate Reductase (ER) from non-conventional yeast

Enoate reductase (ER) is responsible to reduce double bond C=C to single bond. It is also called as old yellow enzymes (OYE). OYE's catalyze the chemo- and stereoselective hydrogenation process of alkenes which break the weak bond which is double bond to the single bond. This bio-reduction can result in important chiral synthons towards synthesis of pharmaceuticals, fragrances, and chemicals (Toogood et al., 2010).

The biotechnological potential of OYE's that have the ability to produce up to two diverse stereocenters by the stereoselective trans-hydrogenation of the C=C, which is highly demanded in asymmetric synthesis, but difficult to perform with conventional methods (Stuermer et al., 2007, Williams and Bruce, 2002). OYE's can reduce various type of substrates, such as conjugated enals, enones, α,β-unsaturated carboxylic acids, imides, nitroalkenes, and ynones (Stuermer et al., 2007).

Although the physiological role of OYE's still remains unknown, and their natural substrates have not been justified, a lot of investigate and research about this family of enzyme is required in order to develop new catalysts. Several OYE's have been isolated from higher plants, bacteria, yeasts and filamentous fungi (Strassner et al., 1999, Kataoka

et al., 2004b, Brigé et al., 2006). However, effect of high demand of optically pure building blocks, the scientific community is still addressing extra efforts at identifying and developing OYE biocatalysts.

2.4.2 Carbonyl reductases (CBRs)

Carbonyl reductases (CBRs) belong to a class of oxidoreductase proteins that are part of the family of short chain dehydrogenases/reductases (SDR) (Wermuth, 1992, Krook et al., 1993). They are ubiquitous in nature and catalyze the NADPH reduction of a large number of biologically and pharmacologically active substrates, including a variety of endogenous and xenobiotic carbonyl compounds (Wermuth, 1985, Felsted and Bachur, 1980). CBRs have been detected in every tissue analyzed. They have been reported in non-mammalian tissue of plants, bacteria, yeast, fish and insects.

Generally, CBRs are low molecular weight, monomeric, cytosolic, NADPH-dependent enzymes that reduce aldehyde and keto groups of prostaglandins, steroids, pterins, biogenic amines, and quinones derived from polycyclic aromatic hydrocarbons. Many keto drugs and the aliphatic keto side chain of the anthracycline anticancer drugs, daunorubicin (dn) and doxorubicin (dox), are also reduced. Carbonyl reduction is the main reaction and in many cases the reaction is irreversible. However, in some reactions, oxidation of secondary alcohols and hydroquinones can occur with the $NADP^+$ cofactor. (Jarabak and Harvey, 1993)

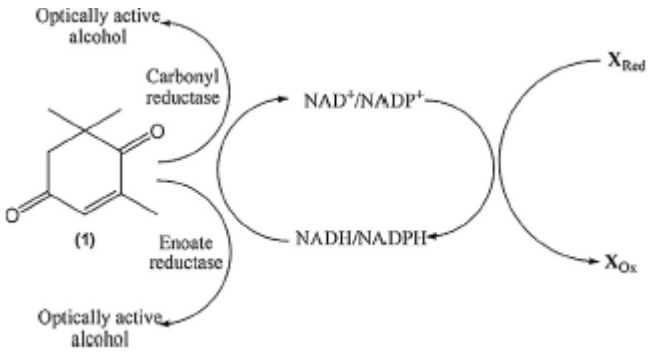

Figure 2.2 The use of both cofactor NADH/NADPH in the reduction of ketoisophorone (Uzir and Najimudin, 2018)

2.5 Cofactor vs coenzyme

A cofactor is a non-protein chemical compound. It is bound to the protein and it is needed in the biological activity of the protein. Another term for them are 'helper molecules' because they help in the biochemical transformations (Celine, 2013). There are two types of cofactors:

- Coenzymes
- Prosthetic groups

Coenzymes are cofactors that are attached to an enzyme loosely. Prosthetic groups are cofactors that are attached tightly to an enzyme. As additional information, an enzyme can be without a cofactor, and this is called apoenzyme. An enzyme is considered complete if it has the cofactor and it is called a holoenzyme (Celine, 2013).

A coenzyme, on the other hand, is a small, organic non-protein molecule. It carries chemical groups between enzymes. It is not regarded as a part of the enzyme's structure. Vitamins are good examples of a coenzyme. They carry chemical groups between the enzymes. Another term for them is co-substrates.

To summarize, here are the differences between a cofactor and a coenzyme:

- A coenzyme is a type of cofactor. It is the loosely bound cofactor to an enzyme.
- Cofactors are chemical compounds that are bound to proteins.
- A cofactor is a non-protein chemical compound, while a coenzyme is a non-protein molecule.

It is important to understand that, in our body, enzymes are very important. They help in regulating metabolism. They help in controlling the chemical reactions in the body. This is why knowing about coenzymes and cofactors is quite essential in the processes of our body. Initially, coenzymes and cofactors combine with enzymes to alter and bring about change to the body by making, offering, and doing changes to the chemical reactions. At the same time, to achieve certain chemical reactions, cofactors and coenzymes are needed. Cofactors serve the same function as coenzymes, as they regulate, control, and adjust how fast these chemical reactions would respond and take effect in our body. The big difference is that coenzymes are organic substances, while cofactors are inorganic. Coenzymes function as intermediate carriers. Cofactors, on the other side, as they are classified as inorganic substances, are needed and required to increase how fast the catalysis would take place (Celine, 2013).

2.6 Chirality

Stereoisomers are isomers that differ in spatial arrangement of atoms, rather than order of atomic connectivity. One of their most interesting type of isomer is the mirror-image stereoisomers, a non-superimposable set of two molecules that are mirror image of one another. The existence of these molecules are determined by concept known as chirality (Vollhardt et al., 2003). The word "chiral" was derived from the Greek word for hand, because our hands display a good example of chirality since they are non-superimposable mirror images of each other. The opposite of chiral is achiral. Achiral objects are superimposable with their mirror images. For example, two pieces of paper

are achiral. In contrast, chiral molecules, like our hands as shown in Figure 2.3, are non-superimposable mirror images of each other (Atkins et al., 2006).

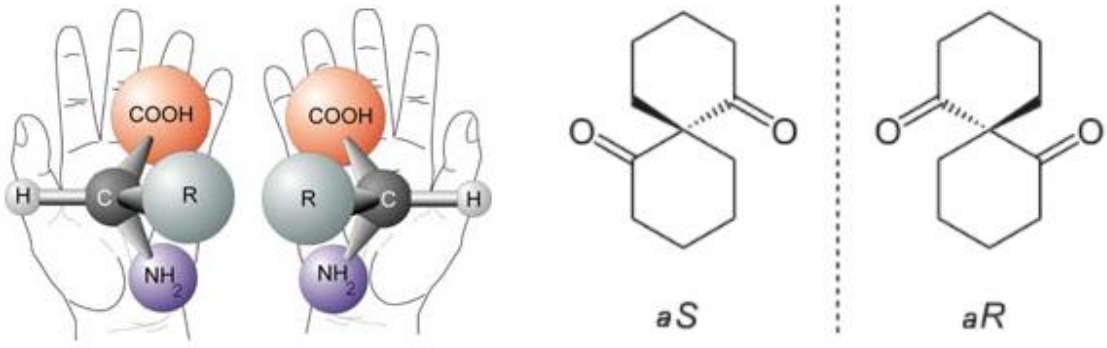

Figure 2.3 Mirror image of chiral molecules (Wagniere and George, 2007)

A chiral molecule has a mirror image that cannot line up with the mirror images are non-superimposable. The mirror images are called enantiomers. A chiral molecule and its enantiomer have the same chemical and physical properties (boiling point, melting point, polarity, density etc...). It turns out that many of our biological molecules such as our DNA, amino acids and sugars, are chiral molecules. It is very interesting that our hands seem to serve the same purpose but most people are only able to use one of their hands to write. Similarly, this is true with chiral biological molecules and interactions. By applying with your left hand will not fit properly in your right glove, one of the enantiomers of a molecule may not work the same way in your body. This must mean that enantiomers have features that make them unique to their mirror images. One of these properties is that they cannot have a plane of symmetry or an internal mirror plane (Croft and William, 2006). So, a chiral molecule cannot be divided in two mirror image halves. Another property of chiral molecules is optical activity (Vollhardt et al., 2003).

Optical activity

As mentioned before, chiral molecules are quite similar to each other since they have the same components to them. The only thing that differ from the others is their

arrangement in space. As a result of this similarity, it is very hard to differentiate chiral molecules from each other when we try to compare their properties such as boiling points, melting points and densities. However, we can distinguish them by their optical activity. A chiral molecule that molecule rotates light in a certain direction when a plane-polarized light is passed through one of the two enantiomers. Then, that enantiomer rotates light by the same amount but in vice versa when the same plane polarized light is passed through the other enantiomer. If one enantiomer rotates the light counter clockwise, the other would rotate it clockwise because chiral molecules are able to rotate the plane of polarization differently by interacting with the electric field differently, they are said to be optically active. On top of that molecules are rotate light in different directions are called optical isomers (Wagniere and George , 2007).

- Dextrorotatory (+ enantiomer) is when it rotates the plane polarized light clockwise (when viewing towards the light source).
- Levorotatory (- enantiomer) is when it rotates the plane polarized light anti-clockwise (when viewing towards the light source).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Flowchart of procedures

Figure 3.1 shows the schematic flow chart of the experimental procedures.

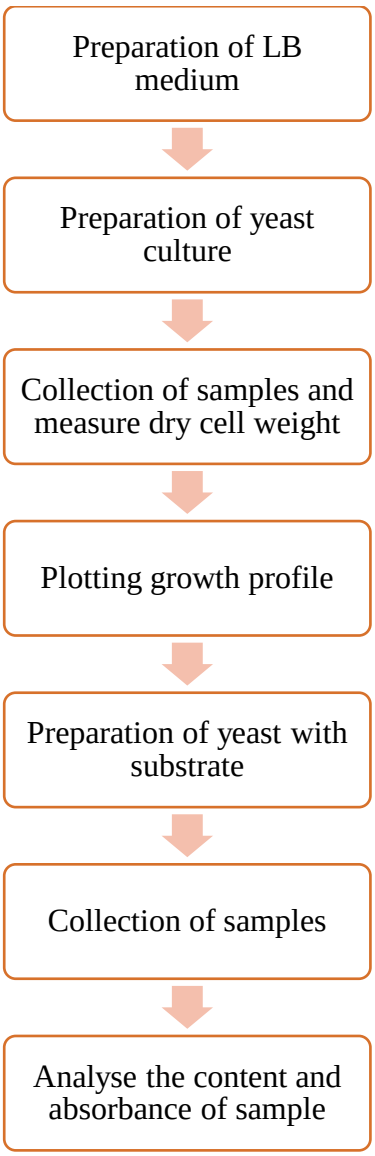

Figure 3.1 Schematic flow chart of experimental procedures

3.2 Microorganism

Baker's yeast type-II (*S. cerevisiae*) in dried yeast form purchased from Sigma-Aldrich (USA).

3.3 Materials

The chemicals used during experiment: standard ketoisophorone substrate (98%) purchased from Sigma-Aldrich (USA); Ethyl acetate from Sigma-Aldrich (USA); Potassium hydrogen phosphate (K_2HPO_4) (99%) purchased from QRe˙C; Potassium dihydrogen phosphate (KH_2PO_4) (99.9%) from Fisher Scientific; Glucose (≥99.5%) from Sigma-Aldrich; Tri-Hydrochloride from Calbiochem and Bovine serum albumin (BSA) from Sigma-Aldrich (USA); Luria Bertani, (LB) medium including peptone, sodium chloride, yeast extract were also purchased from Sigma-Aldrich, USA; Pure enantiomer of (S) -4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (>98%); 2,2,6-trimethylcyclohexane-1,4dione, (>95%) were purchased from AAP Pharma Technologies (India) and Angene International Limited (Hong Kong) respectively.

3.4 Experimental procedures

3.4.1 Preparation of LB medium

The Luria-Bertani (LB) medium was prepared using 10 g of sodium chloride, 10 g of peptone and 5 g of yeast extract. The preparation of LB medium with glucose content was done by adding 5 g of glucose to get 5 g/L of glucose concentration. Next, the 1 L of media bottle was filled with 1 L of deionized water. The bottle was then shaken up and down until all the powder was fully mixed with deionized water. Meanwhile, the 500 mL of shake-flasks was dried using oven one day before using it. The volume of 250 mL of LB media was measured using a measuring cylinder and poured into each of the shake-flasks. The mouth of each shake-flasks were covered with a thick cotton wool and wrapped with aluminium foil tightly to ensure no contamination inside the shake flask. The shake-flasks were placed in autoclave and set the temperature of 121ºC for 30 min in order to eliminate the contamination inside the shake flasks. Then, it was allowed to be cooled down to room temperature around 4 h.

3.4.2 Growth of Baker's yeast, *S. cerevisiae* without substrate.

The preparation using yeast was conducted in laminar flow cabinet in clean room to avoid any contamination in the shake flasks. The media bottle contained yeast must sterile first before putting yeast inside the media bottle. The yeast cells were weighed on the folded aluminium foil and the transferred into the shake-flask together with the aluminium foil. The mass of yeast cells was measured using electronic mass balance according to the concentration of yeast cells required. Therefore, 0.25 g of yeast cells were required to get 1 g/L of yeast cells concentration. After that, the shake-flasks was placed in the incubator shaker which operated at the temperature of 37ºC at 150 rpm and the stopwatch was started simultaneously after the incubator shaker was started to shake the shake-flasks as shown in Figure 3.2.

Figure 3.2 Incubator shaker

3.4.3 Collection of sample and dry cell weight

The dry cell weight was calculated using the difference in mass of Eppendorf tube which is before and after using the Eppendorf tubes. The Eppendorf tubes were labelled before drying in the oven for one day at 60ºC to remove any contaminant in the Eppendorf

tubes. After that, the mass of empty Eppendorf tubes were weighed using a mass balance and the data was recorded.

When taking the sample, the incubator shaker was stopped and 1 ml of sample was taken from each of the yeast culture in the shake flasks using a pipette and then, transferred to Eppendorf tube. The Eppendorf tube was immediately centrifuged for 5 min. When centrifuged was done, the liquid was poured out while the cell residue were remained in the Eppendorf tube. The Eppendorf tubes were dried in the oven for 2 h at 60ºC. The mass of Eppendorf tubes with cells were weighed again and the data was recorded. Then, the cell concentration in the Eppendorf tubes was calculated. The steps was repeated every 2 h in the duration of 6 days of fermentation of yeast.

3.4.4 Plotting of Baker's yeast growth profile

The tabulated data from several samples with the presence of glucose were used to plot the growth curve. The growth curve was compared and the suitable time for substrate injection was determined. The suitable time for substrate injection was the initial stage where yeast cells undergo exponential growth.

3.4.5 Growth of Baker's yeast, *S. cerevisiae* with substrate.

The preparation of yeast culture was same with procedure 3.4.2. The substrate used was ketoisophorone (KIP). The concentration of substrate was 0.2 g/L, hence 0.03 g which is equivalent to 50 µL volume of substrate. The substrate was added to yeast culture in incubator shaker at the time initial stage of dead phase was observed from the growth of fermentation of yeast. Then, the yeast culture was continued to rotate at 150 rpm at 37ºC in the incubator shaker.

3.4.6 Collection of samples

The incubator shaker was stopped and 0.5 mL of sample was taken from each yeast culture and transferred to the respective labelled Eppendorf tubes, and this step was done twice. Hence, two Eppendorf tubes with 0.5 mL samples were prepared for each yeast culture. Later, 0.5 mL of ethyl acetate were added to each Eppendorf tubes respectively. Another 1 mL of sample was taken from each yeast culture and transferred to the respective labelled Eppendorf tubes. The collection of sample was taken every 1 h duration time of dead phase of 22 h before the yeast cells were totally dead.

3.5 Analytical methods

3.5.1 Substrate and product quantification

0.5 mL of samples were taken out from the shake flasks in the incubator shaker using pipette into Eppendorf tube. Then, 0.5 mL of ethyl acetate was added into the Eppendorf tube and let the mixture mixed vigorously using vortex at least 5 min. After that, the Eppendorf tube was centrifuged at 4,000 rpm for 10 min to separate the aqueous and the organic phase. After separation was completed, the top layer which is organic phase was removed using pipette and kept in glass vials for gas chromatographic analysis.

3.5.2 Gas chromatographic (GC) analysis

The organic samples extracted from the previous subsection 3.5.1 were kept in glass vials for chromatographic analysis. Before testing the organic samples, the standards solution were performed using 7820A Gas Chromatography, (Agilent Technologies, USA) equipped with flame ionization detector and a capillary column (0.25 µm, 0.25 mm, 30 m, MEGA-DEX DMT Beta, Italy) as shown in Figure 3.3. The oven was initially set at 120^{o}C for 2 min and ramped to 210^{o}C at a rate 2^{o}C/min. The

injector temperature was maintained at 220^{o}C with helium as a carrier gas with a flow rate of 1.0 mL/min. The pure ketoisophorone which is substrate was injected from glass vials using 10 µL syringe and then 1µL of substrate was injected into the gas chromatography and make sure that there was no bubble inside the syringe. After that, the gas chromatography was run for 47 min and let the temperature of oven to cool down around several minutes before injecting the next organic samples. This step was repeated to inject the intermediates and standard solution from the concentration of 2 g/L, 4 g/L, 6 g/L, 8 g/L and 10 g/L. Then, this step was repeated three times to get the average result in order to plot the calibration curve of standard solution. The compound present in the sample can be determined based on the peak produced from GC reading.

Figure 3.3 Gas chromatography

3.5.3 Cofactor (NADH/NADPH) analysis

The procedure was started by taking out 1 mL of sample from the reaction medium in incubator shaker into the labelled Eppendorf tube. Then, the sample was centrifuged for 10 min at 4,000 rpm. After that, the supernatant was removed and the cell pellet was remained in the Eppendorf tube. 1 mL solution of BSA and Tris-HCL buffer was added

into Eppendorf tube. The mixture was vortexed for 5 min until the cell pellet was completely mixed with buffer. After that, the mixture was carried out sonication process using Misonix Sonicator 3000 (Cole Palmer, USA) as shown in Figure 3.4 in order to break out the cell membrane in the mixture. During the sonication process, the ice (<5^{o}C) is required to prevent the cell from damaged. This process required 10 cycles (5 s on and 5 s off) at the sonication amplitude of 8 µm and later centrifuged at 4,000 rpm for 10 min to remove the cell debris. After that, the supernatant inside the Eppendorf tube was carefully transferred into a quartz cuvette and quickly checked for absorbance using UV-vis spectrophotometer (Cary 60, Agilent Technologies, USA) in Figure 3.5. The absorbance wavelength was set at 340 nm wavelength of NADH/NADPH. These steps were repeated in the interval of 1 h during the dead phase of fermentation of yeast.

Figure 3.4 Sonicator

Figure 3.5 UV-Vis spectrophotometer

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 Growth profile of yeast, *Saccharomyces cerevisiae*

From the result obtained during 6 days of fermentation process, the phase of growth profile have been classified based on their yeast cell concentration. At the first 5 h, the lag phase was taken in the fermentation of yeast where the yeast cell concentration is same with the initial cell concentration. The lag phase is the period after the medium has been inoculated with yeast and before the exponential growth phase. The length of the lag depends on the change in the nutrient, concentration, pH and temperature from the inoculating medium to the fresh medium, and the age and size of the inoculum (Glenn C. B., 1985). A change in nutrient concentration can cause several effects on the yeast. If the new medium is richer in nutrients, the cells must create a larger concentration of metabolizing enzymes. A decrease in the nutrient level may result in no lag phase at all (Bailey and Ollis, 1977).

After the first 5 h, the exponential phase was started until 14 h of fermentation before stationary phase immediately takes place. This phase when the nutrient level can no longer support exponential growth and/or the toxic level inhibits the exponential growth, this phase will end and the stationary phase will begin. Normally, the duration of exponential phase was around 10 h -15 h of yeast fermentation.

The stationary phase is when the total viable cell count remains constant. The effect of any particular nutrient of the medium upon the growth rate is dependent on the importance of the nutrient in cell metabolism. Exhaustion of a carbon source or important nutrients will cause a rapid decrease in the growth rate. For non-essential nutrients in the medium, the yeast can synthesize the nutrients and little effect on the growth rate will be