STEREOSPECIFIC BIOTRANSFORMATION OF 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE (KETOISOPHORONE) IN A NON-GROWING WHOLE CELL *SACCHAROMYCES CEREVISIAE*

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by

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Nur Zaherra Binti Zakaria

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LIST OF SYMBOL

	Symbol	Unit
C	Concentration	g/L
M	Molarity	M
pH	Potential of hydrogen	-
ΔT	Rate of change of temperature	$^{o}C/min$
RPM	Revolution per minute	r.p.m
T	Temperature	^{o}C
t	Time	hr
L	Volume	L
λ	Wavelength	nm

LIST OF ABBREVIATIONS

(6R)-levodione	2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione]
(4S)-phorenol	4-hydroxy-2,6,6-trimethylcyclohex-2-ene-1-one
(4R,6R)-actinol	4-hydroxy-2,6,6-trimethylcyclohexane
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
ERs	Enoate reductases
FID	Flame ionization detector
GC	Gas chromatography
GMHP	Genetically modified herbicide plant
HCO_2H	Formic acid
IUB	International Union of Biochemistry
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KIP	2,6,6,-trimethycyclohex-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
OYEs	Old yellow enzymes
S.cerevisiae	*Saccharomyces cerevisiae*
Tri-HCL	Tri-hydrochloric acid
UV-vis spectrophotometer	Ultraviolet visible spectrophotometer
X	Electron-withdrawing substituent

STEREOSPESIFIK BIOTRANSFORMASI 2, 6, 6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE (KETOISOPHORONE) DENGAN MENGGUNAKAN SEL KESELURUHAN *SACCHAROMYCES CEREVISIAE* YANG TIADA PERTUMBUHAN

ABSTRAK

Biotransformasi ketoisophorone dengan menggunakan sel yang tiada pertumbuhan telah dicadangkan dalam kajian ini. Tujuan kajian ini adalah menyiasat kesan penggunaan semula kofaktor dengan menggunakan kepekatan yis yang berbeza dan untuk mengkaji masa kursus bagi 2,6,6,-trimethycyclohex-2-ene-1,4-dione (ketoisophorone) menghasilkan pertengahan yang sepadan seperti 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione] dan 4-hydroxy-2,6,6-trimethylcyclohex-2-ene-1-one [(4S)-phorenol] termasuk produk utama, 4-hydroxy-2,6,6-trimethylcyclohexane [(4R,6R)-actinol] dengan menggunakan sel keseluruhan *Saccharomyces cerevisiae* yang tiada pertumbuhan. Biotransfromasi fasa cecair telah dijalankan dalam kelalang goncang bersama media sebagai penampan tindak balas pada 37^{o}C dan 150 rpm dengan menggunakan sel *S.cerevisiae* yang tiada pertumbuhan. Penggunaan semula kofaktor daripada nikotinamide adenina dinukleotida koenzim (NADH/NAD^{+}) and derivatifnya (NADPH/NADP^{+}) telah dikaji dengan menggunakan spekta penyerapan ultraviolet-yang boleh dilihat. Spektrum kofactor telah dipantau panjang gelombang kepada 340nm dan sampel telah dianalisis setiap satu jam sepanjang kajian dijalankan. Bagi penghasilan produk pertengahan, (6R)-levodione mempunyai kepekatan yang lebih tinggi berbanding kepekatan (4S)-phorenol disebabkan persaingan di antara koenzim dan kadar tindak

balas ikatan pengurangan karbon-karbon kembar lebih tinggi berbanding tindak balas pengurangan karbonil.

STEREOSPECIFIC BIOTRANSFORMATION OF 2, 6, 6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE (KETOISOPHORONE) IN A NON GROWING WHOLE CELL SACCHAROMYCES CEREVISIAE

ABSTRACT

Biotransformation of ketoisophorone by non-growing cells was proposed from the study. The aim of this research is to investigate the effect of cofactor regeneration by using different yeast's concentration and to investigate time courses for 2,6,6,-trimethycyclohex-2-ene-1,4-dione (ketoisophorone) to produce the 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] as well as the main product, 4-hydroxy-2,6,6-trimethylcyclohexane [(4R,6R)-actinol] in a non-growing whole cell *Saccharomyces cerevisiae*. The liquid-phase biotransformation was carried out in shake-flask with buffer as the reaction medium at 37^{o}C and 150 r.p.m using non-growing cells of *S.cerevisiae*. The cofactor regeneration of nicotinamide adenine dinucleotide coenzyme (NADH/NAD^{+}) and its derivatives (NADPH/NADP^{+}) was investigated using changes in ultraviolet-visible absorption spectra of these compounds. The spectrum of cofactor was monitored at 340nm wavelength and the sample was analysed for every one hour along the experiment. For the production of intermediates, (6R)-levodione has higher concentration as compared to the concentration of (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

In the past, governments and industry focused on reducing risk by minimizing exposure to the workers and release of chemicals into the environment. Hence, green chemistry is one of the incentives to prevent environmental problem. Green chemistry defines as integration of industrial manufacturing practise with the natural world. For example, Baker's yeast is not only used to make bread but it can also catalyse the reduction of various carbonyl compounds. The advantages of these incentives are higher yield and eliminate some of the wastes even though the reaction is carried out in the smaller scale. Besides, it can also reduce the cost of raw materials needed and cost for waste treatment (Tao and Kazlauskas, 2011). Table 1.1 shows the list of 12 principles of green chemistry on how biocatalysis gives bigger contribution to green chemistry.

Chemists gradually recognized the potential of biochemical reactions in which practical and conceptual problems can also be solved because of the recent advances in biotechnology. One example of practical problems is enzyme stabilization, while the examples of conceptual problem are difficulty of enzyme to react with narrow range of biochemical intermediate. These problems exist due to substrate and complex enzyme properties such as stability, stereoselectivity, substrate range and reaction type (Tao and Kazlauskas, 2011).

Table 1.1: 12 principles of green chemistry (Tao and Kazlauskas, 2011).

1. Design safer chemicals and products
2. Prevent waste
3. Design less hazardous and chemical syntheses
4. Use renewable raw materials and feedstocks
5. Use catalysts, no stoichiometric reagents
6. Avoid chemical derivatives
7. Maximize atom economy
8. Use safer solvents and reaction conditions
9. Increase energy efficiency
10. Minimize potential of risks
11. Design chemicals and products to degrade after use
12. Analyse in real time to prevent pollution

1.1.2 Class of biotechnologies

Biotechnology is a very broad discipline, which employs biological processes to develop new tools, products and technologies that improve our daily live. These technologies have been used for over 6,000 years by mankind and contribute in biological research, agriculture, industry and medicine. These wide fields of biotechnologies have been organized based on colour coded classification with white, green and red biotechnology. Blue, yellow, grey, black and even gold biotechnology leads this other major technologies due to constant technological advancement (Frankenberg-Dinkel, 2015). Table 1.2 describes class of biotechnologies based on the colour codes.

Table 1.2: Colours of biotechnology (Frankenberg-Dinkel, 2015).

Colour classification	**Description**
White	Industrial processes involving microorganisms
Green	Processes improving agriculture
Red	Medicine, health, diagnostics
Blue	Marine and aquatic systems
Yellow	Food biotechnology and nutrition
Grey	Environmental biotechnology
Black	Biowarfare, bioterrorism
Gold	Bioinformatics

White technology refers to technologies that employ microorganism in chemical production. This technology is gene-based technologies that used to generate efficient production strains. Red biotechnology is related to pharmaceutical industry. Examples of red biotechnology are production of vaccines and antibodies, drug development and genetic engineering for disease treatment. Blue biotechnology is involving environmental and industrial applications. It applies molecular biological methods to marine and freshwater organisms. Blue technology helps the growth of seafood in aqua-culture and the use of drugs, enzymes and other products from marine organisms (Frankenberg-Dinkel, 2015).

Green biotechnology has several aims, these include; creates new crop varieties resistant to disease and pests, to produce crop varieties with improved nutritional properties and to modify plants that can be used as bio-factories to produce biomedical substances (Frankenberg-Dinkel, 2015). For example, the uses of bio-fertilizers to defend crops against pests, extreme weather as well as enhance growth and development of the plant. One of the innovations in green biotechnology is genetically modified herbicide plant (GMHP). The advantages of GMHP are the

need to plough fields can be reduced and help to protect the soil structure due to use of tractor for plant crops preparation. The agricultural practice of ploughing is known as “tillage”. The purposes introducing no tillage systems are to reduce use of fuel and lower carbon dioxide emission (Yashveer et al., 2014).

1.1.3 Biocatalysis

Biocatalysis is becoming one of the most powerful tools in biotechnology that influence social impact on health, food supply, environmental protection and sustainable fuel production. They have been used for hundreds of years and developed in several fields as genetics, molecular biology, fermentation biotechnology, biotransformation, nanotechnology, material sciences, advanced spectroscopy and others (Illanes et al., 2012). In 1980 and 1990s, microbial enzymes was widely used compare to plants and animal enzymes in many industries including food, detergents, textiles, leather, pulp and paper, diagnostics and therapy (Sanchez and Demain, 2010).

European Federation of Biotechnology defined biocatalysis as the integration of natural sciences and engineering science (Grunwald, 2009). According to the International Union of Biochemistry (IUB), enzymes can be divided into six classes that are; oxidoreductases, transferases, hydrolases, isomerases and ligases (Sanchez and Demain, 2010). However, the use of enzymes in organic synthesis has been difficult to adopt in industrial scale. 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).

1.1.4 Biocatalysis versus chemical catalysis

Biocatalysis may be broadly defined as the use of enzymes or whole cells as biocatalyst for industrial synthetic chemistry (Zhao, 2006).The availability of suitable enzyme gives good activity and stability. Hence, substrate selectivity and high enantioselectivity is required for the efficient application of biocatalysts (Ye et al., 2010). The most important advantage of biocatalyst in chemical reactions is high selectivity. There are three types of selectivity, and these include; chiral (i.e., stereoselectivity), positional (i.e., regioselectivity), and functional group specific (i.e chemoselectivity) (Zhao, 2006). Many biocatalysts with high chemo-, regio-, and stereo-selectivity at room temperature, making them superior as compared to chemical catalysts (Ye et al., 2010).

Besides that, there are some disadvantages using biocatalyst. Some serious drawbacks may occur due to limited operating conditions, substrate or product inhibition and reactions in aqueous solutions. In contrast, many of these drawbacks turn out to be misconceptions. For example, enzymes that shows excellent stability can also accept non-natural substrate to convert into desired products (Zhao, 2006). Table 1.3 shows advantages and disadvantages of biocatalysis in comparison to the chemical catalysis.

Table 1.3: Advantages and disadvantages of biocatalysis in comparison with chemical catalysis (Grunwald, 2009; Zhao, 2006).

Advantages	**Disadvantages**
More efficient because lower concentration of enzymes is needed.	Susceptible to substrate or product inhibition.
Can be modified to increase selectivity, stability and activity.	Protein molecules are rather instable in aqueous media.
More selective (chemoselectivity, regioselectivity, diastereoselectivity and enantioselectivity).	Enzymes found in nature only one enantiomeric form.
Milder reaction condition and low consumption (typically in a pH range of 5-8 and temperature range of 20-40^{o}C.	Limiting operating region (enzyme can be denatured at high temperature and pH).
Environmental friendly.	Enzymes can cause allergic reactions.
Low production of by-products.	

1.2 PROBLEM STATEMENT

Most chemical reactions go pretty slowly at room temperature. In order to speed up the reaction, catalyst is required. A catalyst is any substance that speeds up a reaction without taking part in it and at the end of the reaction the same amount of catalyst can be reuse and recycled.

Industrial catalysts are mostly made up of metals. Metal catalysts have large number of electrons to help out in reactions before claiming them back once the reaction is over. Examples of chemical catalysis are iron-based catalysts that used for making ammonia via the Haber-Bosch process and the nickel catalyst is used for

making saturated fats. Unfortunately, chemical catalyst can give negative consequences when take place naturally. For example, nitric oxide can cause depletion of ozone layer. Hence, most industries use biocatalysts as compared to chemical catalyst due to serious environmental problem.

Biocatalysts are useful for pharmaceutical industry because they can catalyse reactions under mild conditions. These reactions require less energy and generate limited greenhouse gas emissions. Enzyme-catalysed reactions are also ideal for the synthesis of chiral pharmaceutical compounds because of their high efficiencies and stereo- and regioselectivities.

As a result, while the demand using biocatalysis is increasing, the use of enzymes especially in pharmaceutical industry remains limited compared with the use of chemical catalyst. Generally, biocatalyst also has alternative routes in manufacturing process although development times are longer.

In this study, Baker's yeast is used as biocatalyst for production of alcohol because of its speciality in stereospecific reduction process. Some research works on biotransformation of ketoisophorone in a growing whole-cell *S. cerevisiae* was reported (Hoshino et al., 2005). Meanwhile, the effect of ketoisophorone in a non-growing whole-cell *S.cerevisiae* is still unknown. Hence, this research investigates the stereospecific biotransformation of ketoisophorone in a non-growing whole-cell *S. cerevisiae* at different cell concentration.

1.3 RESEARCH OBJECTIVES

This research aims,

i. To determine the availability of cofactor using three different yeast concentrations in a non-growing whole-cell transformation.
ii. To investigate the time courses for biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (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] as well as desired product 4-hydroxy-2,6,6-trimethylcyclohexane [(4R, 6R)-actinol] in a non-growing whole-cell *S. cerevisiae*.

1.4 SCOPE OF STUDY

In this work, Baker's yeast was utilized as useful biocatalyst to catalyse compounds with carbonyl groups or carbon-carbon double bonds for biotransformation of ketoisophorone. Yeast is capable of converting 2,6,6-trimethylcyclohex-2-ene-1,4-dione (ketoisophorone) to 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione] and (6R)-levodione to 4-hydroxy-2,6,6-trimethylcyclohexane [(4R,6R)-actinol] simultaneously with the help of ketoisophorone reductase and levodione reductase. The phosphate buffer solution with the presence of glucose acts as a medium. The culture broth must be maintained at 37^{o}C and speed of 150 rpm in the incubator shaker for 24 h. The supernatant consists of solvent, substrate and product was used to analyse using gas chromatography (GC). Sonication was conducted in this experiment to break apart the cells for further analysis by using UV-vis spectrophotometer in order to determine cofactor availability.

The entire sample with three different cell concentrations; 5g/L, 10g/L and 15g/L of *S. cerevisiae* were analysed using UV-vis spectrophotometer and GC. UV-vis spectrophotometer was used to determine the cofactor availability of the non-growing whole cell *S. cerevisiae* using three different of yeast concentrations. Gas chromatography was used to analyse production of intermediates and main products along the reaction time.

CHAPTER TWO

LITERATURE REVIEW

2.1 BIOTRANSFORMATION

Biotransformation is a process in which organic compounds are transformed from one form to another with the aid of organism such as bacteria, fungi and enzymes (Kebamo et al., 2015).

2.1.1 Isolated enzyme and whole cell biotransformation

Biocatalyst refers to an enzyme, an enzyme complex, a cell organelle or a whole-cell (Santen, 2000). Biocatalysts can be microbial, plant or animal origin. The whole-cell can either be growing or non-growing (Drauz, 2012).

Factors to consider in deciding whether an enzyme or whole-cell should be used include; the availability, the number of steps involved, yield, cofactor required, byproduct formation, required product purity and price. If the enzyme involved is unstable in isolated form and electron transport chain is utilized in complex coenzyme regeneration, whole-cell biotransformation method is used (Santen, 2000). The most widely used whole-cell biocatalysts is baker's yeast. The reason is it contains many kinds of dehydrogenases such as S-selective and R-selective. Therefore, enantioselectivities can be low to high depending on the substrate structures (Drauz, 2012).

On the other hand, isolated enzyme systems also have many advantages. The problem associated with the product isolation and over metabolism can be avoided using an isolated enzyme. More importantly, types of stereoselectivities such as chemo-,regio-, and enantioselectivities of isolated enzyme systems are usually higher

than the whole-cell because two competing enzymes with different stereoselectivities are absent. However, the product isolation may be difficult due to large amounts of biomass and metabolites (Drauz, 2012). Some parameters which differentiate the form of biocatalyst between whole cell and isolated enzyme is summarized in Table 2.1 (Drauz, 2012).

Table 2.1: The form of biocatalyst: whole cell versus isolated enzyme (Drauz, 2012).

Parameter	**Whole-Cell**	**Isolated Enzyme**
Kinds of enzymes	Many	One
Kinds of reactions	Many	One
Regio- and enantioselectivity	Low to high	High
Coenzyme	Unnecessary	Necessary
Catalyst preparation	Easy	Difficult
Work up	Difficult	Easy
Example	Baker's yeast	Horse liver alcohol dehydrogenase

Besides that, biosynthesis of enzymes is strictly regulated in microorganisms. Biological variability depends on activity of enzymes, nutrients, inducing agents, growth condition, time of harvest and condition of storage. The differences between whole-cell and cell-free enzymes as biocatalyst are summarized in Table 2.2.

Table 2.2: Advantages and disadvantages using isolated enzymes or whole-cell biocatalysis (Drauz, 2012).

	Advantages	**Disadvantages**
Isolated enzymes	Catalyst concentration as free process viable.	Limited stability.
	High catalyst concentration possible.	Require cofactor regeneration.
	No side reactions.	
	Simple product recovery.	
	Multienzyme reactions possible.	
Whole-cell biocatalysts	Unlimited availability exploiting growth.	Side reactions.
	Cofactor recycling by cellular machinery.	Transport limitation.
	Possible multistep conversions.	Complex product recovery.

In summary, whole-cell and isolated enzyme biocatalysts both have various advantages and disadvantages.

2.2 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the most significant forms of yeast that commonly known as baker's yeast and brewer's yeast (Moyad, 2007). *S. cerevisiae* is commonly applied to whole-cell biocatalysis in biotransformation (Rehm et al.,

1993). The growth development of green plants undergo photosynthesis process, but yeast consumes carbohydrate and potential nutrient for growth as well as producing alcohol as the main product (Moyad, 2007).

The advantages using the yeast are easy to handle, broad substrate acceptability and production of enzymes belonging to different classes (Białecka-Florjańczyk and Kapturowska , 2012). Baker's yeast is very economical source of enzymes because it can easily grow in open jar without sterilisation. In addition, it has high reducing capacity, thus, addition of cofactors is not required (Rehm et al., 1993). The disadvantages of biotransformation using yeast are denaturation of biocatalyst due to high temperature. Yeast cannot survive in temperature higher than 60^{o}C. Yeast also can inactivate in freezing temperature, grow slowly at cold temperature and steadily at moderate temperature (24^{o}C) and without limit at 38^{o}C (Moyad, 2007).

Application of yeast is mostly used in food and beverages industries (Moyad, 2007). Baker's yeast and brewers 'yeast (*Saccharomyces carlsbergensis*) are examples of industrial organisms produced for the food industry (Rehm et al., 1993).

2.2.1 *Saccharomyces cerevisiae* as stereospecific bioreduction tool

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 (Matsumoto et al., 2001). *Saccharomyces cerevisiae* particularly shown that they can catalyse compounds with carbonyl groups or carbon-carbon bonds (Khor and Uzir, 2011).

Important method for preparation of chiral building blocks is bioreduction of prochiral carbon-carbon (C=C) double bonds and carbon-oxygen (C=O) conjugated

double bonds. Two important enzymes that responsible for catalytic activity in the reduction of carbonyl groups into hydroxyl groups are dehydrogenases and reductases. Function of alcohol dehydrogenases (ADHs) is reduction of carbonyls, while enoate reductase (ERs) is responsible for the reduction of 'activated' carbon-carbon double bonds. These enzymes are enabled to produce chiral product from prochiral substrate (Engelking et al., 2006 ; Friberg et al., 2006 ; Kaluzna et al., 2005 ; Katz et al., 2002 ; Watanabe et al., 2005).

The help of nicotinamide co-enzyme, NADPH, is required for ADHs and ERs. Hydride from NADPH is transferred to substrate carbonyl carbon. The oxidised form of the co-enzyme, $NADP^+$, will be transformed back into reduced form for the next cycle of the reduction process. Co-substrates such as alcohols and glucose are serving as hydrogen source. Figure 2.1 illustrates the cofactor regeneration using ADH and ER in biocatalytic reduction process with baker's yeast (Khor and Uzir, 2011).

Regeneration process of cofactor alongside the presence of co-substrate is depleted and causes the reduction process to stop. Hence, the rates of both reactions should be equilibrium to provide an optimum bioreduction system. Reduction process such as asymmetric reduction of prochiral ketones is widely used due to microorganism like yeast is inexpensive (Khor and Uzir, 2011).

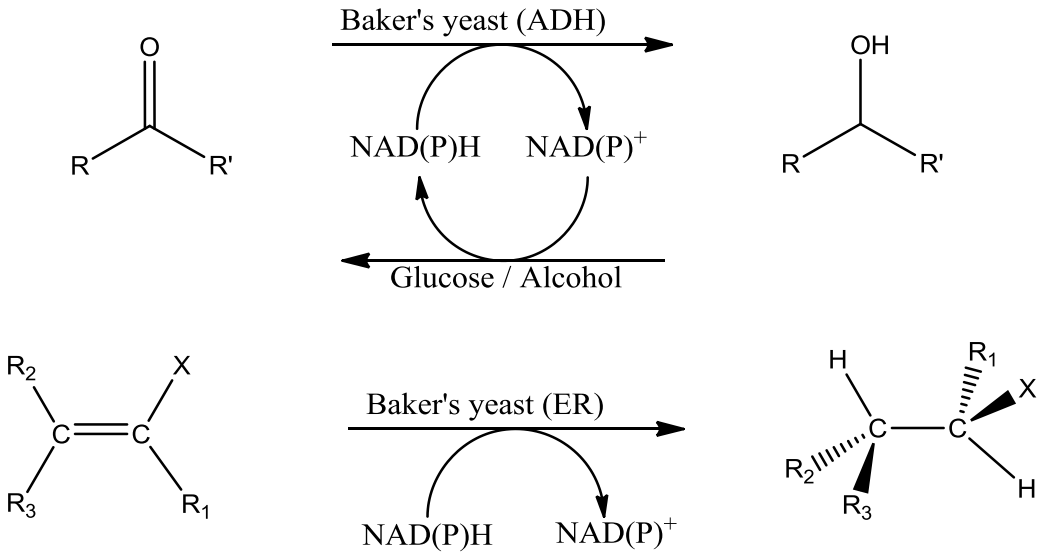

Figure 2.1**:** Schematic diagram of cofactor regeneration in baker's yeast reduction process (Khor and Uzir, 2011). Note that $NADP^+$ is oxidized form, NADPH is reduced form, ADH is alcohol dehydrogenase, ER is enoate reductase, and X is electron-withdrawing substituent such as carbonyl-, carboxyl-, imide-, nitro-.

2.3 Glucose as Energy Source

Fermentation is a chemical process that has been used in biotechnological applications for thousands of years. This process involved breakdown of sugar molecules when there is no oxygen around. Variety of applications such as productions of beer and biofuels are used fermentation process. Most living organisms need glucose as energy source. Embden-Meyerhof pathways are the glycolytic pathway and it takes places into two phases that are investment phase and payoff phase.

The first phase in glycolytic pathway is the investment phase. The investment phase is defined refers to the steps of glycolysis that require energy input. The energy obtained is obtained through the hydrolysis of a high-energy phosphate in an

adenosine triphosphate (ATP) molecule. The first step is the conversion of glucose into glucose 6-phospahte, which can be written as;

$$\text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}$$

Phosphate from ATP molecules is attached to the initial glucose molecule and it supplies the energy need to drive the reaction forward with the help of hexokinase enzyme. Figure 2.2 shows the conversion of glucose to glucose-6-phosphate with the help of hexokinase enzyme and presence of ATP.

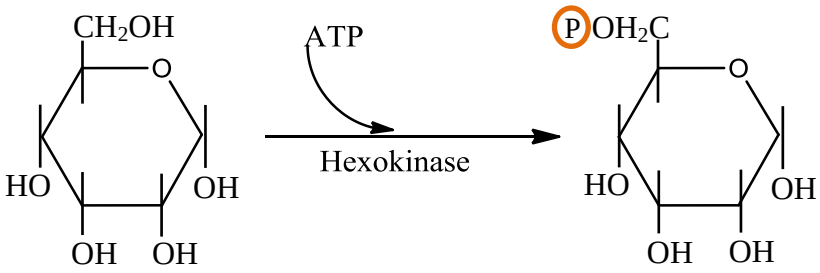

Figure 2.2: Conversion of glucose to glucose-6-phosphate with help of hexokinase enzyme.

Then, glucose-6-phosphate undergoes isomerization from a 6-carbon sugar into the 6-carbon sugar fructose as fructose-6-phosphate.

$$\text{Glucose-6-phosphate} \rightarrow \text{Fructose-6-phosphate}$$

As shown in the Figure 2.3, another ATP molecule is invested to yield a fructose in which two phosphates are attached on carbon 1 and carbon 6 respectively by using phosphofructokinase enzyme.

$$\text{Fructose-6-phosphate} + \text{ATP} \rightarrow \text{Fructose-1,6-biphosphate}$$

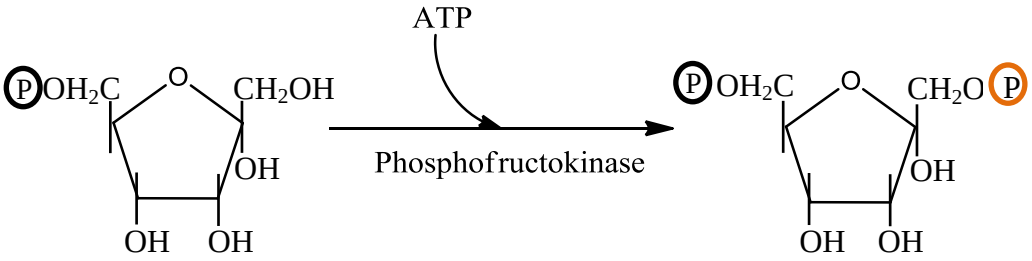

Figure 2.3: Conversion of fructose-6-phosphate into fructose-1,6-biphosphate with the help of phosphofructokinase enzyme.

The yield of the payoff phase will be four molecules, leaving the cell with a net gain of two ATP molecules for the conversion of one glucose molecules into two pyruvates. Figure 2.4 shows the first step for payoff phase of glycolysis involving the conversion of 1, 3-bisphosphoglycerate, which can be refer as a deprotonated glyceric acid with the two high energy phosphates. An ATP molecule is formed from the cleavage of a high energy phosphate.

1,3-bisphosphoglycerate + ADP → 3-phosphoglycerate + ATP

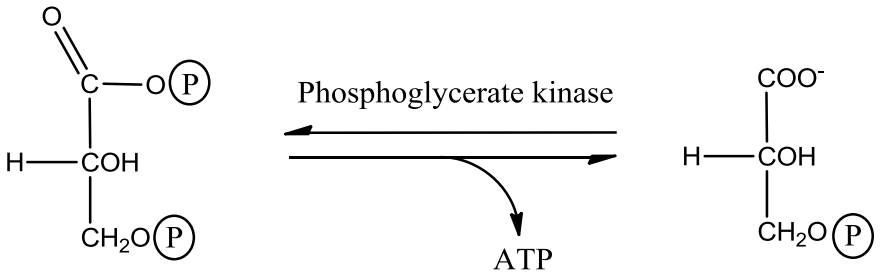

Figure 2.4: Conversion of 1,3-biphosphateglycerate into 3-phosphoglycerate with the help of phosphoglycerate kinase enzyme.

Another ATP molecule is produced from the removal of the final high-energy phosphate. The reaction is shown as below and the removal of ATP molecules is shown in Figure 2.5,

Phosphoenolpyruvate + ADP → Pyruvate +ATP

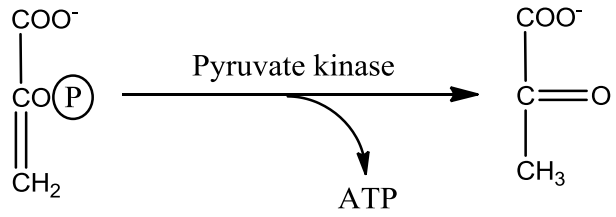

Figure 2.5: Removal of ATP molecules from the conversion of phosphoenolpyruvate to pyruvate with the help of pyruvate kinase enzyme.

Production of 6-carbon molecule fructose 1,6-biphosphate is split into three carbon molecules, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Each of these 3-carbon products will progress separately down the remainder of the glycolytic pathway to yield two ATP molecules and one pyruvate molecules each. From the pathways, the total of four ATP molecules and two pyruvate molecules. Therefore, the net yield is two ATP molecules and 2 pyruvate molecules with 2 NADH molecules (Godbey, 2014). Figure 2.6 shows the summary of Embden-Meyerhof pathway of glycolysis.

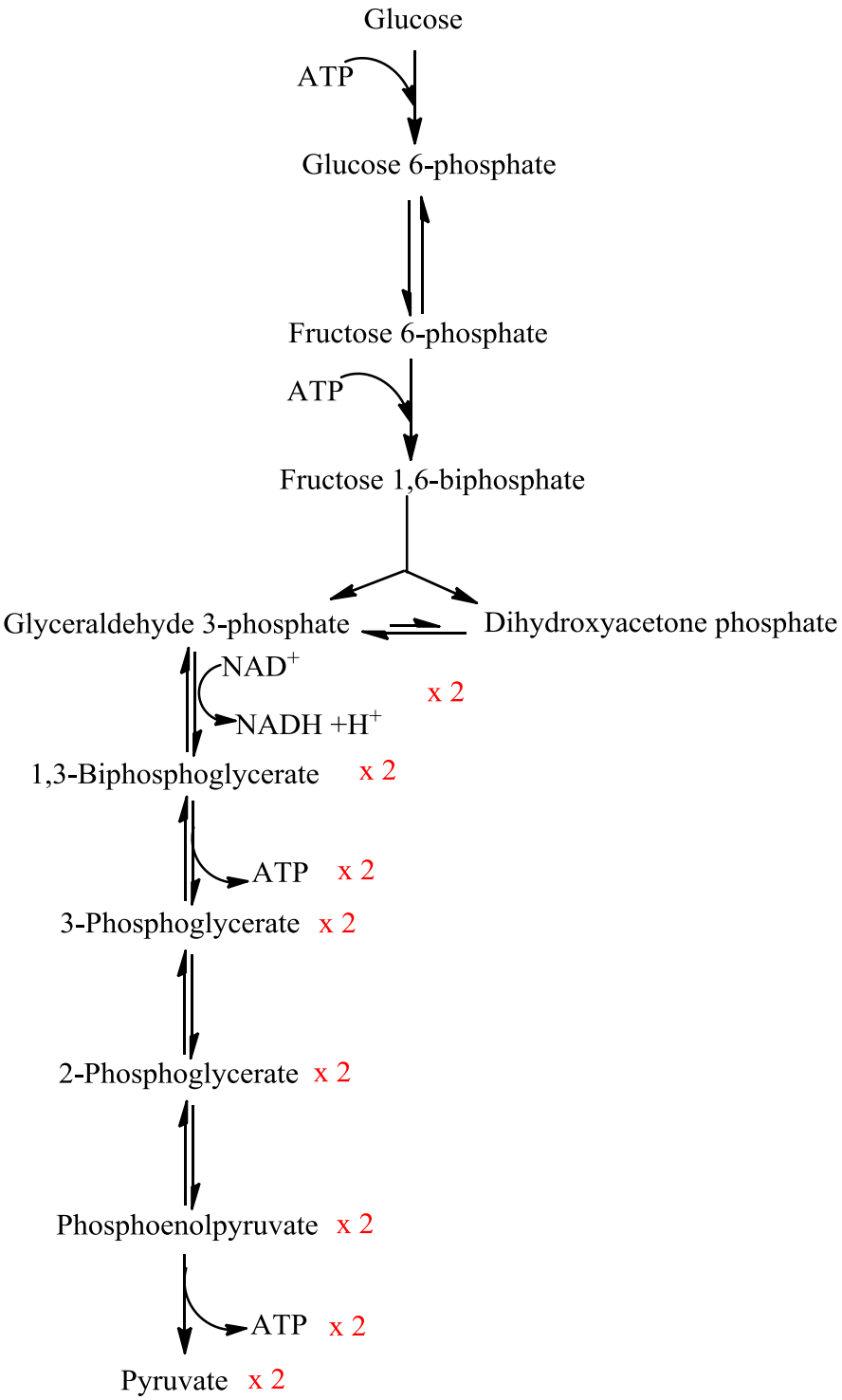

Figure 2.6: The Embden-Meyerhof pathway of glycolysis

2.3 ENZYME MEDIATING THE REACTION

2.3.1 Enoate Reductases (ER)

Old yellow enzymes (OYEs) plays important role in biocatalysis. OYEs are flavin-dependent oxidoreductases that catalyse the asymmetric reduction of electron-poor alkenes by enoate reductase activity which can result in important chiral synthons towards synthesis of many pharmaceuticals and chemicals. Yeast that express high enoate reductase (ER) activity and good acrolein resistance give high-throughput. Acrolein is a toxic aldehyde resulting from endogenous lipid peroxidation as a consequence of oxidative stress (Raimondi et al., 2010).

OYEs gives alternative pathway to conventional chemical reaction by exhibiting some improved performances such as thermal stability, activity within broader range of pH, stereoselectivity, activity in organic solvents. The outcome of the reaction is the stereoselective trans-hydrogenation of the double bond. OYEs are able to reduce a wide variety of substrates, such as conjugated enals, enones, and α,β-unsaturated carboxylic acids (Raimondi et al., 2010).

In this reaction, Bi Bi Ping Pong mechanism is applied. This mechanism defines as the cofactor and the substrate use the same binding site. β-NADPH is recognized as the physiological reductant. However, OYEs are loosely specific and can also be reduced by α-NAPDH and NADH cofactors (Raimondi et al., 2010).

Regeneration of the reducing cofactor is a crucial step and has been only partially overcome by whole-cell biocatalysts, since microbial conversion displays low chemoselectivity, mainly due to presence of competing alcohol dehydrogenases. The catalytic efficiency generally declines as the substrate size increases. OYEs

possess identical stereoselectivity product formation, and this dramatically limits their application (Raimondi et al., 2010).

2.3.2 Alcohol dehydrogenase (ADH)

Alcohol dehydrogenases (ADHs) are enzymes responsible for the reversible oxidation of alcohols to aldehydes with the associated reduction of NAD^+ or $NADP^+$. Two major pathways are involved in the energy metabolism of *S. cerevisiae* are glycolysis and aerobic respiration. Ethanol is an example of key metabolite in energy metabolism and serves as carbon substrate during glycolysis and ethanolic fermentation. Alcohol dehydrogenases (ADHs) catalyse the interconversion of acetaldehyde and ethanol during aerobic respiration (De Smidt et al., 2008).

In 1937 and 1948, Negelein and Wuluff, Bonnichsen and Wassen respectively were succeed discovered the first ADH to be isolated was from brewer's yeast or Saccharomyces cerevisiae in crystallising ADH from horse liver. Dehydrogenases are mostly NAD(P)H-dependent. Baker's yeast is often used for the reduction of aldehydes and ketones. Properties of ADH are ADH is dimeric protein, optimum pH that suit with ADH reduction process, and ADH will only remove the pro-R hydrogen form alcohol group because of specific configuration of active site (Voss et al., 2008).

2.3.3 Ketoisophore (KIP) Biotransformation

Figure 2.7 shows reduction of ketoisophorone that catalysed by ADHs or known as OYEs and ERs. From the reduction process, OYEs activity at **2, 5, and 6** can be expressed and KIP conversion as percentage of the initial KIP molar concentration (% OYE products). The extent of KIP conversion was higher than the

sum of OYE products whenever competing ADH reduced the ketone without complete further reduction of the double bond (Raimondi et al., 2010).

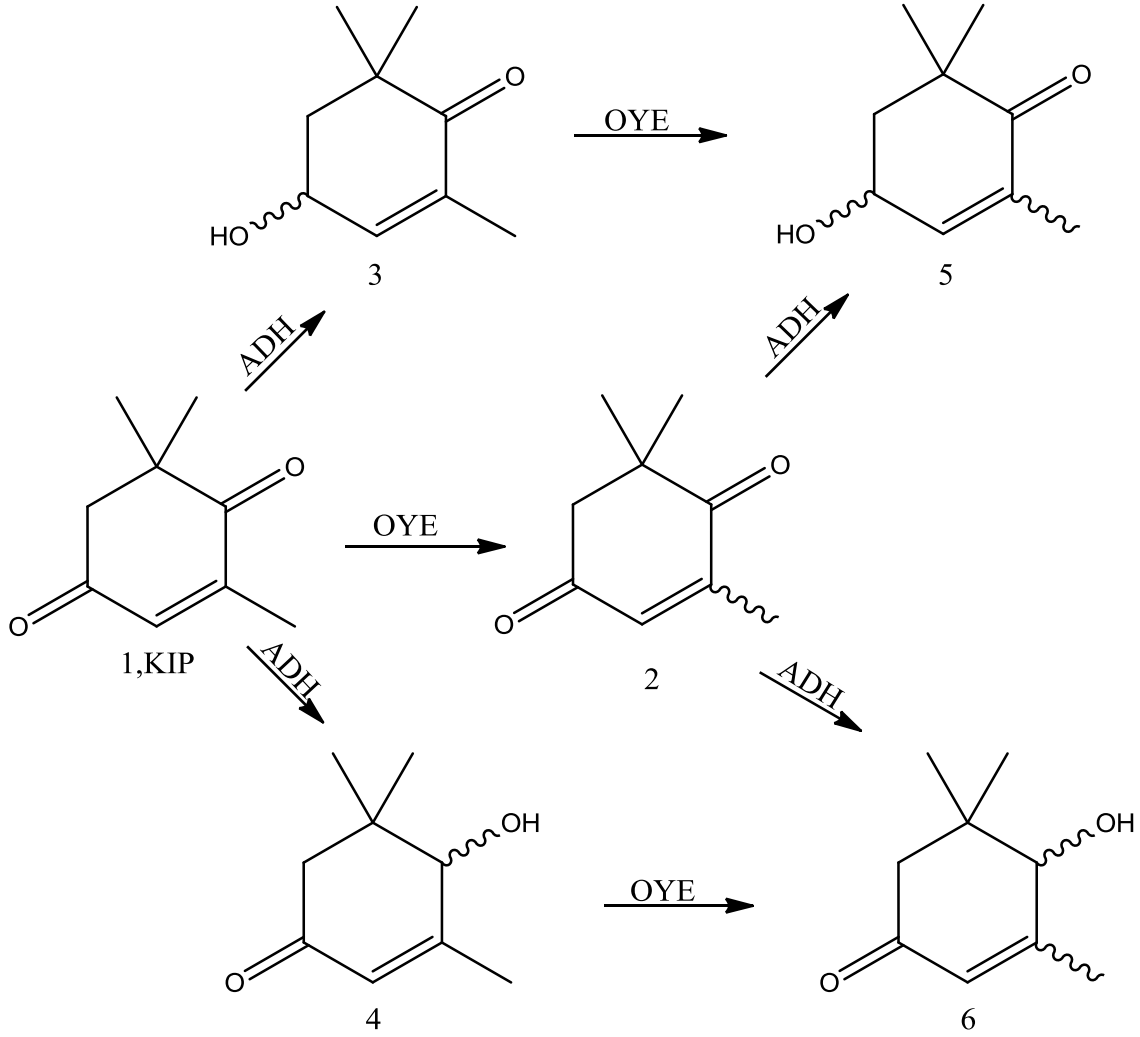

Figure 2.7: Reduction of KIP: reactions catalyzed by enoate reductases (OYEs) and alcohol dehydrogenases (ADHs). The products observed during bioconversion are reported:**1**:2,6,6-trimethylcyclohehex-2-ene-1,4-dione [ketoisophorone]; **2**:2,6,6-trimethylyclohexane-1,-dione[(6R)-levodione]; **3**:4-hydroxy- 2,6,6-trimethylcyclohex-2-en-1-one[(4S)-levodione]; **4**:4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one[Hydroxyisophorone]; **5**:4-hydroxy-2,6,6-trimethylcyclohexanone[(4R,6R)-actinol]; **6**:4-hydroxy-3,5,5-trimethylcyclohexanone.

2.4 COFACTOR

Fermentation processes are performed by cells to extract energy from a starting material such as glucose. A complete absence of oxygen is not a strict requirement for fermentation to occur but oxygen is not involved on the chemical reactions. In addition, the NADH/NAD^+ ratio is unchanged by the process and the hydrogen to carbon ratio is unchanged between reactant and product.

Two pyruvate molecules produced from glycolysis will be converted to lactate in anaerobic respiration. This step is to remove NADH that was produced earlier in glycolytic pathway. In aerobic respiration, the NADH would be able to enter the electron transport chain, which would indirectly responsible for the hydrogenation of oxygen to form water. Higher concentration of NADH turn off certain glycolytic enzymes and breakdown of glucose will be halted. Therefore, NADH will be siphoned off by fermentation to produce lactate and in order to prevent potentially deadly effect. Figure 2.8 below illustrates the fermentation of glucose into two lactic acid molecules.

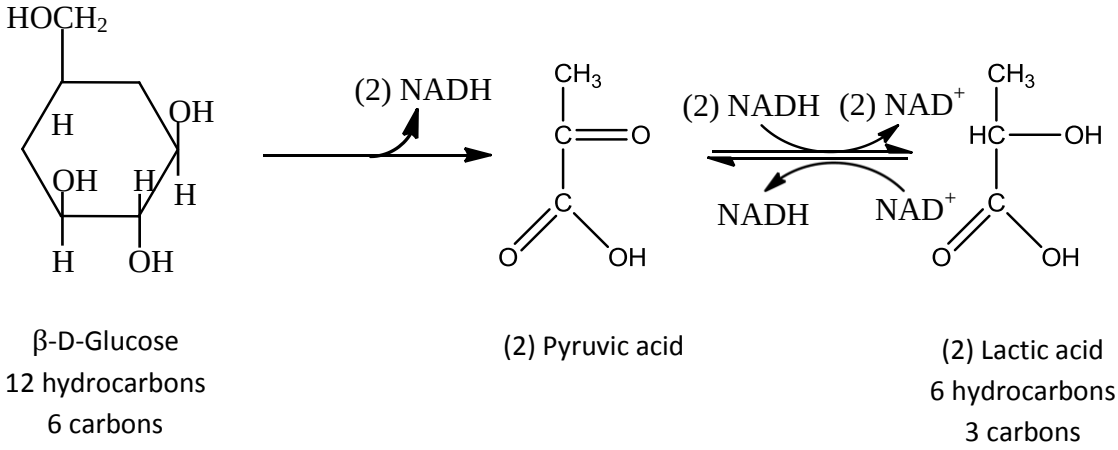

Figure 2.8: Fermentation of glucose into two lactic acid molecules.

NADH in aerobic respiration is serves as an energy source. The cell uses the concentration of NADH to help determine its own energy state. If there is an excess

of NADH, the cell behaves in a way consistent with being in an energy- rich state, thus, all of its energy needs are met. In addition, glycolysis and citric acid cycle will be inhibited because the cell will resort to other pathways designed for energy storage (Godbey, 2014).

2.4.1 Cofactor Regeneration

Oxidoreductases are particularly attractive for biosynthesis due to their high efficiency and specificity. The majority of enzymes need to interact with cofactors that are not permanently bound to the enzymes. The most widely involved cofactors are several organic compounds, which are often referred to as coenzymes, such as NADH, NADPH and ATP (Liu and Wang, 2007). Cofactor is a small dissociable molecule that helps enzymes catalyse oxidation and reduction reactions as well as helps equilibrium the redox reaction (Moulijn et al., 1993). Cofactors acts as stoichiometric in biotransformation reactions and undergo chemical reactions with substrates(Liu and Wang, 2007). The advantage introducing cofactor regeneration is to reduce costs (Moulijn et al., 1993).

Many methods for the regeneration of the reduced form of coenzyme, NADPH have been developed. There are two types of coenzyme regeneration methods that are two-enzyme system and one enzyme system. Two- enzyme system is define as different enzyme is reducing substrate and $NADP^+$ while one enzyme system is define as substrate and $NADP^+$ are both reduced by the same enzyme (Drauz, 2012). Two figures below show regeneration of NADPH in both systems (Drauz, 2012).

From Figure 2.9(a), two-enzyme system uses a formate dehydrogenase for the recycling of coenzyme. Reduction of NAD^+ to NADH by these two enzymes is oxidized HCO_2H to CO_2. The advantages of these systems are enzyme is