ON THE 2,6,6 – TRIMETHYLCYCLOHEX-2-ENE 1,4-DIONE BIOTRANSFORMATION BEYOND THE EXPONENTIAL PHASE OF BAKER'S YEAST TYPE-II GROWTH CURVE.

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Universiti Sains Malaysia

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by

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LIST OF ABBREVIATION

ACT	Actinol
ADH	Alcohol dehydrogenase
DOIP	Dihydro-oxo-isophorone
GDH	Glucose dehydrogenase
KIP	Ketoisophorone
LDR	Long chain reductase
MDR	Medium chain reductase
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
OYE	Old Yellow Enzyme
SDR	Short chain reductase
UV-Vis	Ultra violet-visible

2,6,6 TRIMETILKLOROHEK-2-ENE 1,4 DIONE KE ATAS LENGKUNG TERBINA FASA EKSPONEN YIS ‘BAKER’ JENIS II

ABSTRAK

Pengurangan ‘ketoisophorone’ melalui biotransformasi telah lama menjadi minat kerana ianya merupakan produk berharga serta mekanismenya yang menarik. Satu kajian tentang biotransformasi ketoisophorone oleh sel tidak berkembang telah dijalankan. Tujuan utama kajian ini dijalankan adalah untuk mengkaji profil pertumbuhan *Saccharomyces cerevisiae* dan untuk mengkaji jumlah produk yang terbentuk iaitu 4R,6R actinol semasa biotransformasi fasa pegun. Selepas profil pertumbuhan diperolehi, pengenalan substrat disimpulkan untuk dibuat pada jam ke-12. Biotransformasi telah dilakukan dalam satu bekas dengan menambahkan sel-sel tidak berkembang. Spektrum kofaktor sampel sampel dianalisis pada setiap satu jam. Sebuah model 7820 Gas Chromatography digunakan untuk menganalisis penggunaan substrat, pembentukan perantaraan dan produk. Sebagai rumusan, didapati bahawa pengeluaran perantaraan, (6R) -levodione hanya dihasilkan dalam jumlah yang sedikit. Hal ini berkemungkinan berpunca daripada sedikit atau ketiadaan aktiviti reductase karboksil, ataupun penukaran substrat kepada produk terlalu cepat. Untuk pembentukan produk pula, (4R,6R)-actinol amat bergantung kepada kepekatan substrat, ketoisophorone. Apabila kepekatan ketoisophorone berkurang, pembentukan 4R,6R actinol juga akan berkurangan. Sebagai konklusi, substrat dan pembentukan produk semakin berkurang daripada permulaan sehingga ke akhir eksperimen dijalankan kerana substrat digunakan

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ABSTRACT

The reduction of ketoisophorone as a substrate has long become an interest for its valuable products as well as its interesting mechanism through biotransformation. A study of biotransformation of ketoisophorone beyond the exponential phase by non-growing cells has been conducted since there is lack of number of research had been done about biotransformation of ketoisophorone during this phase. The main purpose of this research is to study the growth profile of *Saccharomyces cerevisiae* and to investigate the amount of product which is actinol formed during stationary phase biotransformation. From the first objective, the growth profile was obtained and substrate introduction (ketoisophorone) was deduced to be made at the 12^{th} hour. Biotransformation was carried out in shake-flask by adding non-growing cells of *S.cerevisiae* into culture medium. A model of 7820 Gas Chromatography was used to analyze the consumption of ketoisophorone, formation of (6R)-levodione as intermediate and (4R,6R)-actinol as the product. The results showed that production of both (6R)-levodione and actinol was only produced in a small amount. It may be due to no or low activity of carbonyl reductase or the conversion of substrate to product was too fast. For the product formation, actinol is strongly depends on the concentration of ketoisophorone. As the concentration of ketoisophorone is lower, the product formation will be lower too. As the time passes, concentration of substrate is reduced as it was consumed by the cells, so the actinol formation was reduced too.

CHAPTER 1

INTRODUCTION

1.1 Research background

1.1.1 Biocatalysis

Chemical industry during this era needs to satisfy the increasing societal requirement of more environmentally compatible processes besides optimizing the typical parameters characterizing a process. Undoubtedly, biocatalysis is the brightest area in order to search for an answer during this challenging demand. The application of biocatalysis has been extended by researcher where they have focused on understanding potential in terms of activity and stability, and also by successfully improving their ability using directed evolution (Liebeton et al., 2000). It is important to understand the advantages of biocatalysts, relative to many chemical catalysts, in terms of their specificity, structure, and activity under mild or extreme conditions, as well as their biodegradability, for advancement (Nakagawa et al., 2007). This is very useful for identifying the factors that currently limit the number and diversity of biocatalytic applications, such as limited enzyme availability, substrate scope, and operational stability. In the past 10 years, the rapid growth of biocatalysis is a direct result of research and development in enzyme engineering technologies, including molecular evolution and associated high-throughput screening methods (Aucamp et al., 2005).

Biocatalysis uphold to be an authoritative device for the decisive synthesis of optically pure pharmaceuticals that are difficult to access via conventional chemistry (Schrittwieser

et al., 2011). Biocatalyst has many applications, where it can be used for regio- and stereoselective reactions, or to introduce chirality (Bezborodov and Zagustina, 2016).

The most efficient application of biocatalysis requires the availability of suitable enzymes with high activity and stability under process conditions (Borchert and Buchholz, 1984). Nevertheless, the borderline stability of biocatalysts in many types of reaction media usually delayed the implementation for industrial-scale syntheses of pharmaceuticals and fine chemicals. Therefore, in order to improve enzyme stability and activity in desired reaction media, there is an interesting way in understanding the effects of solution conditions on protein stability (Mohamad et al., 2015). In addition, for thermostability and tolerance to low pH, mutation is targeted towards regions of protein sequence predicted to have a high propensity for aggregation. However, for retention of biocatalytic activity at high temperatures or pH, the attractive target is the stabilisation of the cofactor binding loops. Figure 1.1 shows biocatalysis development cycle (Schmid et al., 2001).

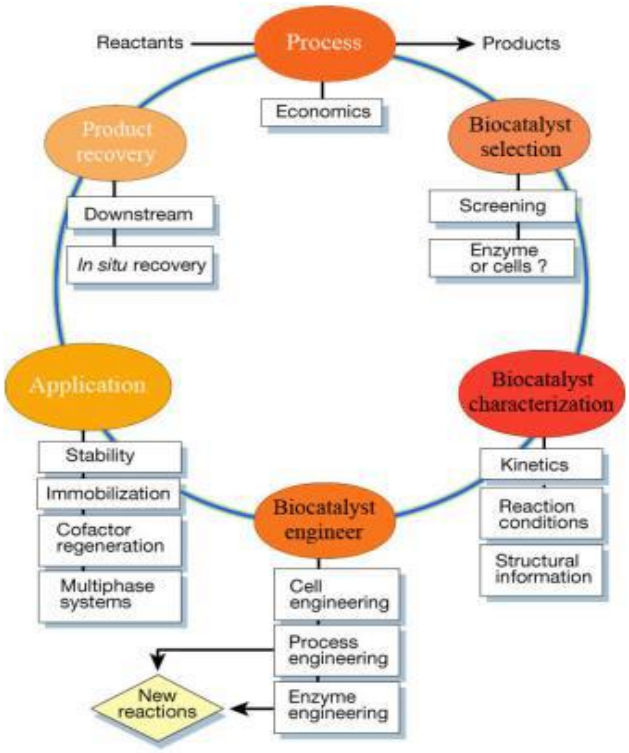

Figure 1.1 : Biocatalysis development cycle (Schmid et al., 2001)

1.1.2 Biocatalyst

Biocatalyst is a biological molecule that having catalytic properties with the ability to perform specific chemical transformations. It is useful for various industries such as bioprocesses in food, agricultural and chemical industries. Generally, for every biochemical reaction in the cell, it is catalysed by specific enzymes. They function in the same manner as do chemical catalysts, which is by lowering the activation energy. Isolation of enzymes, either in a crude or purified form, permits biotransformation directly without the need for whole cells. In fact, biotransformations with isolated enzymes rather than cells as biocatalysts confer some advantages where high selectivity can be obtained with enzyme biocatalysts due to fewer by-products are produced, and many enzymes confer high degrees of regioselectivity (Goswami and Stewart, 2016). Biocatalyst is useful for pharmaceutical

industry because it can catalyse reactions under mild conditions. These reactions require less energy and generate limited greenhouse gas emissions. Biocatalysts provide an excellent opportunity to decrease waste of organic syntheses for green chemistry applications. Their regio-, stereo-, and chemoselectivity provides additional avenues for green syntheses of complex fine chemicals and pharmaceuticals (Himmelberger et al., 2018).

1.1.3 Ketoisophorone

During 1970s, the oxidative conversion of α- and β-isophorone (α- and β-IP) to ketoisophorone (KIP) has received huge attention (Murphy et al., 2000). Interest in this cyclic enedione arises from its use as a key intermediate for the synthesis of various carotenoids and flavoring substances (Mayer, 1979a). Normally, KIP is prepared via allylic oxidation of α-IP and β-IP. α-IP is readily available and thermodynamically the more stable isomer. This show oxidation of α-IP is more attractive route to KIP, effectively eliminating the isomerization step involved in the conversion of α- to β-IP. However, β-IP is now considered a 'readily available' intermediate and liquid phase oxidations of β-IP mediated by manganese Schiff base and porphyrin catalysts afford KIP in 85% and 93% yields, respectively. Among all bioreductions of prochiral cyclic enones, ketoisophorone is probably the most interesting from an industrial point of view. Moreover, *S. cerevisiae* mediated reduction of KIP gave mainly (6R)-levodione, together with a small amount of (4R,6R)-actinol, and the reaction was successfully performed on a multi-kg scale (Murphy et al., 2000).

1.1.4 Actinol

Actinol (ACT), a key intermediate in the synthesis of optically active xanthophylls, xanthoxin and zeaxanthins as well as aroma constituent of tobacco and saffron (Sode et al., 1987) that can be produced by the reduction of ketoisophorone (KIP) with high yield and enantioselectivity. In a few cases, the sequential two-step reduction of OIP to (4R,6R)-actinol (R-ACT) was also explored using two different species of bacteria, namely *T. curvata* and *Bacillus stearothermophilus* (Buque-Taboada et al., 2005).

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. This conversion has been carried out via hydrogenation of the substrate (KIP) over alumina-supported Pt and Pd catalysts (von Arx et al., 1999) or over a Pd/Al2O3 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 as catalyst had a higher yield of 80–85% DOIP and a very attractive enantiomeric excess which is more than 98% (von 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).

1.1.5 Intermediate

Intermediate cell can be defined in terms of cellular phenotype as the quantifiable characteristics of a cell, which include gene expression, protein abundances, post-translational modifications, and cell morphology. For any state that lies between two traditionally defined cell types, it is considered as intermediate and it is referred to a generic intermediate cell state. Figure 1.2 shows cell states that have accompanying functions to be intermediate (MacLean et al., 2018).

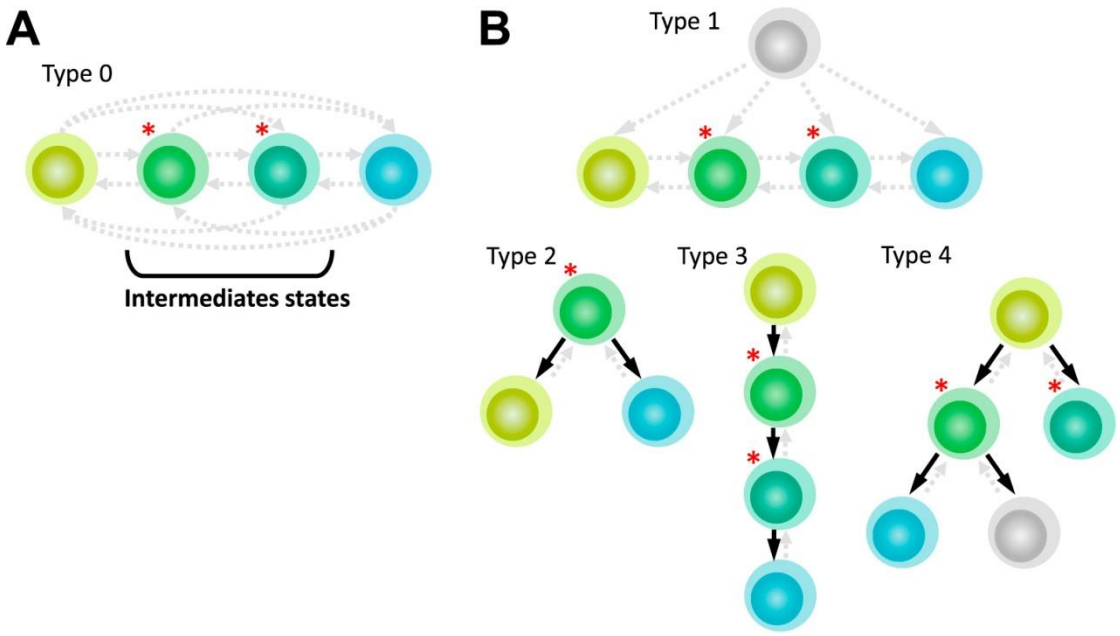

Figure 1.2 : Identities of intermediate cell states (ICSs). (A) An ICS (green, asterisk) refers to any phenotypic state lying between traditionally defined cell types (yellow or blue); generic ICSs are referred to as Type 0. (B) ICSs can facilitate cell state transitions in many ways, occupying the same (Type 1) or distinct (Types 2&3) hierarchical levels as other cell states. Complex lineage transitions can be mediated by ICSs (Type 4) (MacLean et al., 2018).

A reaction intermediate is transient species within a multi-step reaction mechanism that is produced in the preceding step and consumed in a subsequent step to ultimately generate the final reaction product. Intermediate reactions are common in the metabolism of metabolites and nutrients. The lifetime of an intermediate is usually short because it is usually consumed to make the next product of the reaction sequence (Pounds, 2001). The biotransformation of KIP produces DOIP as intermediate which transforms into ACT as shown in Figure 1 (Wada et al., 2003).

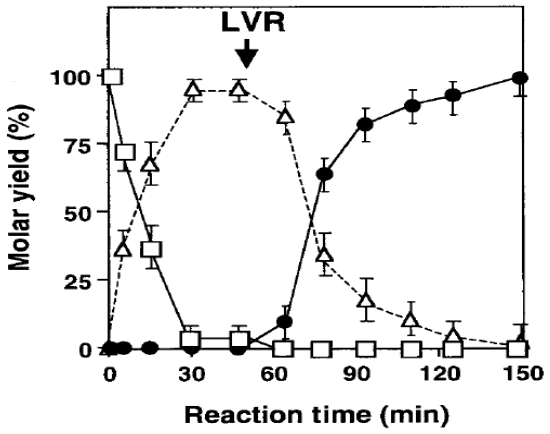

Figure 1.3 : Two-step enzymatic conversion from KIP to ACT; KIP molar residual rate (□), DOIP molar yield (Δ), and ACT molar yield (●) (Kataoka et al., 2004b).

1.1.6 Cofactor

A cofactor is a non-protein chemical compound or metallic ion that is required for an enzyme's activity. Cofactors can be considered "helper molecules" that assist in biochemical transformations. The rates at which these happen are characterized by enzyme kinetics. They are divided into two major groups which are organic cofactors and inorganic cofactors. The example of organic cofactor is NADH and inorganic cofactor is metal ions. Cofactors are involved in numerous intracellular reactions and critically influence redox

balance and cellular metabolism. Cofactor engineering can support and promote the biocatalysis process, even help driving thermodynamically unfavorable reactions forwards. To achieve efficient production of chemicals and biofuels, cofactor engineering strategies such as altering cofactor supply or modifying reactants' cofactor preference have been developed to maintain redox balance (Wang et al., 2017).

The NADH is the cofactors needed for the biotransformation of ketoisophorone which are produced in the Krebs Cycle. The Krebs Cycle is part of glycolysis process which converts glucose into energy. Glycolysis is reduced to three reactions. During the first reaction, the conversion from glucose to glyceraldehyde-3-phosphate (GAP) occurs which it consumes two ATP and produces two GAP. For the second reaction, it contains the GAP dehydrogenase reaction that produces NADH in the cytosol and 1,3-diphosphoglycerate (1,3-DPG) and the third reaction lumps together the remaining glycolysis reactions which produces two ATP molecules per GAP; four per glucose molecule and one molecule of pyruvate; two per glucose molecule (Dynnik et al., 1980). Figure 1.3 shows the overview of Krebs Cycle that consist of Krebs cycle enzymes, intermediates and their connections to the other major energy metabolic pathways.

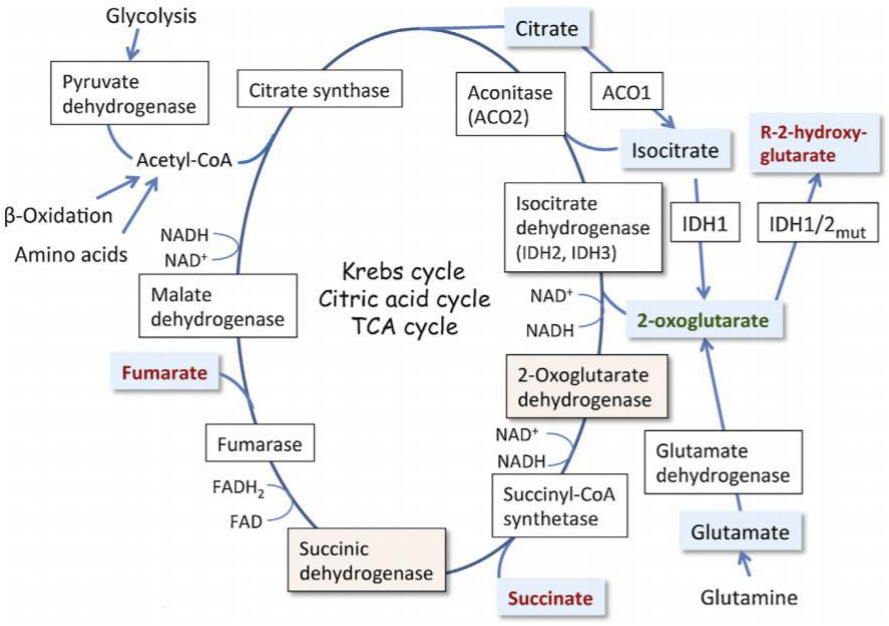

Figure 1.4 : An overview from the Krebs cycle enzymes, intermediates and their connections to the other major energy metabolic pathways (Salminen et al., 2014).

1.2 Problem Statement

Fine chemical industry is increasingly taking advantage of biocatalysts to produce high-value products because enzymes and whole-cell biocatalysts can be sometimes more convenient than synthetic chemistry. In biotransformation of ketoisophorone, *S. cerevisiae* is always been used. So, the injection of substrate into the sample should be controlled to determine substrate utilization rate and to make sure high amount of product is produced. However, less research had mentioned the starting time for the stationary phase in fermentation of *S. cerevisae*. Hence, this research is carried out to study the details during stationary phase of growth of *S. cerevisae.*

1.3 Research Objectives

i. To study the growth profile of *S. cerevisiae*.

ii. To investigate the suitable time to introduce the ketoisophorone as a substarate.

iii. To investigate the amount of (4R,6R)-actinol formed during stationary phase biotransformation.

1.4 Organization of thesis

This thesis consists of five main chapters and each chapter contributes to the sequence of this study. The following are the contents for each chapter in this study:

Chapter 1 introduces research background, problem statement, research objective and organization of thesis.

Chapter 2 discusses the literature review of this study made.

Chapter 3 covers the experiment materials and the details of methodology. Discussion is made on the description of equipment and materials used, experimental procedure and description on types of test made.

Chapter 4 discusses results and discussion.

Chapter 5 provides conclusion about the findings of the experiment.

CHAPTER 2

LITERATURE REVIEW

2.1 Baker's Yeast

Baker's yeast or its scientific name, *Saccharomyces cerevisiae* was reported as a model organism in several experiments, which can be performed under biologically and technically well- controlled conditions after exposure to microwave radiation (Vrhovac et al., 2010). Yeast cells in culture follow a very predictable pattern of growth which is growth curve type II that can easily be divided into four phases (Alsuhaim et al., 2013). The phases are lag phase, exponential phase, stationary phase and death phase. During the lag phase, no growth occurs as newly pitched yeast cells mature and acclimatize to the environment. Then, during exponential phase, cells start to grow and divide rapidly. The third phase in growth of yeast is stationary phase when metabolism starts to slow down and the cells stop from dividing rapidly. The factor affected cells to enter stationary phase is related to change in the environment typically caused by high cell density. The final phase of the growth curve is the death phase, which is characterized by a net loss of culturable cells. In the death phase, individual cells may metabolize and divide, but more cells tend to loss than are gained so there is a net loss of viable cells (Maier and Pepper, 2015).

2.1.1 Growth of yeast

Saccharomyces cerevisiae was reported as a model organism in several experiments, which can be performed under biologically and technically well-controlled conditions after exposure to microwave radiation (Vrhovac et al., 2010). Yeast cells in culture follow a very predictable pattern of growth which is growth curve type II that can

easily be divided into four phases (Alsuhaim et al., 2013). The phases are lag phase, exponential phase and stationary phase and death phase. The cell division of yeast occurs by budding in which a daughter is initiated as an out-growth from the mother cell, followed by nuclear division, cell-wall formation, and finally cell separation. The sizes of haploid1 and diploid2 cells vary with the phase of growth and from strain to strain (Herskowitz, 1988). For routine culture, scientists usually use rich media that supply all the nutrients that cells need to grow. The individual components of rich media are often undefined. For example, yeast is commonly grown in a medium known as YPD, which is simple and inexpensive to prepare (Russell, 2016). However, in this experiment, Luria-Bertani was used as a medium to grow *S. cerevisiae*.

When a culture of yeast cells is inoculated in a fresh growth medium, they enter a brief lag phase where they are biochemically active but not dividing. During the lag phase, no growth occurs as newly pitched yeast cells mature and acclimatize to the environment (Muñoz Flores and Catrilaf, 2013). The lag phase refers the initial growth phase, when number of cells remains relatively constant prior to rapid growth, also referred as adaptation time. During this phase the individual cells are actively metabolizing, in preparation for cell division. The cells usually activate the metabolic pathways to make enough of the essential nutrients to begin active growth. From literature it is seen that the duration and extent of this phase depends on firstly the initial population size and secondly environmental conditions like temperature, pH, alcohol, oxygen, salt concentration, nutrients and etc (Hill, 2016).

Then, entering the exponential phase, cells start to grow and divide rapidly. During exponential growth the rate of increase of cells in the culture is proportional to the number

of cells present at any particular time. This exponential phase depends on several factors: the organism itself, the growth medium, and the temperature are all important factors in determining the generation time (Chien et al., 2012).

The third phase in growth of yeast is stationary phase when metabolism slows and the cells stop rapid cell division. The factors that cause cells to enter stationary phase are related to change in the environment typically caused by high cell density. During this phase, cells may enter into a specialized non-dividing resting state. The entry into such resting states is typically accompanied by a dramatic decrease in the overall growth rate and an increased resistance to a variety of environmental stresses (Herman, 2002).

The final phase of the growth curve is the death phase, which is characterized by a net loss of culturable cells. Even in the death phase there may be individual cells that are metabolizing and dividing, but more viable cells are lost than are gained so there is a net loss of viable cells (Maier and Pepper, 2015).

2.1.2 Difference between baker's and brewer's yeast

Many people get confused with these two popular yeasts; brewer's and baker's yeast. Both of these yeasts are made from strains of the *Saccharomyces cerevisiae* fungus and they will turn sugar into alcohol and carbon dioxide. However, they are differ in their composition, uses and health factors. Baker's yeast is a blend of several strains of *S. cerevisiae* chosen for their flavor and ability to make carbon dioxide, which causes bread to rise while brewer's yeast is made of strains chosen for their alcohol-producing ability and tends to have a bitter flavor. Brewer's yeast is used to brew homemade wines and beers, while baker's yeast makes bread rise (Cespedes, 2017).

2.1.3 Advantages of baker's yeast

Yeast has been used as a stereospecific bioreduction biocatalyst. They possess relatively rigid cell walls that enabled their structures to be retained in the presence of various organic compounds and solvents (Kheng and Hekarl, 2011). *S. cerevisiae* showed that they can catalyse compounds with carbonyl groups or carbon-carbon bonds (Ward and Young, 1990). Then, it is able to utilize sugars in the presence of oxygen (aerobic) and in conditions that are completely absent of oxygen (anaerobic). Aerobic catabolism produces more energy and is called respiration, while anaerobic catabolism produces less energy and is called fermentation. Compared to most other yeast species, baker's yeast is able to perform alcoholic fermentation, and can grow under strictly anaerobic conditions (Dashko et al., 2014). It is one of the few yeast that is able to grow anaerobically. Besides, a researcher at Charles Drew University of Medicine and Science is investigating the potential use of non-pathogenic baker's yeast as a promising, natural therapy for cancer. Dr. Mamdooh Ghoneum presented his findings where there is a theory that cancer cells will destruct themselves when exposed to small amount of yeast. Then, in laboratory tests, cancer cells had been exposed to yeast and it was found as they ingested the yeast-through a process known as phagocytosis and the cancer cells died. There were several times of test had been carry out. Firstly, this phenomenon was investigated in test tubes, introducing yeast to breast, tongue, colon, and skin cancers. Next experiment, yeast was injected inside the tumors of mice and it was observed there was a decrease in the size of the tumor mass. Lastly, yeast was examined whether it could kill cancer cells in mice that had cancer metastasized to the lung and the results also showed significant clearance of the cancer cells from the lung (Ghoneum et al., 2007).

2.1.4 Disadvantages of baker's yeast

Some drawbacks of using baker's yeast have always been some concern for industry. It is due to its sensitivity to temperatures greater than 35°C and is subject to lactic acid bacteria contamination and glucose repression (Barrette et al., 1999). Apart from that, it was found that baker's yeast is not suitable for use in the industrial production as the yields of 6R-dihydrooxoisophorone (DOIP) were too low. There were two problems arise during the production process using baker's yeast and needed to be overcome for developing an industrially feasible production process. First, yeast cells could not be reused because of its short lifetime of the reaction activity of the yeast. Secondly, the purification process was complicated because it was difficult to separate the yeast from the culture solution after the catalytic reaction (Cespedes, 2017).

2.1.5 Optimum condition for baker's yeast

One of the most important factors that affect the growth of baker's yeast is temperature. Thus, we investigated the effect of temperature on the growth rate of *S. cerevisiae*. The optimum temperature for growth is around 30°C (Salvado et al., 2011). At low temperature (1-10°C), the cells are viable but they do not grow well (Arthur and Watson, 1976). At temperatures from 28°C to 37°C, the growth rate increases. However, at temperatures from 39°C to 41°C, the growth rate does not increase, but the cells are still viable. In addition, the viability of cells decreases at 42°C and higher temperatures, which in turn, decreases the growth rate of *S. cerevisiae* (Mensonides et al., 2002).

2.1.6 Fermentation of baker's yeast

Baker's yeast production is a fed-batch fermentation with an inoculum of *S. cerevisiae* (ATCC 32167) culture and as substrate feed (carbon) a glucose solution. There are three metabolic pathways; respirative growth on glucose, fermentative growth on glucose and lastly, respirative growth on ethanol. For respirative pathway, it occurs in presence of oxygen and the fermentative one occurs without oxygen (with production of ethanol) (Pierre, 2017). The production of aker's yeast can be considered as very old food biotechnology and may be known as "ripened technology." During the turn of the 20th century, baker's yeast industry had developed independently from distilleries where high alcohol yield gave little yeast biomass. During the golden age of modern baker's yeast manufacturing, from 1910 to 1940, much effort was put in to find the best and cheapest growth media available. Extra concentration was given on the control of both infection and sugar concentration in such sugar-rich growth media in order to increase biomass yields and reduce costs (Pierre, 2012). Table 2.1 lists some of factors that affecting the fermentation process.

Table 2.1 : Factors that affecting fermentation processs (El-Din and El-Shazly, 1969)

Characteristic	**Explanation**
Temperature of room	24-27°C
Fermentation time	Depends on type of pre-ferment
Amount of yeast	The more the yeast, the more time
Type of yeast	Instant active dry yeast contains fast acting yeast
pH value	4-6

In *S. cerevisiae*, high sugar concentrations and high specific growth rates trigger alcoholic fermentation, even under fully aerobic conditions. Alcoholic fermentation during the industrial production of baker's yeast is highly undesirable, as it reduces the biomass yield on the carbohydrate feedstock. Industrial baker's yeast production is therefore performed in aerobic, sugar-limited fed-batch cultures. The conditions in such cultures differ drastically from those in the dough environment, which is anaerobic and with sugars at least initially present in excess (Kaspar von Meyenburg, 1969).

2.2 Biotransformation

Biotransformation has always been an active participant in the field of biotechnology and, since the number of available cells and enzymes exploitable in performing selective biotransformation into a myriad of desired products continues to (Hilterhaus and Liese, 2009). Biotransformation is organic reactions that utilize biological catalysts. Biocatalysts can be either whole cells or enzymes where they are used under several conditions like free enzymes, whole cells, immobilized enzyme or whole cells, aqueous and two phase systems. Biotransformation was observed by humans well before they were appreciated as having an underlying microbial cause (Parales et al., 2002).

Biotransformation has many advantages when compared to the corresponding chemical methods. Some biotransformation can be cheaper, economic, more direct than chemical analogues and the conversion normally proceeds under conditions that are regarded as environmentally acceptable (Sultana and Saify, 2013). In biotransformation, the enzymes or whole cells provide a significant improvement in reaction rates in order to exhibit high stereo-selectivity over the corresponding reactions. Moreover, microbial transformation offers the advantages of highly selective operation at low pH, room

temperature and low levels of toxic waste products (Parales et al., 2002). Table 2.2 shows the advantages and disadvantages of biotransformation.

Table 2.2 : Advantages and disadvantages of biotransformation

Advantages	**Disadvantages**
Microorganism such as bacteria able to produce large amount of biomass and great variety of different enzymes in a short time.	If the substrate is toxic, it can kill the microorganisms. Therefore, no transformation will be observed.
The chemio-, regio-, and enantioselectivity of enzymes, because of small size of bacteria have by far the largest surface to volume ratio, it allow them to maximize their metabolic rates because of high exchange of molecules and metabolites through their surface.	Alternatively, if the microorganisms use the substrate as an energy source (carbon source food), no transformed or untransformed material will be recovered.
Microorganisms have great potential for inducing new or novel enzyme systems that capable to convert foreign substrates.	Very low chemical yields are obtained due to the involvement of a complex biological system.
Microorganisms are capable of producing unique enzymes that are stable toward heat, acid and alkali.	Many of the ground rules for applying biotransformations are not yet well-defined.
A combination of microbial transformation (chemo and enzymatic synthesis) can be exploited for partial, as well as the total synthesis of organic compound.	Many chemical reactions have no equivalent biotransformations and vice-versa

Biotransformation with recombinant microbial enzymes has been widely used; such as its applications in producing of hormones, antibiotics, and speciality chemicals (Seeger et al., 2003). Table 2.3 gives some randomly chosen milestones in the history of applied biotransformation.

Table 2.3 : Milestones in the history of applied biotransformation (Leresche and Meyer, 2006)

Year	**Process**
5000 BC	Vinegar production
800 BC	Casein hydrolysis with chymosin for cheese production
1670	"Orlean" process for the industrial bio-oxidation of ethanol to acetic acid
1680	Antoni van Leeuwenhoek first to see microorganisms with his microscope
1897	E.Buchner discovers yeast enzymes converting sugar into alcohol
1934	Regioselective biooxidation of sorbite to sorbose for the Reichstein Vitamin C synthesis
1940	Sucrose inversion using an invertase
1950	Bioconversion of steroids
1970	Hydrolysis of penicillin to 6-aminopenicillanic acid
1973	Fist successful genetic engineering experiments
1974	Glucose to fructose isomerisation with immobilized glucose isomerase
1985	Enzymatic process for the production of acrylamide
1990	Hydrolysis by protease (trypsin) of porcine insuline to human insulin
1995	3000 ton pa plant for the biotransformation of nicotinonitrile to nicotinamide

2.2.1 Biotransformation of ketoisophorone

Ketoisophorone (KIP) is an industrially important cyclic endione since 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 and zeaxanthin. (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*, where in situ removal of the product is employed by an external crystallization step (Taboada et al., 2004).

There was an investigation has been carried out where two steps were needed in production of actinol from the commercially available ketoisophorone; by using old yellow enzyme and levodione reductase for enzymatic asymmetric reduction (Wada et al., 2003). It was reported that for the first time, 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. A scheme predicted for the biotransformation of ketoisophorone is strongly supported by re-incubation of one of the products [(6R)-levodione] with the same cultured plant cells under identical conditions (Nishii et al., 1989).

Biotransformation of ketoisophorone by *Marchantia polymorpha* and *Nicotiana tabacum* cultured suspension cells was observed with products isolated from the cultured medium including: (6R)-levodione and (4R,6R)-actinol as major products as well as

(4R,5S)-4-hydroxy-3,3,5- trimethylcyclohexanone, (4R)-hydroxyisophorone and (4S)-phorenol as the identified minor products. The endocyclic olefin ketoisophorone was initially reduced and then followed by carbonyl reduction to produce (4R,5S)-4-hydroxy-3,3,5-trimethylcyclohexanone and (4R,6R)-actinol. Additionally, carbonyl reduction of ketoisophorone was observed generating (4R)-hydroxyisophorone and (4S)-phorenol. Table 2.4 shows the details of the process biotransformation of ketoisophorone with cultured cells of *M. polymorpha* (Hegazy et al., 2008).

Table 2.4 : Properties of biotransformation of ketoisophorone with cultured cells of *M. polymorpha* (Hegazy et al., 2008)

Compound	Conversion (%)[a]		Enantiomeric excess (%)[b]		Configuration
	M. polymorpha	*N. tabacum*	*M. polymorpha*	*N. tabacum*	
2	45	41.6	37	76.4	6*R*
3	20.5	38.8	17.5	56.6	4*R*,5*S*
4	14.8	7.4	> 99	> 99	4*R*,6*R*
5	4.8	7.18	55.7	9.6	4*R*
6	13.8	3.7	> 99	9.1	4*S*

[a] Incubated for 7 days.
[b] e. e. is determined by GLC.

2.3 Reduction of ketoisophorone

The reduction of ketoisophorone or 2,6,6-trimethylcyclohex-2-ene-1,4-dione become an interest for its beneficial products due to its interesting mechanism through biotransformation. Its reduction was first synthesized and reported for more than four decades (Mayer, 1979b). During the reduction of KIP, it could be summarized that the rate of product formation is totally governed by two enzymes. However, in reduction reaction, the case is getting more challenging since the product formation is dependent on the

amount of cofactor available within the cells (Su et al., 2015). In the figure shown below, there is a cofactor that coupled the ketoisophorone reduction. Two compounds shown; compounds X_{Red} and X_{Ox} are the reduced and oxidized formed of X respectively, that occur during the oxidation process. The process of oxidation could take place by many substrates within the cell, so, X represents an arbitrary compound that undergoes such oxidation reactions (Uzir and Najimudin, 2018). Figure 2.1 shows the oxidation process in reduction of ketoisophorone.

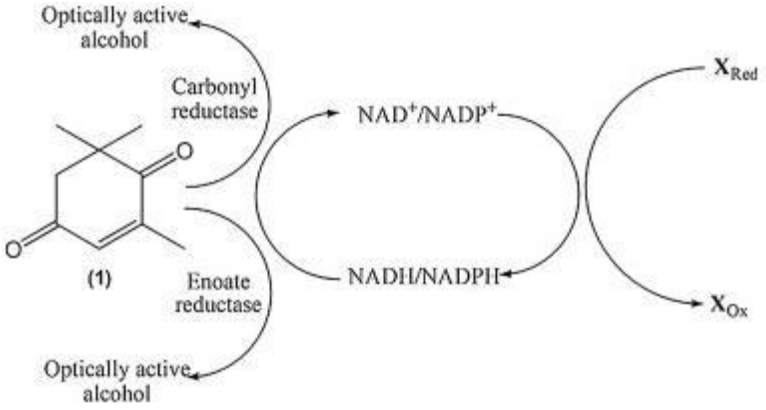

Figure 2.1 : Oxidation process in reduction of ketoisophorone (Uzir and Najimudin, 2018)

2.4 Enzymes involved in reduction of KIP

Carbonyl reductase or commonly known as alcohol dehydrogenase (ADH), is a compound that mediates the reduction of aldehyde and ketone to alcohol, while enoate reductase or normally known as Old Yellow Enzyme, (OYE) is a compound that catalyzes the reduction of the CC bond are the two enzymes that stereoselectively reduced ketoisophorone (Winkler et al., 2012). Both of these enzymes work in parallel and sequential in order to keep the biological system alive. These connections of enzymes could

coup the ability of a microorganism in order to become an important biocatalyst that could lower the cost of a process and maintaining a green environment (Uzir and Najimudin, 2018). Figure 2.2 shows the role of these two enzymes that works together in biotransformation of ketoisophorone and finally leads to formation of final products.

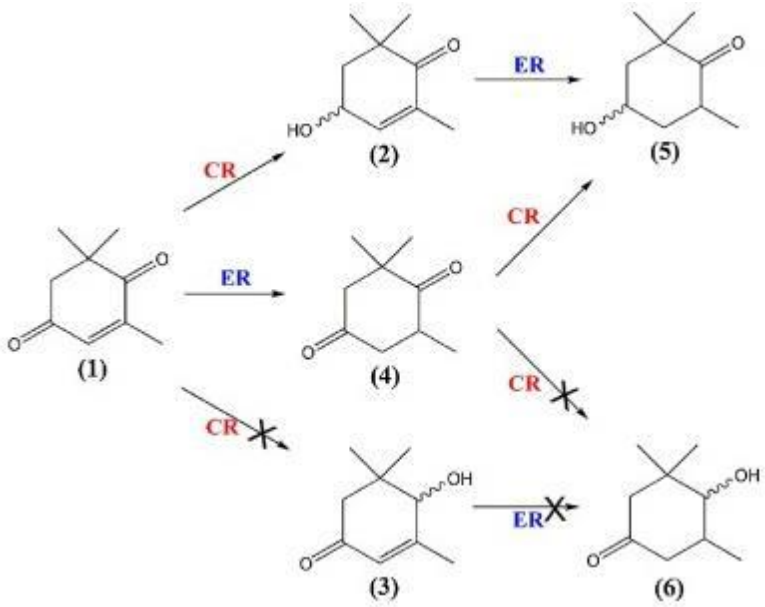

Figure 2.2 : Ketoisophorone bioreduction in yeasts (Uzir and Najimudin, 2018)

Description of figure 2.2

1) 2,6,6-trimethylcyclohex-2-ene-1,4-dione [ketoisophorone]
2) 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 – enoate reductase

CR – carbonyl reductase

2.4.1 Enoate Reductase (OYE)

Old yellow enzyme (OYE) is a group of flavor enzymes that act as catalyst in stereoselective reduction of activated C=C bonds in the presence of nicotinamide cofactor. It was first discovered in 1933 by Christian and Warburg during their investigation on biological oxidations. OYE was detached from brewer's bottom yeast and categorized as the first flavin-dependent enzyme. OYE has been used as a reducing agent during Warburg's first demonstration while he was investigating the reaction between glucose-6-phosphate, $NADP^+$, an enzyme ("Zwischenferment"), and a substance called "Gelbe Ferment", later identified as OYE and permitting the system to form a complete respiratory chain reacting with molecular oxygen. Studies have been extended as an attempt to identify the physiological function of OYE (Brige et al., 2006)

Members of the "Old Yellow Enzyme" family share common catalytic properties as they are able to reduce a variety of different substrates such as nitro esters, nitro aromatics, and α,β-unsaturated compounds (activated by an electron withdrawing group such as aldehyde, ketone, imide, nitro, nitrile, carboxylic acid or ester) at the expense of a nicotinamide cofactor (Stuermer et al., 2007). OYE interacts with phenolic compounds in order to generate long wavelength charge transfer bands in the region of 500-800 nm, which were attributed to the formation of OYE-phenolate complex (Stott et al., 1993). The competing reduction of C=C- and C=O- bonds by enoate reductases often resulted in poor chemoselectivity in the overall reaction and produced the corresponding alcohols of the saturated products (Fronza et al., 1996).