THE EFFECT OF GROWING CELLS AND IN-SITU BIOTRANSFORMATION OF 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE TOWARDS PRODUCTS FORMATION

SIVA SREE HARI A/L MUNIANDY

Universiti Sains Malaysia

2017

THE EFFECT OF GROWING CELLS AND IN-SITU BIOTRANSFORMATION OF 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE TOWARDS PRODUCTS FORMATION

by

SIVA SREE HARI A/L MUNIANDY

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 convey my sincere gratitude to my supervisor, Associate Professor Dr. Mohamad Hekarl Uzir for his precious encouragement, guidance and generous support throughout this work.

I would also extend my gratitude towards all my colleagues for their kindness 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 also like to thank all SCE staffs for their kindness cooperation and helping hands. Indeed, their willingness in sharing ideas, knowledge and skills are deeply appreciated.

Once again, I would like to thank all the people, including those whom I might have missed out and my friends who have helped me directly or indirectly. Their contributions are very much appreciated. Thank you very much.

Siva Sree Hari A/L Muniandy

May 2017

TABLE OF CONTENTS

	Page
ACKNOWLLEDGEMENT	**i**
TABLE OF CONTENTS	**ii**
LIST OF TABLES	**v**
LIST OF FIGURES	**vi**
LIST OF ABBREVIATIONS	**vii**
ABSTRAK	**viii**
ABSTRACT	**ix**
CHAPTER ONE: INTRODUCTION	**1**
1.1 Biotransformation of ketoisophorone	**1**
1.1.1 Actinol	**1**
1.1.2 Yeast	**2**
1.1.3 Biotransformation	**2**
1.2 Problem Statement	**4**
1.3 Research Objective	**4**
1.4 Organization of thesis	**4**
CHAPTER TWO: LITERATURE REVIEW	**5**
2.1 Choice of Yeast	**5**
2.2 Reduction with Enone Reductase	**6**
2.3 Cofactor	**7**
2.4 Experimental conditions	**8**
2.5 Biotransformation of ketoisophorone	**9**
2.6 Intermediate	**9**
2.7 Selectivity	**10**

2.8 Krebs Cycle **10**

2.9 Cofactor **12**

CHAPTER THREE: MATERIALS AND METHODS **14**

3.1 Materials **14**

3.2 Equipment and instrumentations **14**

3.3 Experimental procedures **14**

3.3.1 Preparation of LB Medium **14**

3.3.2 Preparation of yeast culture **15**

3.3.3 Collection of sample and weighing dry mass **15**

3.3.4 Plotting of growth curve **15**

3.3.5 Preparation of yeast culture **16**

3.3.6 Collection of sample **16**

3.3.7 Carrying out required types of testing **16**

3.3.7.1 Calculation of cell concentration **16**

3.3.7.2 Content analysis using gas chromatography **17**

3.3.7.3 Calculation of the concentration of NADPH/ NADH **17**

3.4 Experimental Activities **18**

CHAPTER FOUR: RESULTS AND DISCUSSION **17**

4.1 General observation **19**

4.2 Growth curve of *S. cerevisiae* **21**

4.3 Growth curve of *S. cerevisiae* with ketoisophorone (substrate) **22**

4.4 NADH and NADPH concentration during the growth phase of *S. cerevisiae* **23**

4.5 Substrate consumption and the intermediate formation towards product formation during the growth phase of *S. cerevisiae* **24**

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS **28**

5.1 Conclusion **28**

5.2 Recommendation **29**

REFERENCES **30**

LIST OF TABLES

		Page
Table 1.1	Classification of enzymatic reaction	3
Table 2.1	Classification of coenzyme present within cell	7

LIST OF FIGURES

		Page
Figure 1.1	KIP bioreduction in yeasts	3
Figure 2.1	Two-step enzymatic conversion from KIP to ACT; KIP molar residual rate (□), DOIP molar yield (Δ), and ACT molar yield (●)	10
Figure 2.2	An overview from the Krebs cycle enzymes, intermediates and their connections to the other major energy metabolic pathways	12
Figure 3.1	Schematic flow diagram of experimental procedures	18
Figure 4.1	Growth curve of *S. cerevisiae* (letters A, B, C, and D represents lag phase, exponential phase, stationary phase and death phase respectively)	21
Figure 4.2	Growth curve of *S. cerevisiae* after the substrate injection (letters A, B and C represents batch 1, 2 and 3 respectively)	22
Figure 4.3	NADH/NADPH concentration during the growth phase of *S. cerevisiae* (letters A, B and C represents batch 1, 2 and 3 respectively)	24
Figure 4.4	Concentration of Substrate and Intermediate without glucose (letters A, B and C represents batch 1, 2 and 3 respectively)	25
Figure 4.5	Concentration of substrate and intermediate with glucose (letters A, B and C represents batch 1, 2 and 3 respectively)	25
Figure 4.6	Concentration of KIP without and with glucose (letters A, B and C represents batch 1, 2 and 3 respectively)	26
Figure 4.7	Concentration of DOIP in without and with glucose (letters A, B and C represents batch 1, 2 and 3 respectively)	27

LIST OF ABBREVIATIONS

ACT	Actinol
BVMOs	Baeyer– Villiger monooxygenases
CD	Circular dichoism
CPL	Circular polarization of luminescence
CR	Carbonyl Reductase
DOIP	6R-dihydro-oxoisophorone (Levodione)
FAD	Famines
FID	Flame ionization detector
He	Helium
IBA	Isobutyramide
KIP	Ketoisophorone
KREDs	Ketoreductase
LB	Luria-Bertani
NADH	β-1,4-nicotinamide adenine dinucleotide
NADPH	β- 1,4-nicotinamide adenine dinucleotide phosphate
OR	Oxidoreductase
OYE	Old Yellow Enzyme
PDB	Protein Data Base
r.p.m	Rotation per minute
UV	Ultraviolet

KESAN PERTUMBUHAN SEL DAN IN-SITU BIOTRANSFORMASI 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE KE ARAH PEMBENTUKAN PRODUK

ABSTRAK

Actinol adalah substrat yang diperlukan untuk sintesis xanthophylls optik aktif, xanthoxin dan zeaxanthins serta aroma konstituen tembakau dan kunyit boleh dihasilkan oleh pengurangan ketoisophorone dengan hasil yang tinggi dan enantioselectivity. Actinol boleh dihasilkan dari biotransformasi ketoisophorone. Biotransformasi daripada ketoisophorone membentuk levodione, yang merupakan perantaraan sebelum menghasilkan actinol.Biotransformasi daripada ketoisophorone telah dikaji semasa fasa pertumbuhan *S. cerevisiae* dengan penggunaan dan tanpa glukosa. Semasa fasa pertumbuhan *S. cerevisiae*, biotransformasi ketoisophorone hanya mampu menghasilkan pertengahan dan produk itu belum terbentuk.Kadar biotransformasi daripada ketoisophorone adalah lebih cepat dengan penggunaan glukosa. Hal ini kerana kehadiran glukosa meningkatkan konsentrasi NADH dan NADPH yang merupakan kofaktor penting yang diperlukan untuk biotransformasi daripada ketoisophorone. Oleh itu, kadar pengurangan ketoisophorone adalah lebih tinggi menyebabkan konsentrasi yang lebih tinggi levodione. Apabila kadar biotransformasi adalah tinggi, pengeluaran actinol juga akan lebih cepat. Oleh itu, kehadiran glukosa adalah penting bagi biotransformasi ketoisophorone.

THE EFFECT OF GROWING CELLS AND IN-SITU BIOTRANSFORMATION OF 2,6,6-TRIMETHYLCYCLOHEX-2-ENE-1,4-DIONE TOWARDS PRODUCTS FORMATION

ABSTRACT

Actinol (ACT) is the substrate needed for the synthesis of optically active xanthophylls, xanthoxin and zeaxanthins as well as aroma constituent of tobacco and saffron can be produced by the reduction of ketoisophorone (KIP) with high yield and enantioselectivity. The ACT can be produced from the biotransformation of KIP. The biotransformation of KIP forms levodione (DOIP), which is intermediate before further reduction into ACT. The biotransformation of KIP was studied during the growth phase of *S. cerevisiae* with the presence and absence of glucose. During the growth phase of *S. cerevisiae*, the biotransformation of KIP was only able to produce intermediate and the product was yet to be formed.The rate of biotransformation of KIP was faster with the presence of glucose. This was because presence of glucose increases the concentration of NADH and NADPH which was the essential cofactor needed for biotransformation of KIP. Thus, the rate of reduction of KIP was higher resulting in higher concentration of DOIP. When the rate of biotransformation was high, the production of ACT will also be faster. Therefore, the presence of glucose was important for the biotransformation of KIP.

CHAPTER ONE

INTRODUCTION

1.1 Biotransformation of ketoisophorone

1.1.1 Carotenoids

Carotenoids are currently being used as food colorants, nutritional supplements, and poultry feed additives and in ophthalmology for the treatment of age related ocular diseases *viz.* cataract and dry age related macular degeneration (ARMD). The xanthophyll, zeaxanthin and xanthoxin are types of carotenoids which used for pharmaceutical and medical purposes (Jaswir et al., 2011; Hegazy et al., 2008). These types of carotenoids use actinol to be the key intermediate (Leuenberger et al., 1976; Mayer et al., 1979).

1.1.2 Actinol

The biocatalytic production of fine chemicals used for the synthesis of naturally occurring carotenoids, vitamins, pigments, flavors and fragrances, is becoming the preferred route for commercial-scale production. Biocatalytic processes, occurring at mild conditions using renewable resources, are usually slow but with competitive yields and desirable enantioselectivities. Actinol (ACT), a key intermediate in the synthesis of optically active xanthophylls, xanthoxin and zeaxanthins (Leuenberger et al., 1976; Mayer et al., 1979) as well as aroma constituent of tobacco and saffron (Sode et al., 1987) can be produced by the reduction of ketoisophorone (KIP) with high yield and enantioselectivity.

This conversion has been carried out via hydrogenation of the substrate (KIP) over alumina-supported Pt and Pd catalysts (Arx et al., 1999) or over a Pd/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 (*S. cerevisiae*) as catalyst had a higher yield of 80–85% DOIP and a very attractive enantiomeric excess which is more than 98% (Arx et al., 1999). Enantiomeric excess is a measurement of purity used for chiral substances. *S. cerevisiae* facilitates the bioconversion of KIP to production of a doubly chiral compound, ACT with DOIP as the intermediate (Kataoka et al., 2004).

1.1.2 Yeast

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

1.1.3 Biotransformation

Biotransformation is defined as the transformation of a substrate to a recoverable end-product by either microbial or enzymatic means. The biotransformation that will be considered will be simple analogues of single-step synthetic organic reactions that converts substance to product though the action of a single primary catalyst, either enzyme or microbe, and will not therefore, consider multi-step, single-pot enzyme reactions (Bommarus and Polizzi, 2006; Chen and Zeng, 2016; Grogan, 2009).

Actinol can be produced though the biotransformation of KIP by contacting with baker's yeast, such as *S. cervisiae* , which functions as an enantioselective biocatalyst (Vandamme, 1989). The types of enzymatic reactions are listed in Table 1.1. Biotransformation of KIP utilizes the type of enzymes which classified as oxidoreductase. The oxidoreductases depend on a redox cofactor such as NAD(P)H/NAD(P)+, which functions as an electron donating or accepting co-substrate, and thus are coupled to redox metabolism in whole cells, the most common form in which oxidoreductases are applied for biocatalysis (Blank et al., 2010; Oroz-Guinea and García-Junceda, 2013). KIP is capable of producing other intermediates and products as shown in Figure 1.1 (Fukuoka et al., 2002).

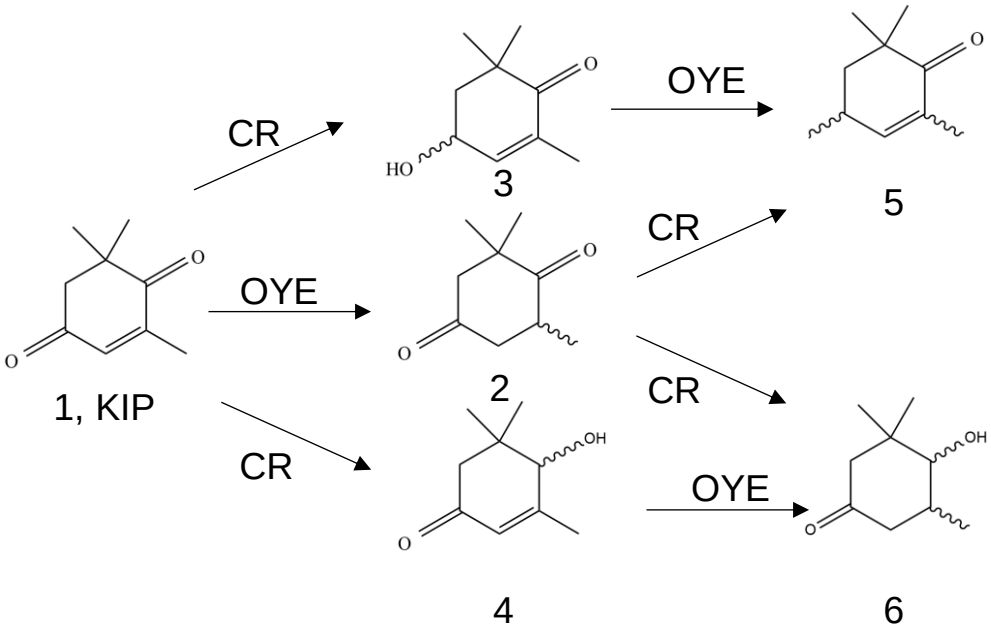

Figure 1.1 KIP bioreduction in yeasts (**1** – 2,6,6-trimethylcyclohex-2-ene-1,4-dione [ketoisophorone]; **2** – 2,6,6-trimethylcyclohexane-1,4-dione [(6R)-levodione]; **3** – 4-hydroxy-2,6,6-trimethylcyclohex-2-en-1-one [(4S)-phorenol]; **4** – 4-hydroxy-3,5,5-trimethylcyclohex-2-en-1-one [hydroxyisophorone]; **5** – 4-hydroxy-2,6,6-trimethylcyclohexaone [(4R, 6R)-actinol]; **6** – 4-hydroxy-3,5,5- trimethylcyclohexanone. OYE – enoate reductase; CR – carbonyl reductase) (Fukuoka et al., 2002).

Table 1.1 Classification of enzymatic reaction

Types of Enzymatic Reactions	**Definition**
Oxidoreductase	Transfer of electron (hydride ions or H atoms)
Transferase	Group transfer reactions
Hydrolase	Hydrolysis reactions (transfer of functional groups to water)
Lyase	Cleavage of C-C, C-O, C-N, or other bonds by elimination, leaving double bonds or rings, or addition of groups to double bonds
Isomerase	Transfer of groups within molecules to yield isomeric forms
Ligase	Formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor

1.2 Problem Statement

The *S. cerevisiae* is used for the biotransformation of KIP. The present invention is related to a process for producing ACT from KIP in non-growing medium. This study primarily focuses on the cell activity during the growth phase of *S. cerevisiae* in growing medium. Hence, the KIP should be injected into sample at the beginning of exponential growth of *S. cerevisiae* to determine the substrate utilization rate throughout the growth phase. The biotransformation of KIP involves enzymes and NADH/NADPH as cofactor. The glucose contributes to the regeneration of NADH/NADPH through Krebs Cycle. The effect of presence and absence of glucose during the biotransformation of KIP in growing medium must be studied.

1.3 Research Objective

i) To determine the correct mechanism of ketoisophorone biotransformation during the growth phase of *S. cerevisiae*.

ii) To determine the effect of presence and absence of glucose during the biotransformation of ketoisophorone.

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 biotransformation of ketoisophorone, 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 Choice of yeast

Actinol (ACT) has previously been prepared though the reduction of the carbon-carbon double bond and carbonyl double bond in ketoisophorone (KIP) by contacting said KIP with baker's yeast, such as *S. cervisiae* , which functions as an enantioselective biocatalyst (Vandamme, 1989) which forms levodione (DOIP) as intermediate. Initially, baker's yeast was found to be not suitable for use in the industrial production as the yields of DOIP were too low. Two problems associated with the production process using baker's yeast and which had to be overcome for developing an industrially feasible production process. Firstly, the yeast cells could not be reused because of the short lifetime of the reaction activity of the yeast. Second problem was a complicated purification process was necessary because it was difficult to separate the yeast from the culture solution after the catalytic reaction.

The result of extensive studies to increase the yield of DOIP produced from KIP and to overcome the other problems it was found that certain yeast strains can produce DOIP much more efficiently from KIP than hitherto. Accordingly, the present invention provides a process for producing DOIP characterized by contacting KIP with at least one kind of yeast capable of converting KIP into DOIP and selected from the group of species consisting of *S. rouxi (Z. rouxii), S. delbrueckii (S. unisporus , T. delbrueckii), S. willianus , Z. bailii* and *C. tropicalis* , and functional equivalents, subcultures, mutants and variants of such species, in water, a water-miscible organic solvent or a mixture of water and said water-miscible organic solvent containing at least one assimilable carbon source, followed by isolating the resulting ACT from the reaction medium after completion of the reaction (Fukuoka et al., 2001).

It was further found that by entrapping such a yeast by certain techniques and using the so-immobilized yeast for said catalytic reaction, DOIP of high purity can be produced in even higher yield. Among these yeasts, *S. rouxii (Z. rouxii)* HUT 7191 (IFO 0494) was the one most preferred for use in the process of the present invention (Fukuoka et al., 2001).

Z. Candida, Gluconobacter, Beneckea, or Vibrio having the same physico-chemical properties, as defined by the International Code of Nomenclature of Prokaryotes. A transformed microorganism, such as Escherichia coli, expressing NADPH dehydrogenase can also be used as a starting microorganism. In one embodiment of the present invention, a commercially available OYE can be used for the catalytic cleavage of KIP into DOIP. Microorganism suitable to produce NADPH dehydrogenase such as OYE is *S. cerevisiae*, preferably *S. cerevisiae S288C* (ATCC 204508) publicly available from the American Type Culture Collection, 10801 University Boulevard Manassas (Shimizu and Wada, 2003).

Temperature is one of the important factors affecting the growth of *S. cerevisiae*. Therefore, 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 temperatures (1-10°C), the cells are viable but they do not grow well (Arthur and Watson, 1976). The growth rate increases at temperatures from 28°C to 37°C and the growth rate does not increase at temperatures from 39°C to 41°C, but the cells are still viable. However, 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).

The effect of temperature on *S. cerevisiae* can be used in pathological industry. *S. cerevisiae* is well known as a Generally Recognized as Safe (GRAS) microorganism, however the number of reported mucosal and systemic infection in human population has increased and even fatal infections have occurred in relatively healthy individuals (Muller et al., 2011).

2.2 Reduction with Enone Reductase

Enone reductase encompasses proteins catalysing the enzymatic reduction of carbonyl activated double bonds according to the Enzyme Nomenclature provided by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). It also relates to proteins having the activities of an enone reductase, which proteins preferably catalyse the conversion of KIP into DOIP. The gene for an enone reductase involved in the biosynthesis of DOIP would be very useful for improvement of DOIP productivity by a microorganism (Kataoka and Shimizu, 2006).

Old yellow enzyme (OYE) was the first flavoprotein discovered in yeast and investigated by Warburg et al. in the 1930s (Warburg and Walter, 1933a,b). The physiological role of this flavoprotein, a single-domain protein of 45 kDa size, is still unclear, however, a number of substrates have been found (Karplus et al., 1995; Vaz et al., 1995). KIP (ketoisphorone; 3,5,5-trimethyl-2-cyclohexene-1,4-dione) was reduced to the optically active (6R)-levodione (2,2,6-trimethylcyclohexane-1,4-dione), catalysed by OYE from *C. macedoniensis* (Kataoka et al., 2002b).

The combination of OYE from *S. cerevisiae* with DOIP reductase, LVR, from Corynebacterium aquaticum M-13, KIP was reduced in a two-step, one-pot reaction to (4R,6R)-ACT (4-hydroxy-2,2,6- trimethylcyclohexanone) (Wada et al., 2003). NADH was regenerated in both steps by adding commercially available glucose dehydrogenase (GDH), glucose, and NAD+. The doubly chiral (4R,6R)-ACT was produced with >95% e.e. and with nearly stoichiometric yield. Subsequently, the genes of both glucose dehydrogenase (GDH) and OYE from *C. macedoniensis* (OYE) were co-expressed in *E. coli* BL21 (DE3) and were utilized to achieve the formation of 627mM (96.6 g/L) of (6R)-DOIP with a molar yield of 95.4% (Kataoka et al., 2004).

A putative enone reductase gene from *C. lusitaniae* was heterologously overexpressed in *Escherichia coli*. This NADPH-dependent flavoprotein was identified with reduction activities toward a diverse range of activated alkenes including conjugated enones, enals, maleimide derivative and α, β-unsaturated carboxylic esters. *Cl*ER was cloned, over-expressed and characterized for its biocatalytic properties, particularly regarding its substrate scope and stereoselectivity. Furthermore, we show its applicability for the production of ACT from KIP (Ni et al., 2014).

2.3 Cofactor

ACT can be formed from KIP by using a NADPH dehydrogenase as a catalyst. The NADPH dehydrogenases for use as catalysts in the process of the present invention are generally known as old yellow enzyme (OYE) and defined by the enzyme class E.C. 1.6.99 according to the enzyme Nomenclature provided by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.

The production ACT from KIP which comprises contacting KIP with NADPH dehydrogenase in the presence of NADH or NADPH in an aqueous medium, and isolating the resulted DOIP from the reaction mixture. As used herein, the term "NADPH dehydrogenase" encompasses proteins capable of catalysing the enzymatic reaction of KIP to DOIP in the presence of NADH or NADPH. Particularly, the invention is related to a process for producing DOIP from KIP wherein the NADPH dehydrogenase catalysing said reaction is OYE defined by the enzyme class EC 1.6.99. A transformed microorganism, such as Escherichia coli, expressing NADPH dehydrogenase can also be used as a starting microorganism.

The microorganism suitable for the production of NADPH dehydrogenase such as OYE is *S. cerevisiae*, preferably *S. cerevisiae* S288C (ATCC 204508) publicly available from the American Type Culture Collection, 10801 University Boulevard Manassas, Va. 20100 2209, USA. The well-known purification process can be used for the preparation of the enzyme (Abramovitz and Massey, 1976).

The purified *Cl*ER was found to catalyse the oxidation of both NADH and NADPH in the presence of molecular oxygen, but a remarkably accelerated rate of NADPH oxidation in the presence of enones was observed. When NADH rather than NADPH was used, the rate of oxidation barely increased with KIP as the substrate, indicating NADPH as the preferred cofactor (Ni et al., 2014).

2.4 Experimental conditions

ACT produced enzymatically or biologically in a reaction mixture as described above may be extracted by an organic solvent such as ethyl acetate, n-hexane, toluene, or n-butyl. The extract may be analysed by known method such as gas chromatography, high performance liquid chromatography, and thin layer chromatography or paper chromatography.

After the reaction, DOIP in the reaction mixture was recovered, by extraction with a water-immiscible organic solvent, which readily solubilizes DOIP, such as ethyl acetate, n-hexane, toluene, or n-butyl acetate. Further purification of DOIP can be effected by concentrating the extract to directly crystallize DOIP or by the combination of various kinds of chromatography, for example, thin layer chromatography, adsorption

chromatography, ion-exchange chromatography, gel filtration chromatography or high performance liquid chromatography (Kataoka et al., 2006).

2.5 Biotransformation of KIP

A KIP-reducing reaction with *E. coli* was performed in a reaction mixture containing a cofactor regeneration system which includes NADP+, GDH and glucose. 100 mg/ml KIP was almost completely converted to (6R)-DOIP in 36 h, and the (6R)-DOIP formed amounted to 98.2 mg/ml, the molar yield being 96.9%. On the other hand, in the reaction without maintenance of the pH at 7.4, (6R)-DOIP was hardly produced (molar yield, 0.48%). Gluconic acid, which was the spontaneous hydrolysis product of gluconolactone produced though cofactor regeneration, i.e. oxidation of glucose, caused a decrease in pH. Therefore, it is necessary to maintain the pH of the reaction mixture at around the neutral range. The effects of glucose and GDH in the reaction with washed cells of *E. coli* were determined cells of the transformant, was incubated at 28 ^{0}C with shaking (300 rpm) for 6 hr.

The KIP-reducing reaction with the *E. coli* transformant overexpressing OYE could proceed even when the cofactor regeneration system, GDH and/or glucose, was omitted from the reaction mixture (molar yield, 13.7% without GDH and glucose; 51.1% without GDH). But the addition of GDH and glucose to the reaction mixture enhanced the efficiency of the KIP reduction reaction (molar yield, 98.7%) (Kataoka et al., 2004).

The asymmetric reduction of KIP is of special interest, since the corresponding product DOIP represents an important chiral intermediate for carotenoid synthesis (Ernst, 2002). Thus, KIP reduction was conducted to evaluate the practical potential of the new enzyme. Using recombinant *E. coli* expressing *Cl*ER which was subjected to ultrasonic disruption, coupled with GDH-mediated NADPH regeneration, as much as 500 mM of KIP was smoothly reduced to DOIP, resulting in a product yield of 97% and an optical purity of 98% e.e. within 1 h (Ni et al., 2014).

2.6 Intermediate

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 (Chang and Raymond, 2005). The biotransformation of KIP produces DOIP as intermediate which transforms into ACT (Winkler et al., 2012) as shown in Figure 2.1 (Wada et al., 2003). Furthermore, in Figure 2.1, the arrow indicates the time of addition of DOIP. Each point represents the mean of thee replicates and includes standard-error bars.

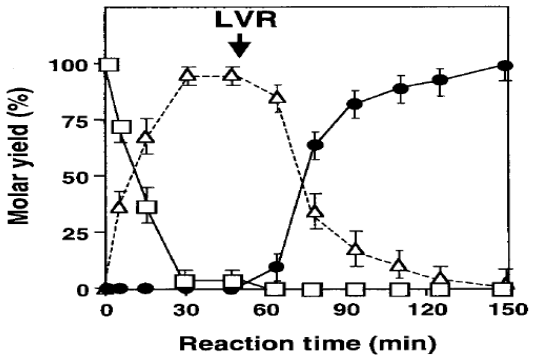

Figure 2.1 Two-step enzymatic conversion from KIP to ACT; KIP molar residual rate (□), DOIP molar yield (Δ), and ACT molar yield (●) (Masaru et al., 2003).

2.7 Selectivity

Selective implies the factors which favours the formation of a specific product. Types of selectivity includes chemoselectivity, regioselectivity, and stereoselectivity. The preferential formation in a chemical reaction of one stereoisomer over another. When the stereoisomers are enantiomers, the phenomenon is called enantioselectivity and is quantitatively expressed by the enantiomer excess. During the biotransformation of KIP, OYE reduced KIP and showed high stereoselectivity, producing DOIP as major product which is later converted into action as shown in Figure 1.1 (Buque-Taboada et al., 2005; Fu et al., 2012).

2.8 Krebs Cycle

Krebs cycle, also known as the citric acid or tricarboxylic acid cycle, is a cyclic pathway of enzymatic reactions which oxidizes the compounds derived from glucose,

fatty acids and amino acids in the matrix of mitochondria (Figure 2.2). The Krebs cycle involves eight steps which produce in different steps CO_2 and NADH and $FADH_2$, reduced coenzymes which transfer electrons to the respiratory chain. Subsequently, the respiratory chain generates ATP for the use in cellular processes. The chemical intermediates of Krebs cycle can penetrate to the cytoplasm where they can be used as precursors for biosynthetic reactions. Moreover, some of the Krebs cycle enzymes have isoenzymes in cytoplasm which can process citrate to 2-oxoglutarate, i.e. via aconitase and isocitrate dehydrogenase (Figure 2.2). On the other hand, cytoplasmic intermediates can enter the mitochondria and be further processed in the Krebs cycle (Salminen et. al., 2014; Zhao and Yang, 2016).

Primarily, the cycle is supplied with new substrates via acetyl-CoA, which can be derived from glycolysis, β-oxidation or amino acid degradation (Figure 2.2). In addition, glutamate dehydrogenase can generate 2-oxoglutarate via the deamination of glutamate (Hudson and Daniel, 1993; Li et al., 2012). In general, Krebs cycle in association with oxidative phosphorylation is the major pathway in cellular energy production (Salminen et al., 2014).

The NADH/NADPH are the cofactors needed for the biotransformation of ketoisophorone which are produced in the Krebs Cycle. The Krebs Cycle is part of glycolysis process which converts glucose into energy. Glycolysis is reduced to three reactions. The first reaction describes the conversion from glucose to glyceraldehyde-3-phosphate (GAP) which consumes two ATP and produces two GAP; the second reaction contains the GAP dehydrogenase reaction that produces NADH in the cytosol and 1,3-diphosphoglycerate (1,3-DPG); the third reaction lumps together the remaining glycolysis reactions which produces two ATP molecules per GAP; which are four per glucose molecule and one molecule of pyruvate; which are two per glucose molecule. From Figure 1.3 ACO1 and IDH1 are cytoplasmic enzymes, others are in mitochondria. Pyruvate can exit, be used for another metabolic pathway, or be converted to lactate with the simultaneous oxidation of NADH as shown in Figure 2.2. The pyruvate dehydrogenase reaction with the production of one NADH molecule in mitochondria. Krebs cycle produces four molecules of NADH (Nazaret and Mazat, 2008; Pollak et al., 2007; Singh et al., 2008).

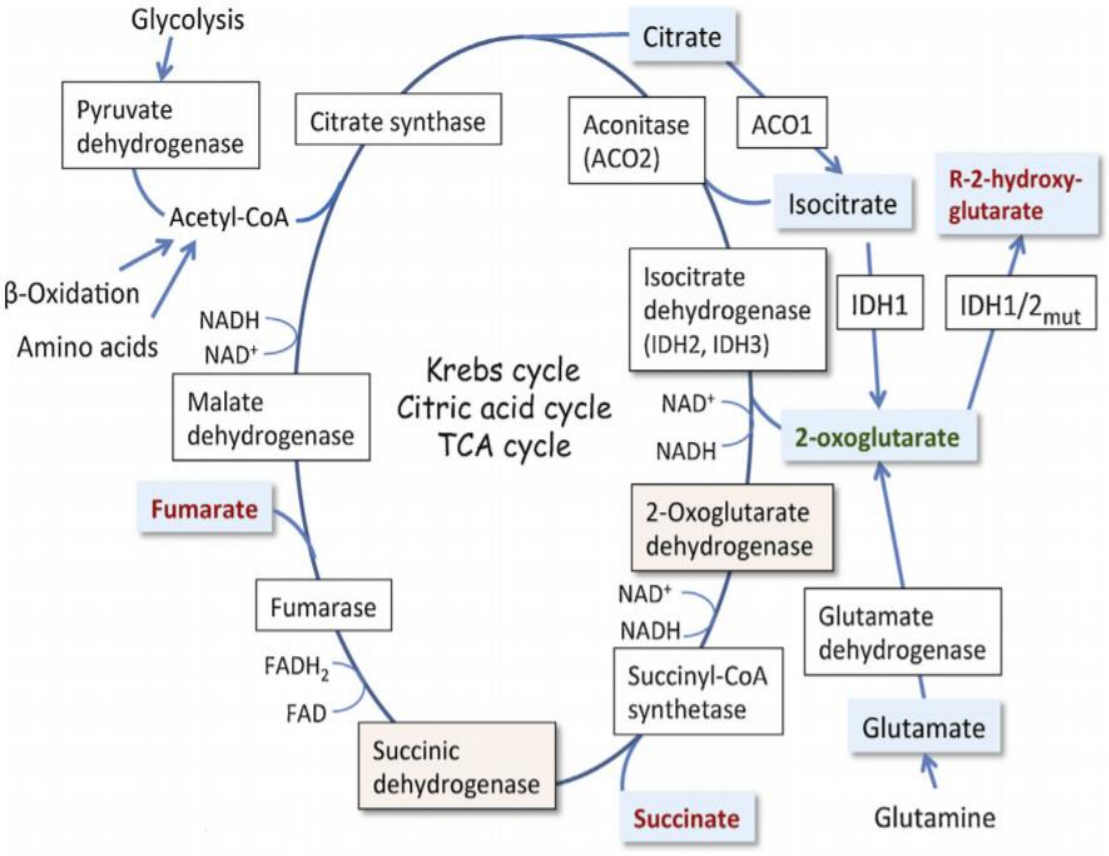

Figure 2.2 An overview from the Krebs cycle enzymes, intermediates and their connections to the other major energy metabolic pathways (Salminen et al., 2014).

2.9 Cofactor

A cofactor is a non-protein chemical compound that is required for the protein's biological activity (Nelson, 2008). The classification of coenzyme present within cell are shown in Table 1.2. Some enzymes or enzyme complexes require several cofactors. The multienzyme complex pyruvate dehydrogenase at the junction of glycolysis and the citric acid cycle (Figure 1.3) requires five organic cofactors and one metal ion: loosely bound thiamine pyrophosphate (TPP), covalently bound lipoamide and flavin adenine dinucleotide (FAD), and the co-substrates nicotinamide adenine dinucleotide (NAD+) and coenzyme A (CoA), and a metal ion (Mg^{2+}) (Sauke et al., 2001).

Table 2.1 Classification of coenzyme present within cell

Coenzyme	Abbreviation	Entity Transferred
Nicotine Adenine Dinucleotide	NAD - partly composed of niacin	electron (hydrogen atom)
Nicotine Adenine Dinucleotide Phosphate	NADP -Partly composed of niacin	electron (hydrogen atom)
Flavine Adenine Dinucleotide	FAD - Partly composed of riboflavin (vit. B2)	electron (hydrogen atom)
Coenzyme A	CoA	Acyl groups
Coenzyme	CoQ	electrons (hydrogen atom)
Thiamine Pyrophosphate	thiamine (vit. B1)	aldehydes
Pyridoxal Phosphate	pyridoxine (vit B6)	amino groups
Biotin	biotin	carbon dioxide
Carbamide Coenzymes	vit. B12	alkyl groups

The NADH and NADPH are important cofactors in the biotransformation of KIP using *S. cerevisiae* is as following (Shimizu and Wada, 2003). In a microbial biotransformation, which involves reduction reaction, living cells either growing or resting, have an advantage of cofactor regeneration cycle within the cells. A reduction reaction by the whole cells requires NAD(H) and/or NADP(H) as cofactors, which are initially available in the cells. However, there is only a limited amount of cofactors inside the cells and co-substrates such as glucose are necessary for recycling the cofactors through the metabolic pathways. Therefore, a sufficient amount of glucose should be supplied in order to achieve an optimum biotransformation rate (Arifin et al., 2011; Meijers et al., 2001; Meijers et al., 2007; Oroz-Guinea and García-Junceda, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Biocatalysts

Yeast from *S. cerevisiae* type II was used in their active dry yeast form (from Sigma-Aldrich (USA)).

3.2 Materials

The following chemicals were used as received: ketoisophorone substrate (98%) purchased from Sigma-Aldrich (USA); Ethyl acetate from Sigma-Aldrich (USA); Potassium hydrogen phosphate (K_2HPO_4) (99%) purchased from QRe˙C; Potassium dihydrogen phosphate (KH_2PO_4) (99.9%) from Fisher Scientific; Glucose (≥99.5%) from Sigma-Aldrich; Tri-Hydrochloride from Calbiochem and Bovine serum albumin (BSA) from Sigma-Aldrich (USA).

3.3 Experimental procedures

3.3.1 Preparation of LB medium

The Luria-Bertani (LB) medium was prepared using 10 g of sodium chloride, 10 g of peptone and 5 g of yeast extract. The preparation of LB medium with glucose content was done by adding 5 g of glucose to obtain the glucose concentration of 5 g/L. Next, the media bottle was filled with 1 L of deionized water. The bottle was shaken up and down until all the solid clumps were dissolved throughout.

Meanwhile, the shake-flasks to be used were prepared and cleaned. The shake-flasks were dried in oven. The LB medium of 250 ml was measured using a measuring cylinder and poured into each of the shake-flasks. The mouth of each shake-flask were covered with a thick cotton wool. Next, the mouth of each shake-flask was wrapped with aluminium foil tightly. The shake-flasks were placed in Autoclave at 121 ^{0}C for 30 min. Then, it was allowed to be cooled down to room temperature.

3.3.2 Preparation of yeast culture

The shake-flask, and the container which contains yeast, were took to the clean room. The yeast was transferred to a smaller bottle from the big bottle to avoid contamination to the source. The yeast cells were weighed on the folded aluminium foil and the transferred into the shake-flask together with the aluminium foil. The mass of yeast cell was measured according to the concentration of yeast cells required. At the concentration of 1 g/L, 0.25 g of yeast cells were required. The shake-flask was then placed into incubator shaker which operated at the temperature of 37 ^{0}C at 150 r.p.m. as shown in Figure 3.1.

3.3.3 Collection of sample and absorbance test

The cell mass was calculated using the difference in mass of eppendorf tube which is empty and after containing cells. Then, the eppendorf tubes were dried in the oven for 24 h at 60 ^{0}C. The mass of empty eppendorf tubes were weighed with a mass balance and recorded.

The incubator shaker was stopped and 1 ml of sample was taken from each of the yeast culture using a pipette. Each sample taken was transferred to eppendorf tube. The eppendorf tube was immediately centrifuged for 5 min. The liquid was poured out while the cell residue were attached to the eppendorf tube. The eppendorf tubes were dried in the oven for 24 h at 60 ^{0}C. The mass of empty eppendorf tubes were weighed with a mass balance and recorded. Then the cell concentration in the eppendorf tubes was calculated using the following equation.

3.3.4 Plotting of growth curve

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

3.3.5 Preparation of yeast culture

The shake-flask, and the container which contained yeast, were taken into the clean room. The yeast was transferred to a smaller bottle from the big bottle to avoid contamination to the source. The yeast cells were weighed on the folded aluminium foil and the transferred into the shake-flask together with the aluminium foil. The mass of yeast cell measured depends on concentration of yeast cells required. At the concentration of 1 g/L, 0.25 g of yeast cells were required. The shake-flasks were then placed into incubator shaker which was at 37 ^{0}C at 150 r.p.m.

The substrate used was ketoisophorone (KIP). The substrate needed was 0.2 g/L, hence 0.03 g which is equivalent to 50 µL of substrate. The substrate was added to yeast culture in incubator shaker at the time initial stage of exponential growth was observed from the growth curve. Then, the yeast culture was resumed to rotate at 150 r.p.m at 37 ^{0}C in the incubator shaker.

3.3.6 Samples collection

The incubator shaker was stopped and 0.5 ml of sample was taken from each yeast culture and transferred to the respective labelled eppendorf tubes, and this step was done twice. Hence, two eppendorf tubes with 0.5 ml samples were prepared for each yeast culture. Later, 0.5 ml of ethyl acetate were added to each eppendorf tubes respectively. Then, 1 ml of sample was taken from each yeast culture and transferred to the respective labelled eppendorf tubes. The collection of sample was done at the interval of 1 h.

3.3.7 Carrying out required types of testing

3.3.7.1 Calculation of cell concentration

The empty eppendorf tubes were labelled and weighed by using mass balance. Then, 1 ml of was added to the eppendorf tube. The eppendorf tube was centrifuged for 5 min and the liquid layer was removed. The remaining residue sample in the eppendorf tubes were dried in the oven at 60 ^{0}C for 24 h. The eppendorf tubes containing biomass were weighed after 24 h of drying period. Then the cell concentration in the eppendorf tubes were calculated using the following equation.

3.3.7.2 Content analysis using GC

The 0.5 ml of sample and 0.5 of solvent were added to the eppendorf tube, and two eppendorf tubes was prepared for each yeast culture. The solvent to be used was ethyl acetate. For even mixing of solution in the eppendorf tube, the vortex mixer was used. Next, the eppendorf tubes were placed in the centrifuge for separation of products by layers. The top layer which contains organic matter was extracted and transferred into a test tube. Extraction of sample was done, and transferred into test tubes using a pipette carefully without extracting any cell residue or aqueous solution. The identical samples were mixed in the test tubes. The extracted organic matter was analysed using gas chromatograph to obtain required data. The gas chromatography capillary column was used. The type of detector used for detection for gas chromatography was flame ionization detector (FID). The carrier gas was helium (He) and its flow rate was 5 ml/min.

3.3.7.3 Calculation of the concentration of NADPH / NADH

The eppendorf tubes used in (3.3.7.2) which contained biomass were collected and Tris-HCl Buffer solution of 1 ml was prepared and transferred to be eppendorf tubes containing biomass. The eppendorf tubes were vibrated using vortex machine for even mixing. The vortex was used until no visible cell residue was observed at the bottom of eppendorf tubes. The identical samples were mixed in the bottles prior to sonication. The sample was sonicated using a sonicator (Figure 3.5) by programming 5 s on and 5 s off, and was run for 2 min. Then, the eppendorf tubes were centrifuged for separation by layers. The buffer solution was extracted and transferred to cuvette without extracting any cell debris. The cuvette was placed in Spectrophotometer (Figure 3.6) and determine the absorbance of NADH / NADPH by finding the absorbance at the wavelength of 340 nm.

3.4 Experimental activities

The overall experimental activities carried out in this study are presented in the Figure 3.7.

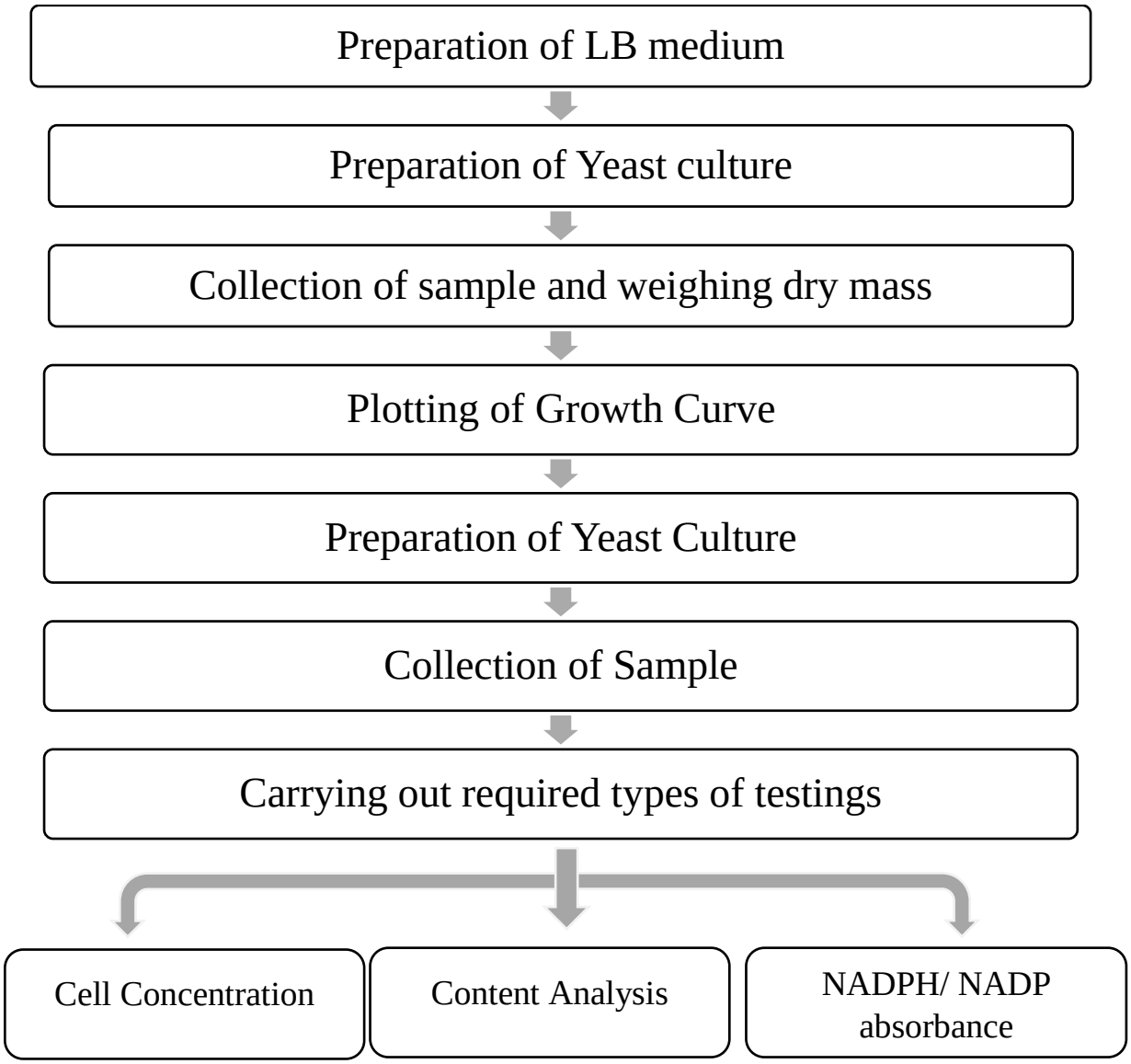

Figure 3.1: Schematic flow diagram of experimental procedures

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 General observations

The biotransformation of ketoisophorone (KIP) towards the formation of product actinol (ACT) during the growth phase of *S. cerevisiae* was the primary goal of this experiment. The exponential growth phase of *S. cerevisiae* until the maximum growth was reached which excludes the lag phase, stationary phase upon reaching maximum growth and the death phase is focused for this experiment. The lag phase was not considered to be growth phase even when minimal growth was observed because the *S. cerevisiae* was still adapting to the new environment introduced (Buque-Taboada et al., 2005).

KIP converts into an intermediate before the conversion into ACT. The ketoisophorone also converts into (4S, 5S)-4-hydroxy-3,5,5-trimethylcyclohexanone which is the other product. The intermediate includes levodione (DOIP), phorenol and hydroxyisophorone. The conversion of KIP into DOIP favors the production of ACT. The biotransformation of ketoisophorone is facilitated by the enzymes which are enoate reductase (OYE) and carbonyl reductase (CR). The OYE reduces C=O double bonds while CR reduces C=C double bonds. The CR for the biotransformation of DOIP into ACT is called levodione reductase (Kataoka et al., 2004; Pscheidt and Glieder, 2008; Raimondi et al., 2011; Yoshisumi et al., 2001).

The selectivity of enzymes determines the intermediates and products formed. The amount of enzymes present were not controlled in the experiment since it requires cloning and expression of selective recombinant strain. When OYE dominates the production of intermediate, DOIP is formed and eventually converted into ACT by the CR. Domination of CR in the formation of product will convert KIP into phorenol or hydroxyisophorone (Kataoka et al., 2004; Pscheidt and Glieder, 2008; Raimondi et al., 2011).

The biotransformation of KIP requires NADH/NADPH, which are the cofactor that links the redox reactions. The NADH/NADPH are oxidized to NAD+/NADP+ while

the KIP was reduced to DOIP. Hence, the concentration of NADH/NADPH are analysed to understand the biotransformation of KIP (Kataoka et al., 2006).

The experiment conducted have analysed the biomass concentration of *S. cerevisiae*, NADH/NADPH concentration in the cells of *S. cerevisiae* and the concentration of substrate, intermediate and product. The experiment was conducted based on the absence of glucose and presence of glucose respectively. The concentration of glucose added was 1 g/L.

The experiment had to be conducted continuously for several hours in order to obtain data throughout the growth phase of *S. cerevisiae*. Due to limitations in time, the experiment was conducted by three different batches of samples at three different time frames which was later combined into one. The time frame of first batch was from 5^{th} hour to 13^{th} hour and 29^{th} hour till 31^{st} hour. The second and third batch is from 14^{th} hour till 22^{nd} hour and 23^{rd} hour till 28^{th} hour respectively. The time frame 3 batches of samples are labelled in the figures used below five hour. Due to the act of combining the three batches of samples, there exists a deviation in the data and continuity of the graph. Furthermore, the time frame for growth phases are labelled in the figure as well.

The product ACT was not observed throughout the experiment. The ACT consists of two enantiomers which are namely R-ACT and S-ACT. The S-ACT are formed even with low concentration of DOIP. The R-ACT are produced only with high concentration of DOIP available. The assumption made for the absence of ACT in experiment was either the concentration of ACT in the solution was too low to be detected or the growth phase favors the formation of R-ACT which are only formed at high DOIP concentration. The ACT will start forming if the experiment was prolonged (Buque-Taboada et al., 2005; Hegazy et al., 2008).

4.2 Growth curve of *S. cerevisiae*

The growth of 1 g/L of *S. cerevisiae* in 250 ml of LB medium was observed and the growth curve was plotted as shown in Figure 4.1. The exponential phase where the *S. cerevisiae* reached highest growth rate until reaching the maximum growth. The time period of exponential phase in the growth curve was observed. The exponential phase period is between at 5th hour to 19th hour from the time *S. cerevisiae* was injected into LB medium.

The graph tabulated shows that the growth rate of *S. cerevisiae* is similar with the presence and absence of glucose. The maximum biomass concentration of *S. cerevisiae* was almost 5 g/L despite the presence or absence of glucose. That shows the maximum growth of *S. cerevisiae* is independent of presence of glucose. The growth of *S. cerevisiae* depends on the concentration of yeast extract (Li et al., 2011). The rate of cell biomass reduction over time was lower with presence of glucose, and the biomass concentration of *S. cerevisiae* is higher during the death phase with presence of glucose. Hence, it was assumed that the glucose utilized by *S. cerevisiae* have been used for the survivability of cell rather than the growth of cells.

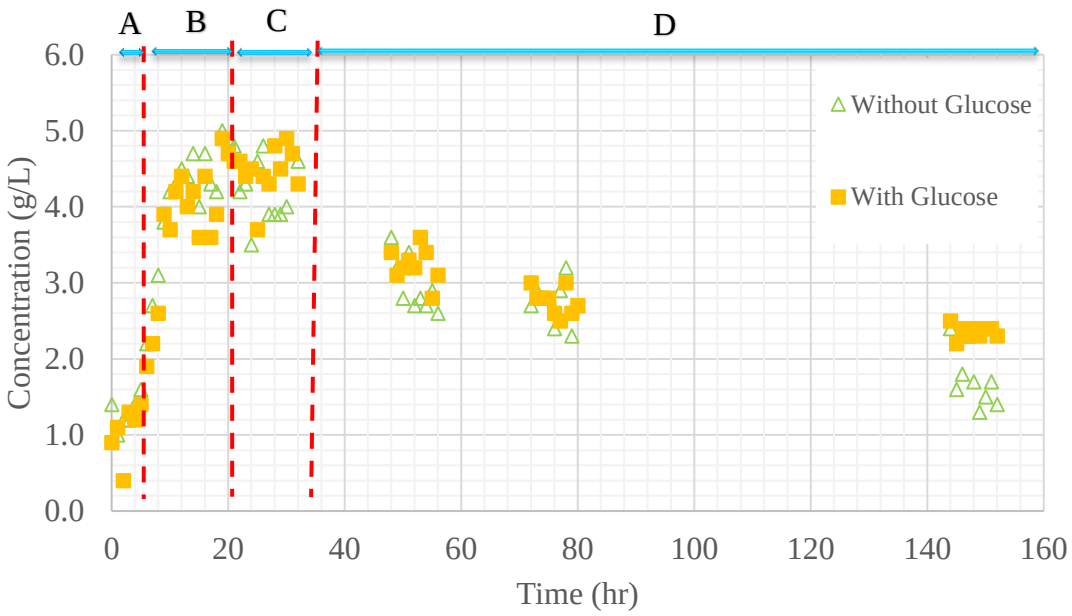

Figure 4.1 Growth curve of *S. cerevisiae* (letters A, B, C, and D represents lag phase, exponential phase, stationary phase and death phase respectively).

4.3 Growth curve of *S. cerevisiae* with KIP (substrate)

From Figure 4.1, the exponential phase of growth of *S. cerevisiae* was observed to occur in between the 5th hour to 19th hour. To observe the biotransformation of KIP towards the formation of product during the growth phase (exponential phase), the KIP was injected to culture at 5th hour and samples were taken each hour.

From Figure 4.2, the 'Substrate Introduction' shows the time at which substrate was injected. The 'Exponential Growth Phase' indicates the time frame of exponential growth phase was observed. the growth rate of *S. cerevisiae* in during each batch is not identical and a slight difference in biomass growth rate was observed. The maximum growth of *S. cerevisiae* with and without glucose are at 22th hour; which is three hours later than growth without substrate injection which was at 19th hour, based on Figure 4.1. The biotransformation of KIP during the growth phase delayed the growth rate by a few hours. Hence, it was assumed that the delay caused was due to the growth of cells and biotransformation, which occurred simultaneously.

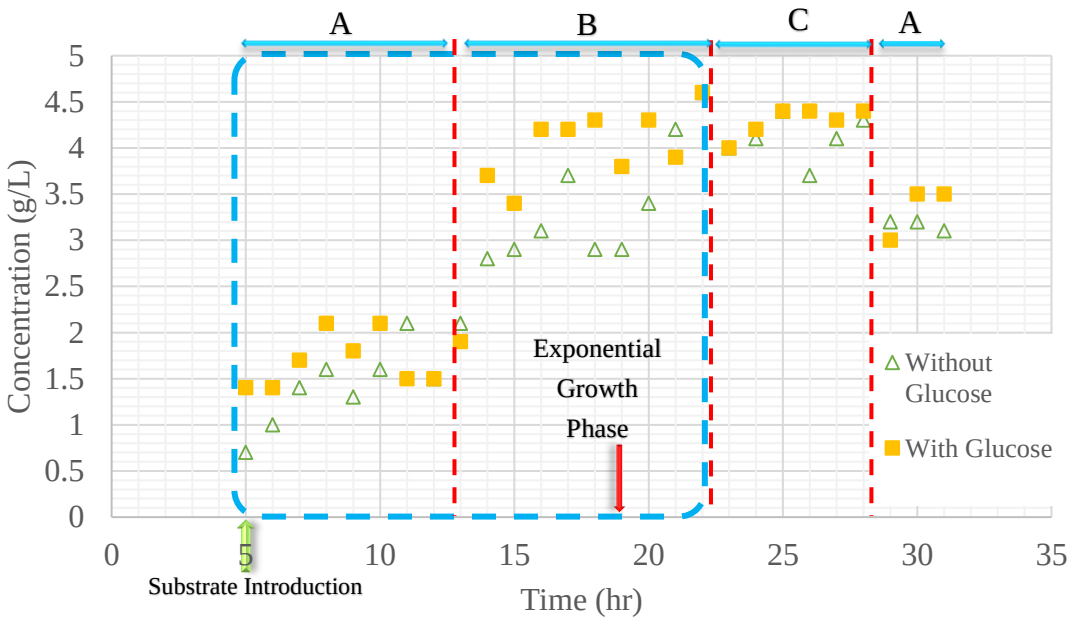

Figure 4.2 Growth curve of *S. cerevisiae* after the substrate injection (letters A, B and C represents batch 1, 2 and 3 respectively).

4.4 NADH/NADPH concentration during the growth phase of *S. cerevisiae*

The concentration of NADH/NADPH have reduced and increased back over time. The biotransformation of KIP oxidized the cofactor NADH/NADPH to NAD+/NADP+. Hence, the decrease in NADH/NADPH concentrations observed are due to the process of biotransformation of KIP towards product formation. (Kataoka et al., 2006; Blacker and Duchen, 2016).

The cofactor recycling includes the redox reactions of the cofactor which alters the concentration NADH/NADPH. The concentration of NADH/NADPH have started to increase at 14th hour which is due to growth of cells and increase in biomass concentration. From Figure 4.2, higher biomass concentration shows higher concentration of NADH/NADPH. The concentration of NADH/NADPH is higher after 14th hour with presence of glucose. The higher concentration of NADH/NADPH are observed due to cofactor regeneration. During glycolysis, Krebs cycle oxidizes glucose intermediates when NAD+/NADP+ acts as the reducing agents reducing them into NADH/NADPH. The glycolysis regenerates the NADH/NADPH which were oxidized during the biotransformation. Glycolysis can only take place in the presence of glucose. Hence the NADH/NADPH regeneration took place due to carbon source from LB medium. However, the presence of glucose had contributed in increasing the rate of cofactor regeneration (Blacker and Duchen, 2016; Spaans, 2015; Toogood and Scrutton, 2014).

From Figure 4.2, the exponential phase of biomass growth is observed within the period of 5th hour to 22nd hour. From Figure 4.3, the 'Substrate Introduction' shows the time at which substrate was injected. The 'Exponential Growth Phase' indicates the time frame of exponential growth phase was observed. From Figure 4.3, the increase in NADH/NADPH is observed from 14th hour. This shows that during the growth of biomass, biotransformation of KIP is active and have oxidized the cofactor NADH/NADPH into NAD+ and NADP+. That explains the decrease in concentration of NADH/NADPH. From 14th hour and beyond, the concentration of NADH/NADPH have increased over time even with biotransformation of KIP taking place as shown in Figure 4.4. Hence, it was assumed that the reason for the increase in NADH/NADPH concentration as shown Figure 4.3 is due to increase in cell biomass concentration which have exceeded the concentration of NADH/NADPH used for the biotransformation.

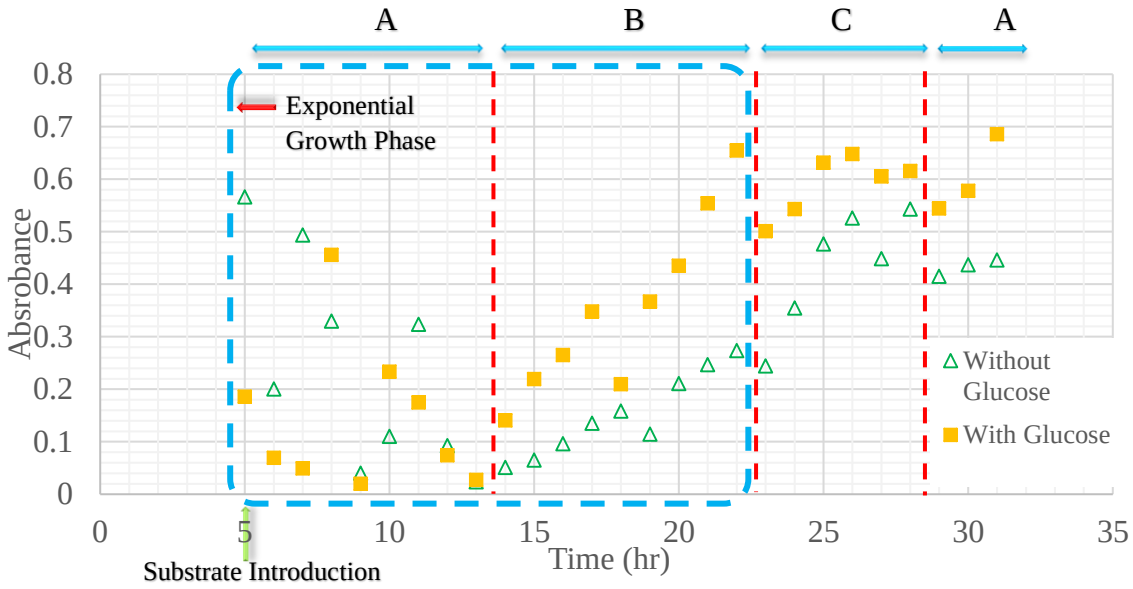

Figure 4.3 NADH/NADPH concentration during the growth phase of *S. cerevisiae* (letters A, B and C represents batch 1, 2 and 3 respectively).

4.5 Substrate consumption and the intermediate formation towards product formation during the growth phase of *S. cerevisiae*

Based on Figure 4.4 and 4.5, 'Substrate Introduction' shows the time at which substrate was injected. The 'Exponential Growth Phase' indicates the period of exponential growth phase was observed.

The Figure 4.4 and 4.5, shows the changes in concentration of KIP and DOIP. The concentration of KIP reduced over time due to biotransformation which in the other hand have increased the concentration of DOIP. The increase in concentration of DOIP shows the biotransformation is active during the growth phase.