THE EFFECT OF GLUCOSE CONCENTRATION ON THE CO-FACTOR RECYCLING IN A NON-GROWING WHOLE-CELL *SACCHAROMYCES CEREVISIAE*-MEDIATED KETOISOPHORONE BIOTRANSFORMATION

NANTHINI A/P UMAPATHY

UNIVERSITI SAINS MALAYSIA

2017

THE EFFECT OF GLUCOSE CONCENTRATION ON THE CO-FACTOR RECYCLING IN A NON-GROWING WHOLE-CELL *SACCHAROMYCES CEREVISIAE*-MEDIATED KETOISOPHORONE BIOTRANSFORMATION

by

NANTHINI A/P UMAPATHY

Thesis submitted in partial fulfilment of the requirement for the degree of Bachelor of Chemical Engineering

May 2017

ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude towards my supervisor Prof. Madya Dr. Mohamad Hekarl Uzir who has been very supportive throughout this whole project. His guidance, constant encouragement and careful monitoring are so great that even my most profound gratitude is not enough. I truly appreciate his recognition on my relentless effort.

Next, I would like to express my deepest appreciation to Muhammad Rasyad who has been generous in sharing his knowledge for the smooth flow of the lab experiment.

I would also like to extend my sincere gratitude to my colleagues for their cooperation and helping hands in guiding me carrying out the lab experiment. They are willing to sacrifice their time in guiding and helping me throughout the experiment besides sharing their valuable knowledge.

Apart from that, I would like to thank all the School of Chemical Engineering staffs for their helping hands in completion of this project. Indeed, their willingness in sharing knowledge and skills are deeply appreciated.

I would like to express my gratitude to my family members for being my pillars of support by giving me endless and unconditional love. Once again, I would like to thank all the people who have helped me directly or indirectly to complete this project. Thank you very much.

Nanthini A/P Umapathy

May 2017

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

e.e	Enantiomeric excess
OYE	Old Yellow Enzyme
NAD(H)/NADH	Nicotinamide Adenine Dinucleotide
NADP(H) / NADPH	Nicotinamide Adenine Dinucleotide Phosphate
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
ThDP	Thiamine Diphosphate
PLP	Pyridoxal Phosphate
ATP	Adenosine-5'-triphosphate
SAM	(S)-adenosylmethionine
ADH	Alcohol dehydrogenase
GM	Genetically modified
KIP	Ketoisophorone
NOS	Nitric Oxide Synthase
H_2S	Hydrogen sulfide
ETC	Electron transport chain
HMP	Hexose Monophosphate Pathway
PPP	Pentose Phosphate Pathway

KESAN KEPEKATAN GLUKOSA TERHADAP KITARAN SEMULA CO-FAKTOR DALAM BIOTRANSFORMASI KETOISOPHORONE DIPERANTARAI SEL *SACCHAROMYCES CEREVISIAE*

ABSTRAK

Kajian ini dijalankan bagi mengkaji kesan kepekatan glukosa yang berbeza terhadap pengitaran semula co-faktor dalam sel rehat *Saccharomyces cerevisiae.* Selain itu, kajian ini juga mengkaji kesan kepekatan glukosa yang berbeza terhadap biotransformasi 2,6,6-trimethylcyclohex-2-ene-1,4-dione atau lebih dikenali sebagai ketoisophorone dalam sel rehat *Saccharomyces cerevisiae*. Biotransformasi fasa cecair dijalankan dalam kultur kelalang goncang. Keadaan kendalian biotransformasi ini ialah 37 °C, 150 rpm, 5 g/L *S. cerevisiae*, 0.2 g/L ketoisophorone dan kepekatan glukosa yang berbeza (5 g/L, 10g/L, 15 g/L). Dalam tindak balas ini, ketiga-tiga kepekatan glukosa menunjukkan kehadiran co-faktor. Ini menunjukkan kewujudan proses kitaran semula co-faktor dalam biotransformasi ketoisophorone. Kepekatan glukosa sebanyak 15 g/L menunjukkan nilai penyerapan yang tertinggi iaitu 0.4100. Terdapat 5.018×10^{22} molekul glukosa dalam kepekatan glukosa 15 g/L. Kepekatan glukosa yang optimum untuk pembentukan actinol ialah 15 g/L. Sebanyak 12 mol % actinol terhasil daripada biotransformasi yang mengunakan 15g/L kepekatan glukosa. Semasa biotransformsi ini, hanya levodione yang terhasil. Kepekatan NAD^+ yang tinggi akan merencatkan alkohol dihidrogenase. Ini akan menghalang reduksi keton untuk berlaku. Tambahan pula, kadar tindak balas penurunan keton amat perlahan jika dibandingkan dengan penurunan carbon yang dihubungakan dengan ikatan kembar oleh reduktase enoate.

THE EFFECT OF GLUCOSE CONCENTRATION ON THE CO-FACTOR RECYCLING IN A NON-GROWING WHOLE-CELL *SACCHAROMYCES CEREVISIAE*-MEDIATED KETOISOPHORONE BIOTRANSFORMATION

ABSTRACT

The aim of this study is to investigate the effect of different glucose concentrations on the recycling of co-factor in a non-growing whole-cell *Saccharomyces cerevisiae*. Besides that, this study also investigates the effect of different glucose concentrations on the biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione also known as ketoisophorone in a non-growing whole-cell *S. cerevisiae*. The liquid phase biotransformation was carried out in a shake-flask culture. The conditions of biotransformation are 37 °C, 150 rpm, 5 g/L *S. cerevisiae*, 0.2 g/L ketoisophorone and varied concentration of glucose (5 g/L, 10g/L, 15 g/L). It was found that level of co-factors were shown at different glucose concentrations. This indicates that co-factor recycling process exist in this reaction.15 g/L of glucose showed the highest value of absorbance which is 0.4100. 5.018×10^{22} number of glucose molecules are present in 15 g/L glucose. The optimum concentration of glucose for the formation of actinol is 15 g/L. 15 g/L of glucose showed a maximum of 12 mole % of actinol formed. Levodione was the only intermediate formed during the biotransformation. Ketone reduction did not occur due to the inhibition of alcohol dehydrogenase caused by high concentration of NAD^+. Besides, ketone reduction has a slower reaction rate as compared to the reduction of carbon-carbon double bond by enoate reductase.

CHAPTER ONE

INTRODUCTION

1.1 Research background

Biocatalytic process has been increasingly used for replacing the traditional plant extraction method and most importantly the conventional chemical processes in producing desired flavor compounds. Interests in applications of enzymes and microorganism as catalyst have been overwhelming. Many reviews on biotransformation describing the advantages of biological catalysts have been reported. Synthesis of optically active compound through biotransformation is preferred as it usually proceeds with high stereospecificities (Forti et al., 2015).

Recently, the reduction of symmetrical and asymmetrical chemical compounds under aerobic condition via *Saccharomyces cerevisiae* was investigated (Mahmoodi et al., 2005). Besides that, research on the reduction catalyzed by the Old Yellow Enzyme from *Candida macedoniensis* had been reported as well (Kataoka et al., 2002).

Ketoisophorone (2,6,6-trimethyl-2-cyclohexene-1,4-dione) is an important precursor for the synthesize of carotenoids and flavoring agents (Mata-Gómez et al., 2014). Reduction of ketoisophorone produces many optically active hydroxycyclohexanone derivatives such as actinol. Figure 1.1 shows the complete reduction of ketoisophorone by *S. cerevisiae*.

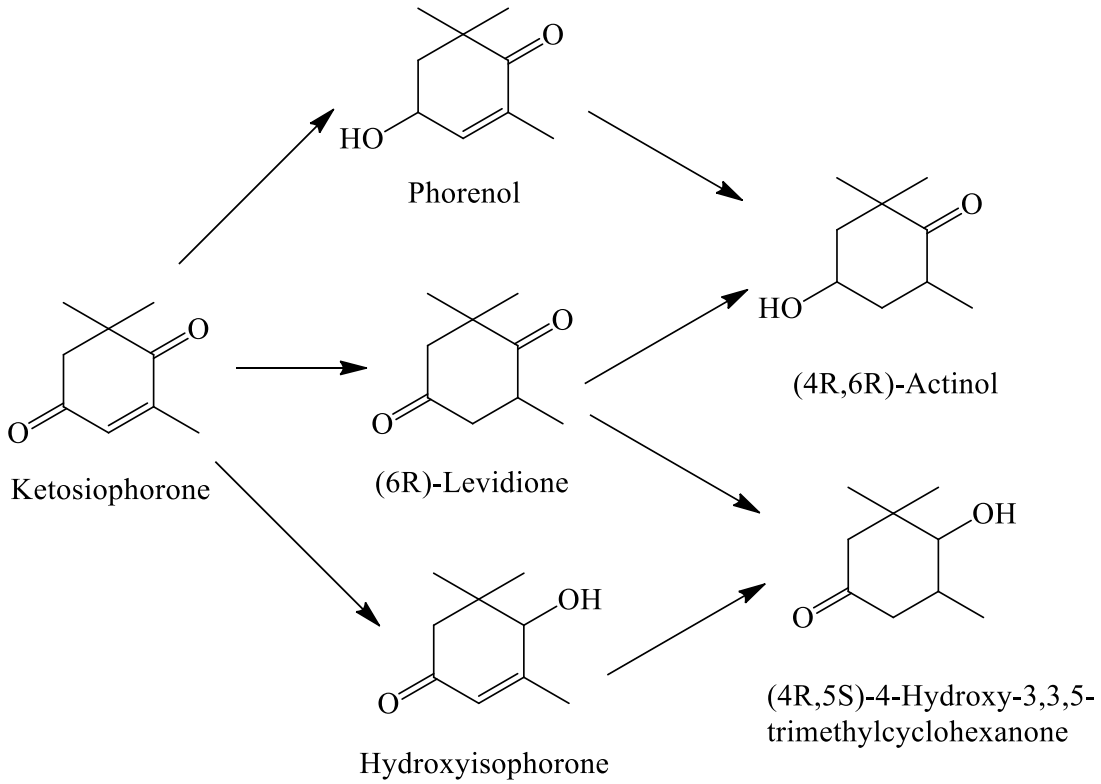

Figure 1.1: Scheme for biotransformation of ketoisophorone by *S. cerevisiae* (Kataoka et al., 2002)

Actinol is the building block of zeaxanthin and xanthoxin. The intermediate compound resulting from the reduction of ketoisophorone is (6R)-levodione (Hegazy et al., 2008). The mechanism of biotransformation of ketoisophorone by S. *cerevisiae* to produce actinol is yet to be demonstrated. However, production of actinol from 2,2,6-trimethylcyclohexanedione had been practiced (Nishii et al., 1989).

1.2 Green biotechnology

Biotechnology is defined as the use of any living organisms or bio molecular processes to produce or modify products for the use of human beings. There are many types of biotechnology including red, blue, white and green biotechnology.

Green Biotechnology deals with the use of environmental friendly solution with an aim of improving the nutritional quality, quantity and production economics.

For example producing plants that are UV-resistance, disease resistance or plants that possesses superior quality through genetic modification. Genetic modification is the most well-known area of application of biotechnology. Cultivation of genetically modified (GM) crops started in 1996 in the USA. This reduces the cost of labor, machines and pesticides, despite considerably expensive GM seeds. Other than that, the yield increases tremendously as the crops are resistant to diseases (Vaghasiya and Shiroya, 2008).

The first green revolution was from the year of 1960-1975. During this period, rice, wheat and maize with higher yield were introduced. However, they were very vulnerable to pest infestation. Other than that, high usage of fertilizer was needed. From the year 1975-1990, the second green revolution took place. During this period, improvement on the resistance of the plant towards pest and diseases were emphasized. The third green revolution is known as biotechnology or gene revolution to further amplify the progress from the first and second green revolution. Areas of biotechnology that were affected include:

i) improving agricultural yields
ii) quickening the pace of plant research
iii) enhancing genetic analysis

Table 1.1 shows some of the chronological development of biotechnology throughout the years. This includes the new development in research and production of new products using biotechnology.

Table 1.1: Historical development of biotechnology (Kumar, 2016)

Year	Development
Before 6000 B.C.	Yeast employed to make beer and wine
4000 B.C.	Leavened bread produced with the aid of yeast
1890	Alcohol first used to fuel motors
1918	Yeast grown in large quantities for animal and glycerol
1953	Double helix structure revealed
1973	Beginning of genetic engineering. Stanley Cohen produced first recombinant DNA organism
1982	The Food and Drug Administration approves the first biotechnology therapy, a human insulin drug made by Genentech
1985	Genetically engineered plant resistant to insects, bacteria and viruses were field tested for the first time
1990	GenPharm international created the first transgenic dairy cow. The cow was used to produce human milk proteins for infant formula
1994	First genetically engineered food product
1996	Dolly the sheep is cloned
2007	Discovering special kind of RNA that can shut down individual genes

1.3 Biocatalysis

Biocatalysis is defined as the use of natural products to speed up chemical reactions. It uses enzyme or whole-cell (containing the desired enzyme or enzyme system) as catalyst for chemical reactions. Enzymes offer much more competitive

processes compared to chemical catalysts. However, there are few drawbacks that restrict the application of enzymes in industry. These include a few undesirable properties in terms of stability, specificity and catalytic efficiency. Screening of enzymes from natural sources, random mutations and immobilization are some approaches to overcome these flaws. The anti-diabetic compound, sitagliptin is one of the successful example of enzymes in pharmaceutical industry. The drawback of substrate size for enzyme was overcome by substrate walking, modelling and mutation approach. This biocatalytic process reduces the total waste and removes the requirement of a rare heavy metal (Rh). Moreover, it increases the overall yield by 10% and the productivity (kg/L/day) by 53% (Choi et al., 2015).

Biocatalysis regulates and controls all metabolic reactions in microorganisms, plants and animals in a very selective way by allowing a high reaction rate. Some microorganisms have been found to grow under unusual environmental conditions. Catalysts created by nature through evolution are mostly proteins and nucleic acids. Up to now, only enzymes are used in applied biocatalysis. The purposes of enzymes catalyzing chemical reactions are mainly for survival and reproduction. A biocatalyst may either be a complete cell itself, employed in a viable, non-viable, growing or non-growing state, or as an individual enzyme. Even though there are few disadvantages, the introduction of the Pollution Prevention Act of 199038 has led to an increased focus on green chemistry. Biocatalysis compliant with the 12 principles of green chemistry.

1.3.1 Advantages of biocatalysts

Enzymes can act as a very efficient catalyst. A reaction with catalyst proceeds way faster as compared to reactions that take place in the absence of catalyst. The rate

of enzyme-mediated processes are usually within the factor of $10^8 - 10^{10}$, which is more than the capability of chemical catalysts (Wolfenden and Snider, 2001). Enzymes are environmentally acceptable as they are completely biodegradable and are compatible with each other. Therefore, similar reactions can be carried out in a single flask. The unfavorable equilibrium can be shifted to the desired product by linking consecutive enzymatic steps (Sheldon, 2008). Other than that, enzymes act under mild condition. This reduces the formation of undesired side-reactions such as racemization, rearrangement and isomerization. The typical optimum condition for enzyme is at the temperature of 30°C and about pH 7. Enzymes catalyze a broad spectrum of reactions. It can proceed in both directions, as the thermodynamic equilibrium is not affected by the enzyme-catalyzed reaction. Enzymes are also not restricted to their natural role. It has high tolerance to substrate, where some of the non-natural substrate does not require water as a medium. A change in medium to organic solvent is possible.

Enzymes display 3 major types of selectivities which are chemoselectivity, regioselectivity and diastereoselectivity and enantioselectivity. Chemoselectivity is when an enzyme acts on a single type of functional group. In chemoselectivity, undesired side reactions can be disregarded. In regioselectivity and diastereoselectivity, enzymes are differentiated between the functional groups by its complex three-dimensional structure. The functional groups are chemically identical but are situated at different places within the same substrate. The chirality of substrate identified from the formation of enzyme substrate complex is defined as enantioselectivity. Both the enantiomers of a racemic substrate react at different rates (Faber, 2011).

Enzymes are highly selective towards the chirality of a substrate. Enantiomers also cause different biological effects in pharmaceutical compounds (Crossley, 1947).

There are 2 different species of enantiomers which are ‘eutomer’ and ‘distomer’. Eutomers are isomers with the highest activity while distomers are isomers with undesired activities. Figure 1.2 shows the examples of different biological effects on enantiomers.

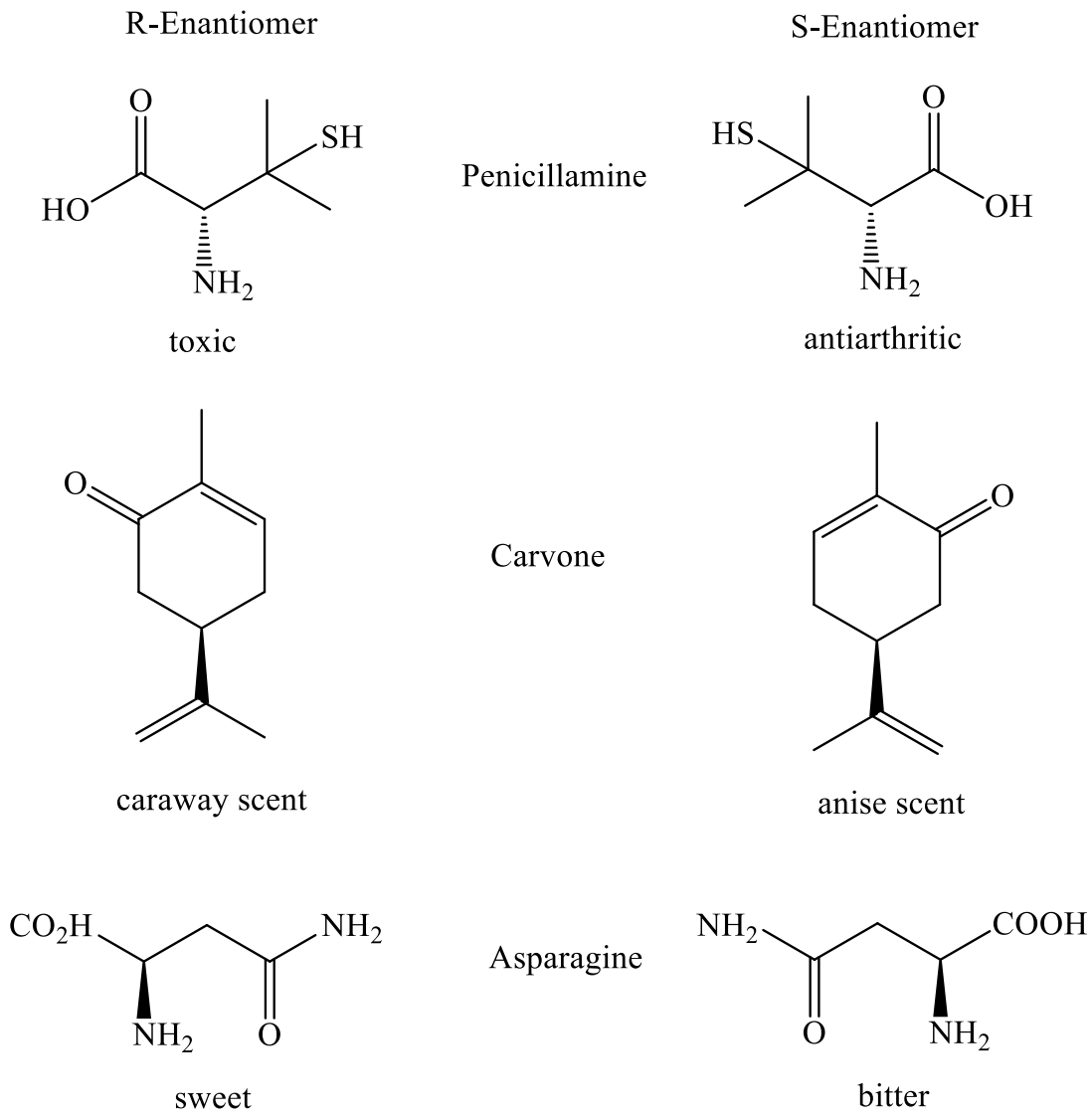

Figure 1.2: Biological effects of enantiomers (Faber, 2011)

1.3.2 Disadvantages of biocatalysts

Enzyme operating in mild conditions can also give drawbacks. This is due to the presence of a very narrow operation of parameter. For example, if a reaction

proceeds too slowly at the given temperature and pH, the alteration will be a challenging process. High temperature and pH can cause deactivation of protein as well (Phillips, 1996). However, there are also enzymes being catalytically active even in ice (Yeh and Feeney, 1996). Enzymes are provided by nature in only one enantiomeric form. There are no general ways to create a mirror image enzyme. Enantiomers are formed only if the enzymes with opposite stereochemical selectivity are found.

Enzymes can cause allergy reactions which can be overcome with proper handling method. Enzymes are prone to inhibition phenomena. In other words, inhibition of substrate or product will cause a drop in the rate of reaction when their concentrations are higher. Another major drawback is that enzymes display their highest catalytic activity in water. Water is usually undesirable in organic reactions as it has a high boiling point. Enzymatic reactions in organic medium can result in loss of catalytic activity (Klibanov, 1990). Although enzymes are extremely flexible, they are bound to their natural co-factor which acts as a storage for chemical energy.

1.4 Biotransformation

Biotransformation is defined as the conversion of a compound to a product of specifically modified structure using biocatalysts such as isolated enzymes, whole-cells and plant cells. Yeast mediated transformation and microbial transformation had been used since the early days of humankind for the production of dairy products, breads and alcoholic beverages. Biotransformations were carried out by pure culture of microorganisms or plant cells with purified enzymes. In 1862, oxidation of alcohol to acetic acid using pure culture of *Bacterium xylinum* was discovered as one of the

earliest achievements in biotransformation (Brown, 1886). Then it was followed by the oxidation of glucose to gluconic acid by *Acetobacter aceti* (Boutroux, 1880).

In biotransformation, only one possible enantiomer is formed as one racemic substrate is attacked. Enzymes are able to operate in mild conditions compared to chemical catalysts. Therefore, various reactions can be catalyzed by enzymes. The initial step of biotransformation is to decide on the microorganism that matches well with the reaction involved. The substrate should be able to pass through the cell membrane and most importantly should not be toxic to the microorganisms. Biotransformation can be carried out in immobilized enzyme, growing cultures or with resting cells. Whole-cell immobilization does not require artificial co-factor regeneration as it carried out within the cell itself (Witholt et al., 1997).

The main goals of biotransformation are resolution of racemates, introduction of chiral center, and functionalization of a certain non-activated carbon and conversion of functional groups (Csuk and Glaenzer, 1991).

1.4.1 Isolated enzymes versus whole-cell systems

Isolated enzymes and whole-cell are two different physical states of biocatalyst used for biotransformations. The decision on using an isolated or whole-cell depends on the type of reactions, whether the co-factor needs to be recycled and the scale of biotransformation. Table 1.2 shows the advantages and disadvantages of isolated enzymes and Table 1.3 shows the advantages and disadvantages of whole-cell. Isolated enzymes usually lead to enantiomeric excess (e.e) in the midst of avoiding issues with competing catalysts. Reduced co-factor has to be regenerated *in-situ* to sustain catalytic activity. Therefore, whole-cell is more attractive because of its own co-factor regeneration system (Schaefer et al., 2013). One of the deciding factor to use

biotransformation in the synthesis is according to the availability of certain microorganisms (Silv, 2013). Other disadvantages of whole-cell for laboratory scale are time consuming and tedious separation process of product from biomass.

Table 1.2: Advantages and disadvantages of using isolated enzymes (Faber, 2011)

Biocatalyst	Advantages	Disadvantages
Isolated enzymes	Simple apparatus, better productivity due to higher concentration tolerance	Co-factor recycling is necessary, limited enzyme stabilities
	High enzyme activities when dissolved in water	Undesired side reactions are possible when dissolved in water
	Easy to perform and recovery of enzyme is easy when suspended in organic solvents	Reduced activities when suspended in organic solvents
	During immobilization, enzyme recovery is easy	Loss of activity during immobilization

Table 1.3: Advantages and disadvantages of using whole-cell systems (Faber, 2011)

Biocatalyst	Advantages	Disadvantages
Whole-cell	No co-factor recycling is necessary	Expensive equipment, low productivity due to lower concentration tolerance
	Higher rate of activities in growing culture	More byproducts and process is tedious to be controlled in growing culture
	Resting cells produces fewer by products	Resting cell can lower the activities
	Cell reuse in immobilized cell	Lower activities in immobilized cell

1.5 Yeast

Yeast is one of the most ancient industrial microorganisms. In 1860, Louis Pasteur discovered yeast as a living microorganism, which is responsible for leavening of bread and fermentation of alcohol (Barnett, 2007). Yeast is actually a single-celled fungus. About 600 different species of yeast is distributed in nature. The size of yeast cell is usually correlated with the size of red blood cell, which is spherical or ellipsoidal in shape (Galbraith, 2002). Most yeasts, including *Saccharomyces* reproduces by budding. Budding is a process where new buds grow from the existing cell walls (Knop, 2011). The grown bud separates from the mother cell to form a daughter cell. Yeast can reproduce every two to three hours under ideal growth condition. Yeast is also very prominent in the production of wine as they influence the fermentation speed and also the quality of wine (Li et al., 2010). Different species of *Saccharomyces* are responsible for bread making industry, wine production and beverages. Examples of those species are *S. cerevisiae, S. carlsbergensis, S. bayanus* and *S. paradoxus* (Mortimer, 2000).

1.5.1 *Saccharomyces cerevisiae*

The most significant whole-cell systems employed in biocatalysis is *Saccharomyces* also known as baker's yeast. Baker's yeast is one of the simplest eukaryotes. *Saccharomyces* by origin carries the definition of sugar mold or fungus. The word *cerevisiae* originated from the old French word cervoise which actually means beer (Mortimer, 2000). Since it is cheap, readily available, and non-pathogenic and widely studied, baker's yeast is an ideal biocatalyst (Bubalo et al., 2015). Baker's yeast is also very economical as it can be grown in an open jar without sterile condition (Servi, 2008). *S. cerevisiae* is usually used to mediate enantioselective reduction due

to its high chemo-, regio- and enantioselectivity. Dumas first observed the reducing action of baker's yeast in 1874 (Csuk and Glaenzer, 1991). High catalytic capacity of baker's yeast as redox biocatalysts play a very important role in the enantioselective reduction of the carbonyl groups (Bubalo et al., 2015). Baker's yeast is an effective biocatalyst used for the reduction of ketone to optically active alcohol, reduction of β-ketoester to β-hydroxy esters, reduction of C=C double bond and reduction of nitro compound (Pratap et al., 2009). Baker's yeast also produces more than 6000 enzymes, which include oxidoreductases. In aerobic condition, *S. cerevisiae* can carry out respiration and fermentative catabolic concomitantly. This is called as respiratory-fermentative catabolism, which is an impact of the Crabtree effect (Perles et al., 2008). The use of baker's yeast in biotransformation has been very common for the past 40 years. Researchers are discovering for solutions on the problem related to chirality. In conclusion, baker's yeast is one of the most important species of yeast in modern biocatalysis (Servi, 2008).

1.6 Problem statement

The consumer demands for varieties of flavorings have been booming. The presence of volatile and non-volatile compounds which possess diverse chemical and physiochemical properties result in flavorings (Sanromán, 2006). There are three common methods to produce flavoring compounds, which are through chemical synthesis, extraction from plant sources and biotransformation. The most traditional method of producing flavor compound is through the extraction from natural sources such as essential oils, fruits or vegetable juices and plant concentrates or extracts (Munch, 1997). However, this method is subjected to a few cons. The raw material does not have sufficient active components that are responsible for flavoring. Other

than that, the desired compounds are usually found in exotic plants, which are very minimum to meet the consumers' needs. This will eventually make the extraction process expensive, thus increasing the price of the flavoring products (Janssens et al., 1992). Besides that, diseases within the plant and weather condition are factors that cannot be controlled by human beings.

The chemical synthesis method is often related to environmentally unfriendly production process. There are high possibilities for the formation of undesirable racemic mixtures due to the lack of substrate selectivity (Sanromán, 2006). This will definitely contribute to the high cost of downstream processing as the process efficiency is being reduced (Janssens et al., 1992).

An increase in demand for flavoring produced naturally without any chemicals gives rise to the growing interest in biocatalysts (Waché et al., 2006). Biotransformation involves the alteration of a precursor to its product (Berger et al., 1999). This method of producing food flavorings is more environmentally friendly in terms of less waste generated, low working temperature and pressure and also a solvent free approach. Other advantages of this method are the production of regio- and stereoselective compounds under mild condition as compared to the chemical routes (Forti et al., 2015).

This work is to investigate the effect glucose concentrations on the co-factor recycling in the *S. cerevisiae*- mediated ketoisophorone biotransformation. Glucose is one of the producers of nicotinamide adenine dinucleotide phosphate (NADPH). The rate of co-factor recycling is affected by the concentration of glucose and thus affects the rate of product conversion.

1.7 Research objectives

This research aims to study the effect of various concentrations of glucose on the co-factor recycling in the *S. cerevisiae*-mediated ketoisophorone biotransformation. The concentration of glucose is varied from 5 g/L, 10 g/L and 15 g/L. In addition, the objective of this research is also to form an interconnection between the co-factor and the product of reduction of ketoisophorone.

CHAPTER TWO

LITERATURE REVIEW

2.1 Reduction reactions

The earliest reducing capacity of yeast was observed through the addition of powdered sulfur in fermenting yeast that results in formation of hydrogen sulfide (H_2S). There were some denials of yeast enzyme being involved in reduction of sulfur as it was observed that proteins containing sulfhydryl group produces H_2S. *S. cerevisiae* is the most widely used microorganism for the reduction of alkene and carbonyl (Csuk and Glaenzer, 1991). Baker's yeast is commonly used to mediate enantioselective reductions mainly due to its abundant bioavailability and ease of treatment as it acts under mild reaction conditions (Schaefer et al., 2013). A very common reaction by baker's yeast is the asymmetric reduction of carbonyl containing compounds (Csuk and Glaenzer, 1991). In 1898, MacLeod and Hub carried out an investigation on reduction of ketone by baker's yeast. The product obtained was secondary alcohol of S configuration. Ketones that were not reduced at all were 4-octanone, *tert*-butyl methyl ketone, isobutyl isopropyl ketone or *n*-amyl phenyl ketone (Csuk and Glaenzer, 1991). A hydrogen transfer to the *re* face of the prochiral ketone was suggested as shown in Figure 2.1. R_S represents the small substituent adjacent to the carbonyl group while R_L represents a large substituent.

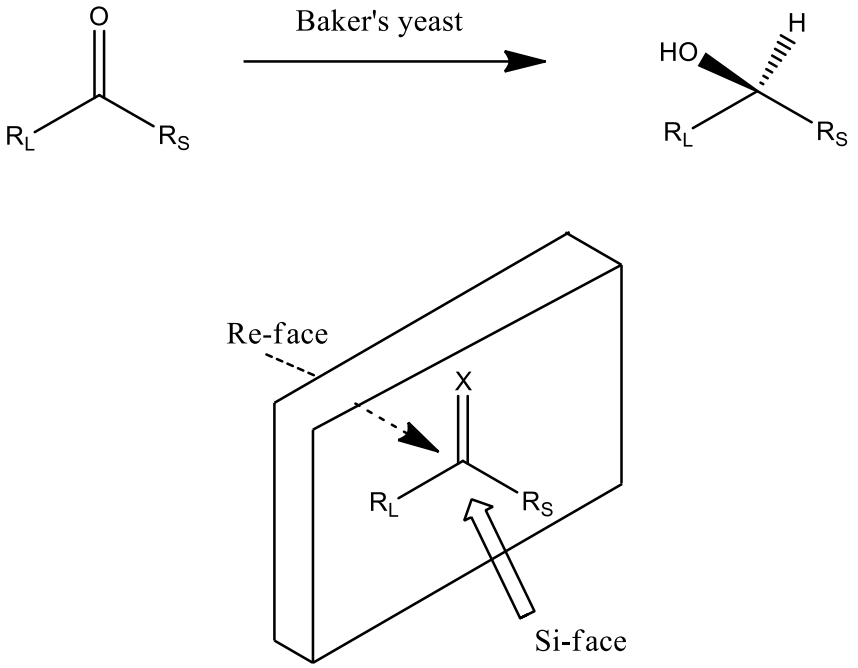

Figure 2.1: Hydrogen transfer to the *re* face of the prochiral ketone (Macleod et al., 1964)

2.1.1 Reduction of aldehydes and ketone using whole-cell

Isolated dehydrogenase requires complex co-factor recycling, whereas whole-cell contains numerous dehydrogenases, which allow it to accept necessary co-factors, metabolic pathway for regenerations and also accepting synthetic substrates. The advantages of whole-cell include affordable carbon sources that can be used as a complementary substrate for reduction. Recycling of own co-factor is readily available in the whole-cell (Faber, 2011). Baker's yeast is also used for asymmetric reduction of ketones. Reduction of simple aromatic and aliphatic ketone is carried out by fermenting yeast according to the Prelog's rule. Prelog's rule ensures the corresponding product of reduction which is (S)-alcohols is in good optical purities. Figure 2.2 shows the general reduction of ketone to alcohol by baker's yeast.

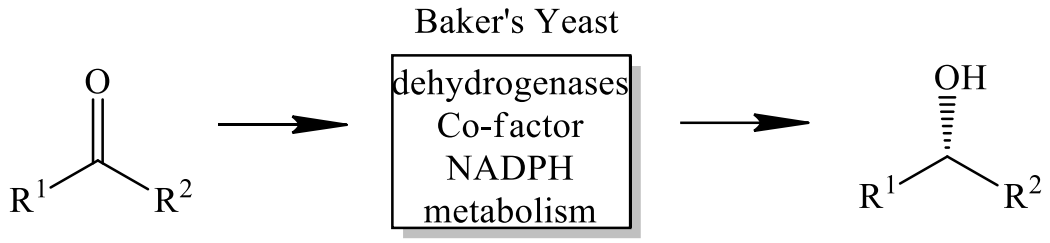

Figure 2.2: Reduction of aliphatic and aromatic ketones using baker's yeast

The reduction of acyclic β-ketoesters (Figure 2.3) which yield β-hydroxyesters serves as the starting material for synthesis of carotenoids and insect pheromones (Gopalan and Jacobs, 1990). For β-ketoester, the enantioselectivity and the stereo chemical preference for the *re*- or the *si*-side depends on the relative size of the alkoxy moiety. This will lead to nucleophilic attack of hydride according to the Prelog's rule. Divergent behavior in baker's yeast is due to the different kind of dehydrogenases. Each dehydrogenase possesses opposite stereochemical preference, which compete for the substrate (Heidlas et al., 1988). A few alternatives have been used to control the stereochemical direction of reduction; these include inhibiting selection of dehydrogenases or precise design of substrate itself.

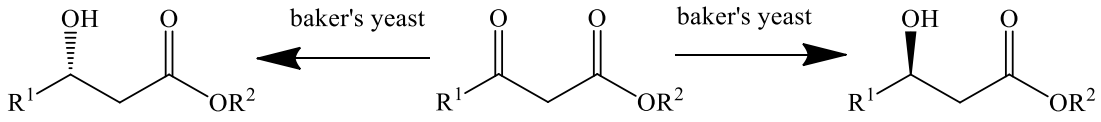

Figure 2.3: Reduction of acyclic β-ketoesters using baker's yeast (Faber, 2011)

The formation of diastereomeric *syn*- and *anti*-products is due to the asymmetrical reduction of living cell of α-substituted ketones. Dynamic resolution due to the rapid *in-situ* racemization occurs because of the ease in altering the α-position of ketone. As a result, the ratio between the diastereomeric *syn*- and *anti*- products is not 1:1 (Katagiri and Uneyama, 2005). A common example of application of diastereoselective yeast-reduction of ketone is the formation of diastereomeric *syn*-

and *anti-β*-hydroxyesters. The presence of α-substituents dominates the formation of *syn*-diastereomers. However, the results are the opposite upon increment in size. Absolute configuration of the secondary alcohol is determined by the Prelog's rule.

In the baker's yeast mediated enantiotopic selective reduction of carbonyl compounds, incomplete stereoselectivity can occur due to different reductase enzyme with different kinetic parameters. Other than that, the reaction might be catalyzed by an enzyme with incomplete stereoselectivity (Egri et al., 1998) .

2.1.2 Reduction of C=C double bond

This is one of the most widely employed strategy to produce chiral compounds as it creates two chiral centers. A detailed mechanism of reduction of asymmetric reduction of alkenes catalyzed by ene-reductase has been studied (Kohli and Massey, 1998). Figure 2.4 shows the asymmetric bioreduction of activated alkenes using flavin-dependent ene-reductases. Hydride (H^-) is stereoselectively transferred onto C_{beta}. Hydride is derived from the reduced flavin co-factor. Tyr-residue which is derived from the solvent adds a proton onto C_{alpha} from the opposite side. (H^+) from Figure 2.4 represents the proton delivered via Tyr-residue. The reaction of addition of H_2 is known as the oxidative half reaction (Ox-HR). The reduction of oxidized flavin co-factor with NAD(P)H is known as the reductive half reaction (Red-HR). The catalytic cycle should consist of both oxidation and reduction. NAD(P)H is normally derived from other external H-source, which has it specific redox reaction. Although this hydride pathway appears rather complex, flavin co-factor are tightly bound to the enzyme and protected from the environment.

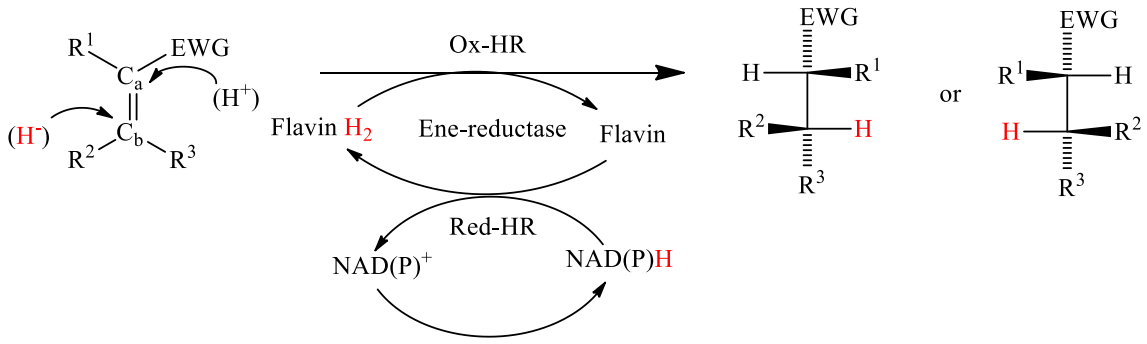

Figure 2.4: Asymmetric bioreduction of activated alkenes using flavin-dependent ene-reductases (Faber, 2011)

These are the few guidelines for the asymmetric bioreduction of the alkenes using enoate- reductases:

i) C=C bond, which are activated by electron-withdrawing group (EWG) are reduced. EWG includes, ester, aldehyde, ketone, carboxylic acid, nitro and cyclic imide (Fuganti and Grasselli, 1989).

ii) Electronically isolated double bonds are not accepted.

iii) The R/S configuration can be controlled by beginning the reaction with a (E)- or (Z)-alkenes.

iv) Cyclic imides are readily reduced without competing side reactions.

v) A good substrate for enoate-reductases is the α,β-unsaturated ketones. The competing of carbonyl-reduction is slower as compared to the enal. The distribution of product solely depends on the rate of competing carbonyl and enoate reductases (Sih et al., 1975).

Yeast-mediated reduction of ketoisophorone produces nonracemic levodione which is the intermediate for the synthesis of carotenoids. Further reduction of the intermediate will produce the product which is actinol. The precursor obtained was 80% and more than 95% of enantiomeric excess (e.e). Reduction of carbonyl moieties will produce

two other minor products as well. As shown in Figure 2.5, no trace of carbonyl reduction was observed using the enoate-reductases OPR3 (Kataoka et al., 2002).

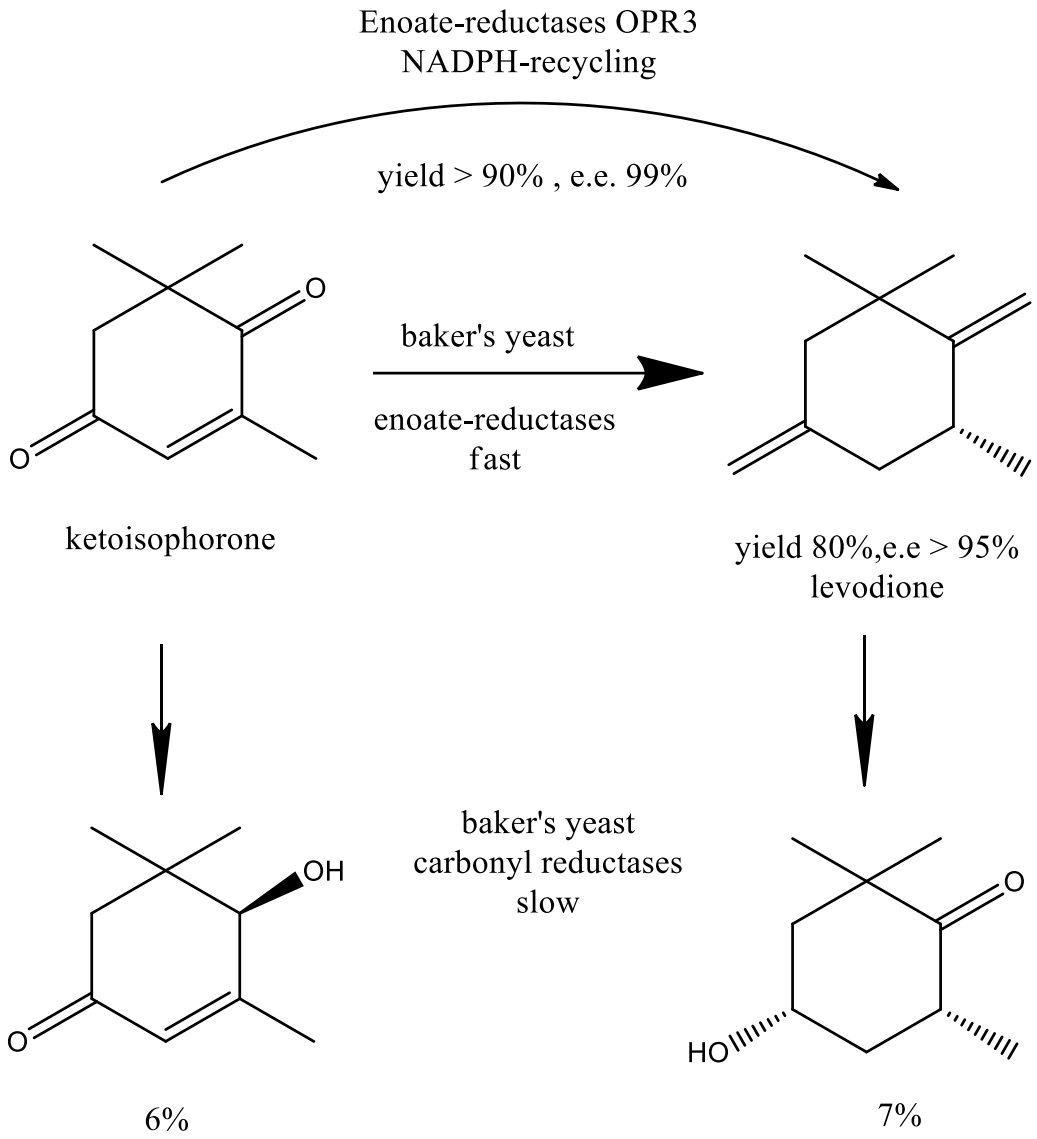

Figure 2.5: Asymmetric bioreduction of α, β-unsaturated diketone (Faber, 2011)

2.2 Co-factors

Co-factor plays a major role in the co-factor dependent enzymes, which are responsible to catalyze several transformations. There are also enzymes which are independent of co-factor like hydrolases, whereby biotransformation occurs via a simple acid-base catalysis (Kara et al., 2014). Requirement of co-factor in a chemical reaction depends on the complexity. Example of complex reactions includes redox reaction or group transfer, which require more than one co-factor (Richter, 2013). Prosthetic groups, which are more commonly known as tightly bound co-factors are self-regenerating and remain in the enzymes active site throughout many catalytic cycles. An example of this co-factor includes flavins, thiamine diphosphate (ThDP) and pyridoxal phosphate (PLP). Dissociable co-factor which are also known as co-enzymes are required in stoichiometric amounts as functional groups are transferred onto substrate during catalysis (Richter, 2013). Examples of these are nicotinamide adenine dinucleotide phosphate (NADP(H)), adenosine-5'-triphosphate (ATP), and (S)-adenosylmethionine (SAM). Table 2.1 shows the common co-factors applied in biocatalysis and their *in-situ* regeneration method.

Table 2.1: Selected co-factor applied in biocatalysis and the most common methods for their *in situ* regeneration (Faber, 2011)

Co-factor	Type of reaction	Regeneration approach	Enzyme or substrate involved in regeneration
NADP(H)	Hydride transfer to substrate	Coupled Enzyme	Glucose dehydrogenase Hydrogenase Formate dehydrogenase
		Coupled Substrate	Isopropanol Ethanol
		Electrochemical	Electrons from anode transferred via a redox mediator
$NAD(P)^+$	Hydride transfer from substrate	Coupled Enzyme	Glutamate dehydrogenase NAD(P)H oxidase
		Coupled Substrate	Acetone Acetaldehyde
		Electrochemical	Electrons to cathode transferred via a redox mediator
ATP	Phosphorylation	Coupled enzyme	Pyruvate kinase Acetate kinase Polyphosphate kinase Carbamate kinase

Determinants of redox state in the cell are the redox couples, which are NAD^+/NADH and $NADP^+$/NADPH. The differences between these two co-factors are the metabolic pathways. ATP is produced in the cytosol by glycolysis while ATP is produced in mitochondria by oxidative phosphorylation. Both the production of ATP in cytosol and mitochondria is driven by NAD (Blacker et al., 2014). In 1960's, it was shown that live tissues emit blue fluorescence when illuminated under ultraviolet light. The nicotinamide moiety of NADH absorbs light of wavelength 340nm and emits fluorescence at 460 nm (Chance et al., 1979).

2.2.1 Roles of NADPH

Four enzymes in mammalian cells are well-known for the production of NADPH; these include Glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (PGD), malic enzyme and isocitrate dehydrogenase. Among all these enzymes, G6PD has an unique importance to numerous cellular processes that utilize NADPH (Stanton, 2012). NADPH oxidases are dependent on the NADPH from G6PD activity. These enzymes portray many physiological roles.

Another example of enzyme dependent on NADPH is nitric oxide synthase (NOS). From a previous research conducted, it was found that endothelial NOS activity is dependent on G6PD-derived NADPH. Therefore, a decrease in G6PD and NADPH will lead to the decrease the production of nitric oxide (Stanton, 2012).

2.2.2 Recycling of co-factors

NADH and NADPH are required for majority of the redox enzyme. Two features of nicotinamide co-factors are:

i) cannot be replaced by other substitutes.
ii) relatively unstable molecule and expensive if used according to stoichiometric amount.

Regeneration can be done *in-situ* by using a redox reaction since the complex structure stays untouched even after oxidation. The total turnover number measures the efficiency of the recycling process before co-factor molecule is finally destroyed. According to the rule of thumb, for technical purposes, the total turnover number must be at least 10^5. The widely used method to recycle NADPH is through the oxidation of glucose catalyzed by the glucose dehydrogenase. Glucose dehydrogenase is very stable and accepts either NAD^+ or $NADP^+$ with high specific activity. Moreover, the

glucose-6-phosphate (G6P) / glucose-6-phosphate dehydrogenase (G6PDH) system complements glucose as an excellent method for regenerating NADPH and NADH.

2.2.3 Glycolysis

Co-factors are produced and recycled through glycolysis, hexose monophosphate pathway, electron transport chain and Krebs's cycle. Glycolysis can occur in both the presence and absence of oxygen. It is a series of reaction that extract energy from glucose by splitting into two 3-carbon molecules known as pyruvate. Figure 2.6 shows the glycolysis pathway of glucose. Glucose is broken down into a 3-carbon molecule known as pyruvate. The cycle will occur twice for one molecule of glucose.

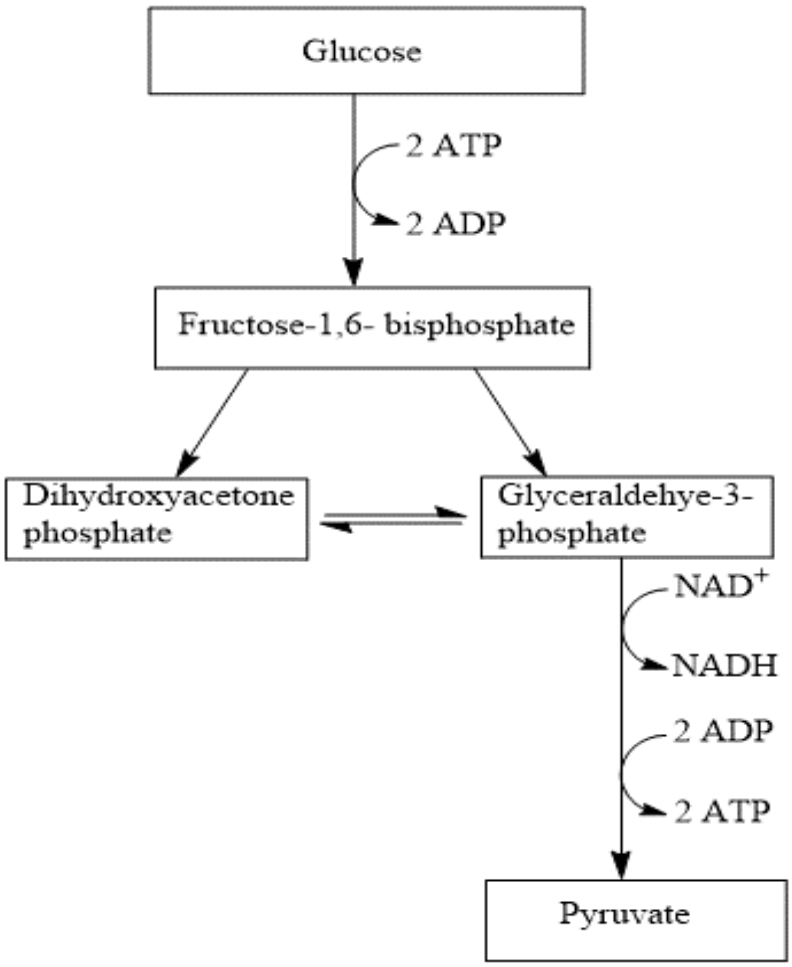

Figure 2.6: Glycolysis pathway of glucose molecule