THE EFFECT OF VALENCENE BIOTRANSFORMATION AT DIFFERENT GROWTH PHASE OF *YARROWIA LIPOLYTICA*

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by

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LIST OF SYMBOLS

C	dry cell weight
T	Temperature
t	Time
t_D	Doubling time
V	Volume
W_0	Weight of Eppendorf tube
W_C	Weight of Eppendorf tube with dry cell
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
[s]	Substrate concentration
K_S	Substrate saturation constant

LIST OF ABBREVIATIONS

$C_{15}H_{22}O$	Nootkatone
$C_{15}H_{24}$	Valencene
DCW	Dry Cell Weight
DNA	Deoxyribonucleic Acid
GC	Gas Chromatography

ABSTRAK

Biotransformasi adalah proses mengubah bentuk sesuatu bahan organik untuk mengurangkan tahap ketosikan dengan menggunakan mikroorganima seperti yis, fungi, bakteria. Biotransformasi valencene ke nootkatone mempunyai nilai pasaran dan permintaan yang tinggi di industri makanan dan wangian kerana sifatnya yang boleh bertindak sebagai perasa dan wangian semula jadi. Oleh kerana permintaan yang tinggi terhadap nootkatone, penyelidikan dan kajian banyak dilakukan dalam mempertingkatkan kualiti dan produktiviti proses penghasilan nootkatone. Namun, penghasilan nootkatone melalui sintesis kimia mengundang ancaman terhadap alam sekitar kerana penggunaan pemangkin kimia yang berbahaya. Oleh itu, penghasilan nootkatone melalui tndak balas biotransmasi merupakan satu initiatif yang bagus dengan menggunakan pemangkin organik seperti yis. Dalam kajian ini, yis *Y.lipolytica* digunakan dalam biotransformasi molekul valencene ke nootkatone. Kajian turut dijalankan untuk mengkaji kesan penggunaan fasa pertumbuhan yis yang berbeza iaitu fasa eksponen dan fasa pegun. Lengkung pertumbuhan direkod menunjukan fasa lag (0-6 hr), fasa eksponen (6-23 hr), fasa pegun (23-60 hr) dan fasa kematian (>60 hr). Hasil daripada kajian, didapati bahawa biotransformasi yang dijalankan semasa fasa pegun lebih berkesan dan mempunyai transformasi yang lebih tinggi berbanding fasa eksponen berdasarkan kepekatan valencene selepas biotransformasi. Analisis sampel terakhir semasa fasa pegun menunjukkan kepekatan valencene yang rendah kerana transformasi yang lebih tinggi berbanding di fasa eksponen.

ABSTRACT

Biotransformation is the process of transforming one organic compound from one form to another form for the purpose of reducing its persistence and toxicity by using microorganism such as yeast, fungi and bacteria etc. The biotransformation of valencene to nootkatone is getting higher demand in the industry due to their properties and characteristic as the flavor and fragrance especially in the food and flavor industries. As the demand for this nootkatone increase by years, many studies and research have been done to improve the quality and productivity of nootkatone synthesis. However, the chemical synthesis of nootkatone is threatening the environment as the process release heavy metal compound due to the hazardous chemical catalyst used. Thus, the biotransformation by using yeast catalyst is another alternative that can be considered to produce nootkatone. In this research, the effect of valencene biotransformation on exponential phase and stational phase was studied by using the biocatalyst *Y. lipolytica*. to produce nootkatone from the reaction. The growth curve shows the division of lag phase (0-6 hr), exponential phase (6-23 hr), stationary phase (23 -60 hr) and death phase (>60 hr). Biotransformation conducted in stationary phase is more favorable and higher conversion compared to exponential phase based on the concentration of valencene after biotransformation. The analysis of final sample in stationary phase shows lower valencene concentration due to higher conversion than in exponential phase.

CHAPTER 1 INTRODUCTION

1.1 Research Background

1.1.1 Nootkatone

Nootkatone with the chemical formula of $C_{15}H_{22}O$ is a sesquiterpenoid and a ketone which have an intense taste of grapefruit (Palmerín-Carreño et al., 2015a). They can be naturally extract from the orange type of citrus especially grapefruit and pummelo. Figure 1.1 shows the chemical structure of nootkatone molecule. The demand for this chemical has increase in recent years due to its properties and benefits as the natural flavour compound. They are highly demanded in food, fragrance, cosmetic and pharmaceutical industries. Another way to synthetically produce nootkatone is by using chemical synthesis. However, this method is less favorable because the nootkatone produced chemically cannot be considered as natural and the used of expensive chemical catalyst. Moreover, this method used the oxidizing agents such as tert-butyl peracetate and tert-butyl hydroperoxide which are environmentally unfriendly and could increase the risk of large amount of heavy metals catalyst released to the environment (Palmerín-Carreño et al., 2015a; Li et al., 2021a).

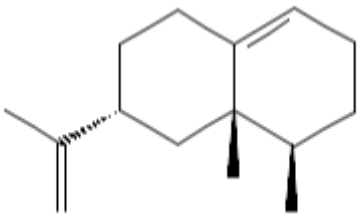

Figure 1.1 Valencene chemical structure

1.1.2 Method of Production

In order to increase the quantity of nootkatone production while protecting the environment, the shift toward biotransformation process is more favorable option instead of relying on natural sources and chemical synthesis. There are several types of microorganism that can acts as the catalyst to produce nootkatone in biotransformation such as fungi, bacteria, yeast, microalgae and plant cells (Li et al., 2021b). Microbial cells are commonly selected as the catalyst for biotransformation due to their special properties of high surface-volume ratio, high growth rate, high metabolism rate and easy to maintain their sterile condition. The transformation may involve the reaction categorized as oxidation, reduction, isomerization, hydrolysis, new carbon bond formation, and functional group introduction (Singh, 2017). Various contribution has been recorded regarding the effectiveness of using microbial biotransformation.

1.1.3 *Yarrowia lipolytica*

Yarrowia lipolytica is one of the yeasts that can be used as the catalyst in for valencene biotransformation which has obtained the Generally Recognized as Safe (GRAS) status by the United States Food and Drug Administration that make it safe to be consumed as food additive. The GRAS status also applied for the mutated or genetically engineered strain of *Y.lipolytica* for the production of some substances such as eicosapentaenoic acid (EPA)-rich triglyceride oil (GRN000355) and erythritol (GRN 000382). Naturally, this compound exists in various type of meats and dairy products such as yoghurts, kefir and cheese. Furthermore, it is used in broad range of biotechnologies applications due to its lipolytic and proteolytic properties which allows it to utilize a diverse type of substances as the carbon source including glycerol, ethanol, alkanes, acetates etc. (Turck et al., 2019).

$$C_{15}H_{24} \xrightarrow[\substack{oxidation \\ catalyst: Y.lipolytica}]{} C_{15}H_{22}O$$

Reaction above shows the oxidation of the molecule of valencene $C_{15}H_{24}$ that is biotransformed into the nootkatone $C_{15}H_{22}O$ by oxidation reaction with *Y.lipolytica* as the catalyst.

1.1.4 Valencene

Valencene ($C_{15}H_{24}$) is a sesquiterpene aroma component from citrus fruit such as 'Valencia' orange that can be used as the substrate to produce nootkatone. Valencene undergo oxidation reaction of the C2 carbon that catalyzed by *Y. lipolytica* yeast to form enantiopure nootkatone. Previously, the research related to the biotransformation of valencene often issued on the different phase systems and partitioning technologies while less discussion has been done on catalytic condition and its optimization (Li et al., 2021a).

This research will focus on the understanding of *Y. lipolytica* growth phase where the experimental work is done to determine the effect of biotransformation of valencene based on different *Y. lipolytica* growth phases. As for yeast, there are four important growth phases of that can be observed from the growth curve which are lag phase, exponential phase, stationary phase and death phase. On the occasion of each phase, the conversion of valencene to nootkatone will be observed in order to determine the optimum phase for the biotransformation which is expressed by the highest concentration and production of nootkatone. The experiment of biotransformation of valencene is done in a baffle shake flask while a gas chromatography will be used for result analysis. The analysis mainly be done only on two phase of *Y.lipolytica* growth which is exponential phase and stationary phase where the cell is still actively dividing and at the highest productivity.

1.2 Problem Statement

Biotransformation is the process of transforming one organic compound from one form to another found in purpose of reducing its persistence and toxicity. Natural and recombinant microorganism such as yeast, fungi and bacteria are the most common catalyst used in biotransformation. The biotransformation of valencene to nootkatone is getting higher demand in industry due to their properties and characteristic as the flavor and fragrance especially in the food and flavor industries. As the demand for this nootkatone increase by years, many studies and research have been done to improve the quality and productivity of nootkatone synthesis. However, the chemical synthesis of nootkatone is threatening the environment as the process release heavy metal compound due to the hazardous chemical catalyst used. Thus, the biotransformation by yeast catalyst is another alternative that can be considered to produce nootkatone. In this paper, the effect of valencene biotransformation on different biocatalyst *Y. lipolytica* growth phase is studied by analyzing the concentration of nootkatone at each growth phase. The time taken for *Y.lipolytica* to reach certain phase is observed based on the dry cell weight and concentration while a gas chromatography analysis is performed to determine the concentration of valencene and nootkatone produce from the reaction.

1.3 Research Objectives

The objective of this research:

i. To study the effect of nootkatone biotransformation at different *Y. lipolytica* growth phase.
ii. To determine the suitable growth phase by observing the maximum concentration and yield of nootkatone.

CHAPTER 2 LITERATURE REVIEW

2.1.1 Natural Extraction and Chemical Synthesis

The production of nootkatone can be synthesized by using either natural extraction, chemical synthesis or biotransformation. The extraction process of this component from its natural sources of grapefruit or Alaska yellow cedar trees is indeed a complicated and tedious process. Moreover, the process involves the use of hazardous chemicals as oxidising agents and could produce low overall yield which is only up to 33%. The amount of nootkatone extract obtained may depend on the type of fruits used and the trace amount of nootkatone obtained is unable to fulfil the high demand of the industry. Due to this reason, the price of nootkatone in the industry is expensive. This method is more suitable for scientific research to study the molecule properties and their stereoisomers instead of being applied as industrial use. Apart from natural extraction process, nootkatone may also be synthesized chemically. In order to increase the production, chemical synthesis method have been introduced by using the valencene compound found in easy and cheap sources which is from 'Valencia' oranges (Fahrenberger et al., 2019). This method involves oxidation reaction with chemical synthesis and is able to generate high amount of valencene at one time.

2.1.2 Utilization of Biotransformation

However, there is the environmental concern arise due to the use of harmful chemical used as the reaction catalyst that release the heavy metal compound to the environment. Moreover, the consumer also worried about the amount of the synthetic chemical in aroma or flavour added to their cosmetics, foods and other household products (Palmerín-Carreño et al., 2015a). Some of the hazardous catalyst that have been used in chemical synthesis is tert-butyl chromate (Hunter and Brogden,1995), sodium dichromate (Shaffer et al., 1975) and tert-butyl peracetate and chromic acid (Wilson and Shaw, 1978). Therefore, the focus shift to the use of

biotransformation for nootkatone production. In biotransformation, some microorganisms are capable to be used as the biocatalyst in the oxidation reaction such as fungi, bacteria, green algae and plant cell (Palmerín-Carreño et al., 2015b; Li et al., 2021b). From all of these types of microorganism, yeast have been chosen as biotransformation carrier in many researches due to their good performance in producing high concentration of nootkatone beside of their variety, low nutritional requirement, and easy cultivation (Li et al., 2021b).

2.1.3 Technologies to increase the production of nootkatone

Li et al., 2021a in their recent work, had studied the effect of catalytic condition optimization in order to increase the nootkatone concentration which is by considering the aspects such as of growth condition, culture condition and co-solvent on conversion of valencene to nootkatone. In their experiment, the biotransformation in a shake flask by using *Y.lipolytica* obtained the highest nootkatone concentration of 628.41 ± 18.6 mg L^{-1} with conversion rate for the reaction is 68.30 ± 2.02 %. Meanwhile, in the study by Palmerín-Carreño et al., (2015a), the *Y.lipolytica* are able to produce 420.9 mg L^{-1} from orange essential oil source in their preliminary experiment in a shake flask. Alternatively, biotransformation can be done in a bioreactor where the stirred tank bioreactor is most common reactor used. In recent research, the phase system and partitioning of the bioreactor has been focused by Palmerín-Carreño et al., (2015a), Palmerín-Carreño et al., (2015b), Castillo-Araiza et al., (2017), Palmerín-Carreño et al., (2016a) to increase the production of nootkatone from yeast culture.

According to Palmerín-Carreño et al., (2015a), the bioreactor in a two-phase system is able to produce 619.8 mg L^{-1} of maximum nootkatone concentration while three-phase system partitioning using orange essential oil can produce up to 852.3 mg L^{-1} of nootkatone which is higher than the product inhibition concentration. Castillo-Araiza et al., (2017) in their

experiment, has proposed the design of four-phase partitioning bioreactor by using whole cells of *Yarrowia lipolytica 2.2ab* that achieved the total production of approximately 850 mg L^{-1} compared to their three-phase reactor, 620 mg L^{-1}. On the other side, biocatalytic process using whole cells which involve the selection of an optimal cellular enzyme, reaction engineering, product recovery and process scaling up are some of the interesting topic that have been emphasized in the valencene biotransformation in academia and industry (Palmerín-Carreño et al., 2015a).

Table 2.1Biotransformation of nootkatone by Y.lipolytica based on different research methods

Microorganism	Maximum nootkatone concentration	Method of production	Reference
Y.lipolytica	628.41 ± 18.6 mg L^{-1}	Catalytic condition optimization	(Li et al., 2021a)
Y.lipolytica	420.9 mg L^{-1} 619.8 mg L^{-1} 852.3 mg L^{-1}	Preliminary experiment Two-phase partitioning bioreactor Three-phase partitioning bioreactor	(Palmerín-Carreño et al., 2015a)
Yarrowia lipolytica 2.2ab	850 mg L^{-1}	Four-phase partitioning bioreactor	(Castillo-Araiza et al., 2017)
Yarrowia lipolytica ATCC 201249.	978.2 µg/L,	Overexpression of the MVA pathway	(Guo et al., 2018)

Many studies and researches have been done to increase the concentration and optimize the yield of nootkatone products especially by using phase partitioning bioreactor and gene overexpression (Guo et al., 2018). However, less study been conducted based on the catalytic condition and optimization such as growth period, substrate concentration, temperature, pH, co-solvent etc. (Li et al., 2021a). Therefore, this study is going to focus on the effect of valencene biotransformation based on the growth phase of *Y.lipolytica*.

2.1.4 Growth phase of yeast *Y.lipolytica.*

The growth of the yeast can be represented by four important phases which are lag phase, exponential phase or log phase, stationary phase and death phase. Yeast cells divide by budding at which the daughter cell is emerged from mother cell, then undergo nuclear division, cell-wall formation and separation of cell (Asaduzzaman, 2007). The first phase which is lag phase occurred when the yeast cells are inoculated in the medium, they are said to be biochemically active but not dividing. During this period, the cell adapts to their new environment and the number of cells remains constant while they are actively metabolizing. The period of this phase depends on the size of initial population and environment condition such as temperature, pH, oxygen, nutrients and mores.

Then, the cell will be entering the exponential phase that begins when the actively metabolizing cells start the DNA replication and followed by cell division. The cells grow rapidly and generation time or doubling time can be observed. The growth during this phase is depending on the organism itself, the medium of growth and operating temperature. The next phase is stationary phase where the metabolism slows and the rapid cell division stops. The cells enter this stage due to the change in the environment as the cell density become high. (Asaduzzaman, 2007) The rate of cell division become similar with the rate of cell death which result in considerably constant number of cells for a period of time. Lastly, the cell will eventually enter the death phase if conditions do not improve (Anon, 2021).

2.1.5 Effect of growth phase toward nootkatone production

Stationary phase and exponential phase are the important phase that will be focused in this experiment based on their performance to produce highest product concentration. A research by Pénicaud et al., (2014) was done by studying the effect of harvesting phase to the

dehydration where the cell harvested at stationary phase and exponential phase. This research provides a brief information and comparison between these two growth phase of *Y.lipolytica*. The result from the experimental analysis shows that, the *Y.lipolytica* cell harvested from stationary phase have more nucleic acid content, thicker cell wall, produce and store longer lipid chains compared to cell harvested in exponential phase. Li et al., 2021a has reported that their highest concentration of nootkatone (628.41 ± 18.6 mgL-1) is produced when the biotransformation was done at exponential phase of *Y.lipolytica* at which the yeast is cultured in medium for 36 hr and growth curve of the yeast is as indicated in Figure 2.1. Meanwhile, Palmerín-Carreño et al., (2015a) have performed their biotransformation process in three-phase bioreactor and obtained the highest product concentration by adding orange essential oil at beginning of stationary phase at 33 hr of growth (Figure 2.2). This indicates that the biotransformation of valencene can be done in both stationary and exponential phase. Therefore, this study will determine the suitable growth phase of *Y.lipolytica* that could be utilized to produce the optimum conversion performance and highest concentration of nootkatone via biotransformation.

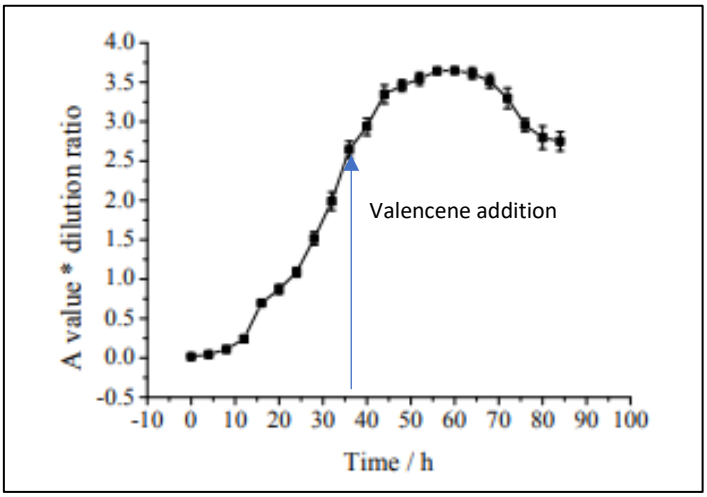

Figure 2.1 The growth curve of Y.lipolytica at 30°C and 100 rpm. (Li et al., 2021a)

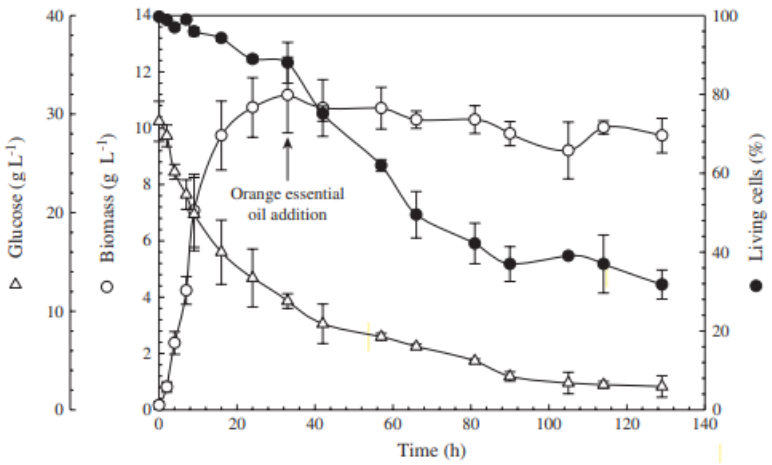

Figure 2.2 Kinetics of cell growth, glucose consumption, and cell viability in the tri-phasic system. (Palmerín-Carreño et al., 2015a)

For GC analysis, the analysis can be used to determine the retention time for valencene and nootkatone. The retention time will be used as the reference to detect the presence of valencene and nootkatone in the sample product. According to Xie et al., (2009) the retention time for nootkatone appeared after the retention time of valencene (Figure 2.3) as Retention Indices (RI) and NIST 02 mass spectra library stated that the retention time for sesquiterpenes supposed to be in the range of 9.00- 12.00 min meanwhile 12.31-24.00 min for oxygenous sesquiterpenes.

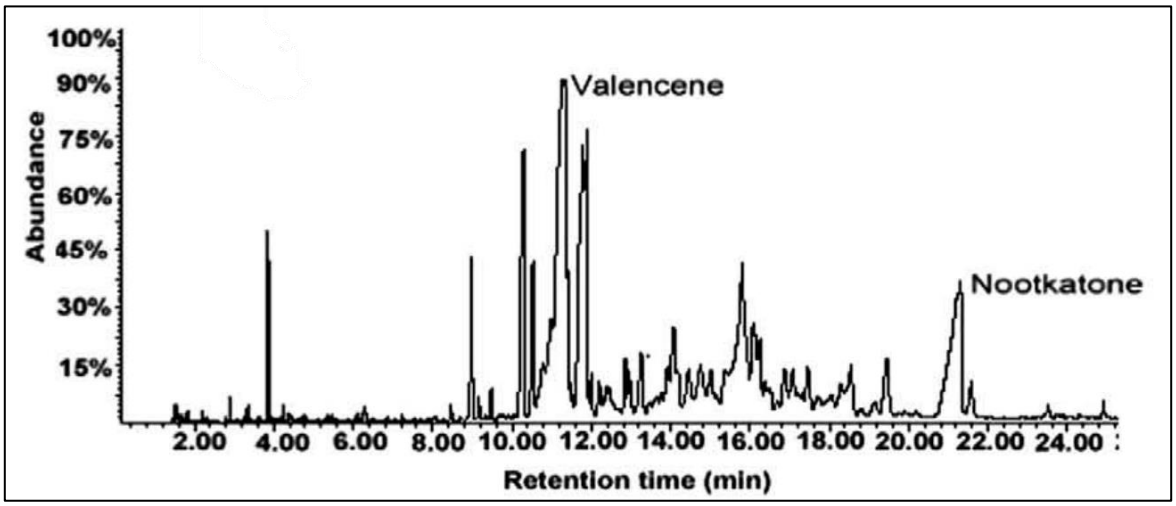

Figure 2.3 Retention time for valencene and nootkatone (Xie et al., 2009)

CHAPTER 3 METHODOLOGY

3.1 Research Flow

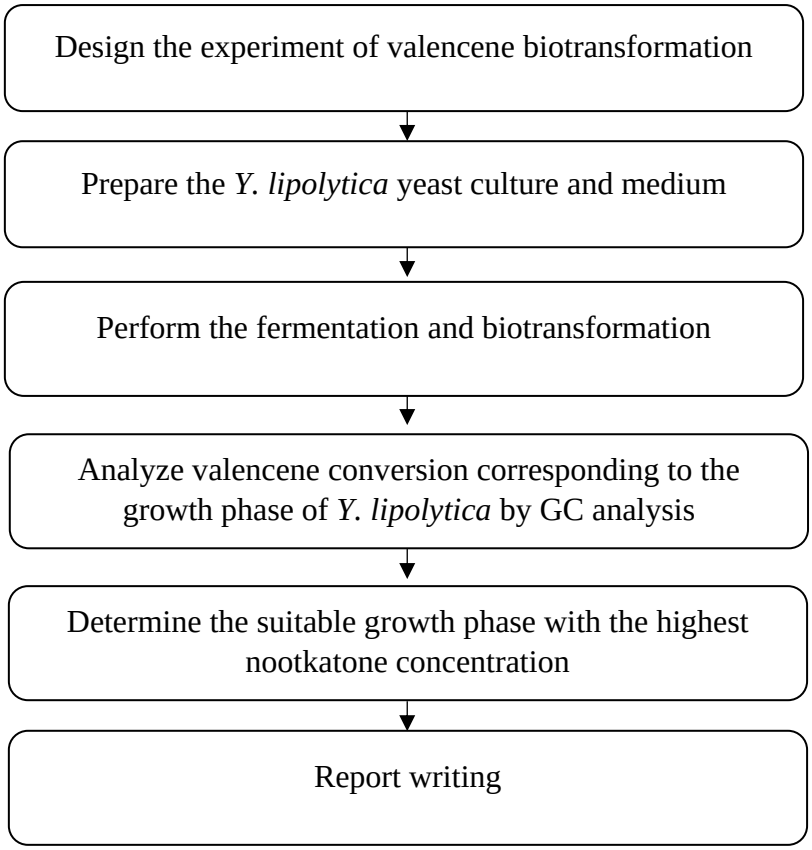

Figure 3.1 Research flow chart

3.2 Material and Equipment

Table 3.1 List of material

Material	Manufacturer	Purpose
Glucose	Sigma-Aldrich, USA	Used to prepare the medium of Luria-Bertani for yeast fermentation.
Peptone	Sigma-Aldrich, USA	
Yeast extract	Sigma-Aldrich, USA	
Agar	Sigma-Aldrich, USA	Used to prepare the agar solution for yeast cultivation.
Ethyl acetate	Sigma-Aldrich, USA	To stop the biotransformation reaction and separate the mixture into two layers of solution.
Valencene	Sigma-Aldrich, USA	Addition of valencene to media perform biotransformation of valencene to nootkatone.
Nootkatone	Sigma-Aldrich, USA	Used to determine the nootkatone retention time in GC analysis
Y.lipolytica.	Sigma-Aldrich, USA	Acts as biocatalyst in valencene biotransformation into nootkatone

Table 3.2 List of equipment

Equipment	Manufacturer	Purpose
Autoclave	Tuttnauer 5057 MLV	Used for sterilization of media
Analytical balance	Shimadzu, Japan	To weight the LB media composition and dry cell.
Incubator shaker	IKA, KS4000i Control, Korea	Used for fermentation of cell culture at certain temperature and rotation speed.
Centrifuge	Eppendorf 5702R, Germany	To separate the media mixture into three layer of ethyl acetate, media and cell.
Oven	Memmert	Used for drying purposes of cell and Eppendorf tube.
Magnetic stirrer	Heidolph MR Hei-Tec, Germany	To perfectly mix the LB media ingredient with deionized water
Vortex mixer	Heidolph REAX 2000, Germany	To perfectly mix the media solution and ethyl acetate. To extract the nootkatone and unreacted valencene
Gas Chromatography	Agilent Technologies, USA	To analyze the concentration of valencene and nootkatone.

3.3 Method

3.3.1 Culture Preparation

The agar solution is prepared in a 500 mL media bottle by using the ingredient of Luria-Bertani media with addition of 15 g/L of agar. The concentration of the agar solution must 17-18 g/L. Then, the solution is autoclaved for 2 hours. The sterilized solution is poured into the agar plates and the yeast of *Y.lipolytica* from fermented medium is cultured on the agar.

3.3.2 Medium Preparation

The medium used is Luria Bertani (LB) medium that compose of three important chemicals. The medium includes 20 g/L peptone, 20 g/L glucose and 10 g/L yeast extract. A 1 L media bottle is used to prepared the media which is then being transferred into 500 mL shake flasks. For a reaction volume of 250 mL, a 500 mL shake flask is used.

3.3.3 Sterilization

The shake flask that contain 250 mL medium is covered with a cotton plug and aluminum foil to prevent the contamination. Then, it was autoclaved at 121°C and 1.5 bar for 20 min. The whole sterilization process consumed at least 2 hours. One colony of *Y.lipolytica* is picked from the culture by using a sterilized loop streak and put into the shake flask together with the medium. It is necessary to done this step in the clean room where the air would not contaminate the sample.

3.3.4 Fermentation

In fermentation process, the shake flash containing the medium together with a colony of yeast is put into the incubator shaker at 30°C and 150 rpm. The 1 mL sample is taken every 1 hour by using pipette and transferred into an Eppendorf tube. The empty of Eppendorf tube is weighed in the first place and labelled as W_0. The tube is then centrifuged in 500 rpm for about 2 to 3 min. The two layers of media divided as supernatant and cell are obtained. The supernatant layer is removed which left the only cell in the tube that will be further dried in oven at 70 °C for 15-20 min. The weight obtained is label as W_c. The dry cell weigh (C) can be calculated by subtracting W_C to W_0.

$$Dry\ cell\ weight\ (C) = W_C - W_0$$

After obtaining the C at different time interval, a graph of C against time (t) is plotted to determine the growth curve of *Y.lipolytica*. Based on the pattern of the graph, the lag phase, exponential phase, stationary phase and death phase are determined and the time to reach each phase are recorded. For a better result, the plot of growth curve is repeated for at least 3 times to ensure the curve is valid and reduce the error.

3.3.5 Biotransformation

In this study, the biotransformation only focuses on two phases of growth curve which is exponential phase and stationary phase where the yeast is actively dividing and in high productivity which is suitable for biotransformation. The process starts at time which the yeast reaches exponential phase, t_E. A 1 g/L valencene is introduced into the flask containing the media and 0.5 mL sample is taken every 30 minute or 1 hour by using a pipette. The mixture of media and valencene sample is then transferred into Eppendorf tube. To stop the reaction, a 0.5 mL of ethyl acetate solvent is put into the solution which resulting the mixture to separate and become two-layer solution. The tube is then put into a vortex to ensure the perfect mixing where the solution is now returns to be one layer. Another purpose of using vortex is to extract the nootkatone and unreacted valencene. Next, it is centrifuged and the solution becoming three layers of ethyl acetate, media and cell. The ethyl acetate which is the top layer (organic phase) is carefully removed from the tube into a gas chromatography (GC) bottle for further chromatographic analysis. Meanwhile, the remaining solution of supernatant and cell in the eppendorf tube is dried at 70°C for 15-20 min. After that, the tube weight is measured to determine the dry cell weight.

3.3.6 Sample Analysis

The first gas chromatography analysis is done to obtain the standard curve of valencene where a sample of different valencene concentration are used which are 0.5 g/L, 1.0 g/L, 1.8 g/L and 3.76 g/L. The GC bottles are used to perform the analysis and a calibration curve of valencene is obtained. Theoretically, a straight-line graph of area (Y) against concentration (X) will be obtained where the relation is given by Y= mX and m is the gradient of the graph.

Another analysis is done to determine the chromatogram by using the earlier sample containing ethyl acetate collected in the GC bottles. The sample is injected into the GC which resulting a chromatogram graph that can be used to determine the peak for valencene and nootkatone. By observing the area under the peak, the valencene concentration (X_C) can be obtained by using correlation of $Y = m\,X_C$. The biotransformation and sample analysis steps are repeated for other time intervals at different growth phase.

From the analysis, the graph of valencene and nootkatone concentration can be plotted together with the cell dry weight against time to determine their relationship and the efficiency of the nootkatone yield by using *Y.lipolytica*.

CHAPTER 4 RESULT AND DISCUSSIONS

4.1 Determination of the *Y.lipolytica* Growth Curve

The growth curve of microorganism can be determined by plotting the graph of dry cell weight (dcw) against time of fermentation process. Based on the research done by Li et al, (2021a), the growth of the yeast would approximately require 90 hours of fermentation with the condition of 30°C and 150 rpm to obtain the complete curve from lag phase to the death phase. The growth curve of Li et al., (2021a) as shown in Figure 2.1 is taken as the standard growth curve for the reference of this experiment.

As the fermentation for this experiment was performed at 30°C and 150 rpm which used higher speed for shaking, the experimental curve was expected to have a shorter fermentation period compared to the theoretical curve. In this experiment, the yeast is observed to be in the lag phase for the first 6 hour of fermentation process. Then, the media started to become cloudy with a thin layer of bubbles formed after entering the exponential phase as the amount of yeast start to grow and increase. As it entering stationary phase, a cloudy sample with a thick layer of bubbles indicating the high number yeast cell can be observed. Eventually, the yeast enters the death phase.

Figure 4.1 shake flask of 250 mL media a) before fermentation b) after 23 hr fermentation

There were four sets of experiment done at different timeframe to complete the whole fermentation data in target of 60 hours experiment. In each set of experiment, 1 mL sample was taken every 1 hours to measure the cell dry weight in which the whole process needs approximately 3 days to complete the growth curve. The fermentation data of dry cell weight and concentration is shown in Table 7.1. By combining all the fermentation data set, a curve that represents the dry cell weight distribution of the *Y.lipolitica* can be illustrated. The experimental curve was then compared to the standard growth curve obtain by (Li et al., 2021a) as shown in Figure 2.1. However, there are still insufficient data recorded for the interval of 30 to 47 hours due to time constraint. Figure 4.2 shows the finalized growth curve of Y.lipolitica in 60 hour duration for fermentation.

Next, the growth curve (Figure 4.2) was categorized into four different phases of lag phase, exponential phase, stationary phase and death phase. The beginning of the curve represent the lag phase of *Y.lipolytica* which is at 0-6 hours. Then, the exponential phase starts at 6 to 23 hours and the curve maintain at stationary phase at 23 to 60 hours. At the end of the curve, there is a declining in dry cell weight observed which the yeast is assumed to enter the death phase after the 60 hours of the growth. After obtaining the growth, the time at where the yeast begins its exponential phase and stationary phase should be noted for valencene addition. In this experiment, valencene is added to the sample at the specific time of growth phase for the conversion of valencene to the nootkatone.

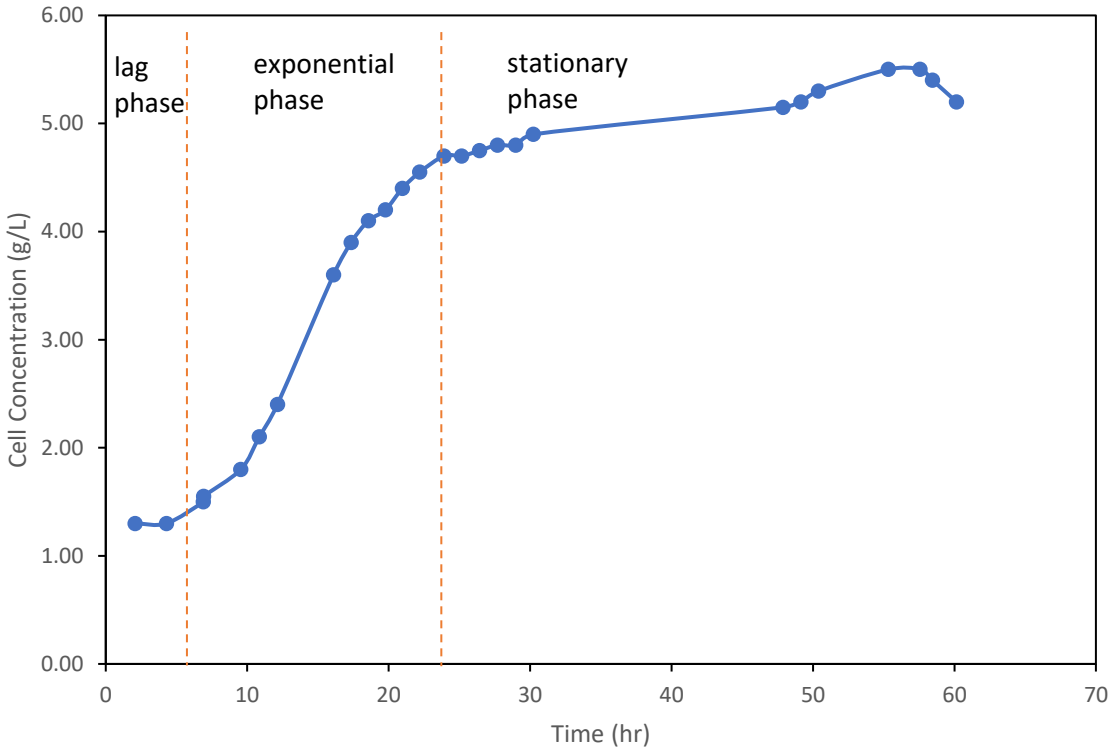

Figure 4.2 Growth curve of Y.lipolytica based on cell concentration

Monod growth kinetics explains the relationship between cell concentration and time where the growth rate is directly proportional to the cell concentration and can be represented as a linear equation of;

$$\frac{dx}{dt} \propto x$$

$$\frac{dx}{dt} = \mu x \ldots (1)$$

The cells are diving at the maximum rate during exponential phase and μ is known as a constant indicates the specific growth rate at the maximum value possible. Equation (1) can be further derived to determine the doubling time for the cell;

$$\ln x = lnx_0 + \mu t \ldots (2)$$

Let

$ut = \mu t_D \; where \; t_D is \; the \; doubling \; time$ and $x = 2x_0$

$$\ln 2x_0 = lnx_0 + \mu t_D$$

$$\ln 2x_0 - lnx_0 = \mu t_D$$

$$\ln \frac{2x_0}{x_0} = \mu t_D$$

$$\ln 2 = \mu t_D$$

$$t_D = \frac{\ln 2}{\mu} \ldots (3)$$

The value of the μ can be determined by using Monod equation in the function of specific growth rate against substrate concentration where μ equal to;

$$\mu = \frac{\mu_{max}[S]}{K_S + [S]}$$

Based on the kinetic study by Li et al., (2022), the value for parameters of maximum specific growth rate (μ_{max}) and substrate saturation constant (K_S) are 0.19 hr^{-1} and 0.818 g/L respectively. With the experimental substrate concentration of glucose [S] of 20 g/L, the specific growth rate of *Y.lipolytica* is given by;

$$\mu = \frac{0.19 \; hr^{-1} \; (20 g/L)}{0.818 \; g/L + 20 \; g/L} \quad \mu = 0.1825 \; hr^{-1}$$

Hence, the time taken for the quantity of the cells to double in constant growth rate, t_D is;

$$t_D = \frac{\ln 2}{0.1825 \; hr^{-1}}$$

$$t_D = 3.8 \; hr$$

4.2 Biotransformation on Valencene to Nootkatone

After obtaining the growth curve of the *Y.lipolytica,* the next step of the experiment is to perform the biotransformation of valencene. Based on the data observed in growth curve, the biotransformation was conducted at the beginning of exponential phase which is at 6 hr meanwhile for stationary phase, the biotransformation was performed at the time where the highest cell concentration is recorded which is at 55 hr. After 250 µL of valencene introduced to the media, a 0.5 mL sample was collected every one hour of growth and the 0.5 mL ethyl acetate used was collected for the GC analysis.

At the same time, the GC analysis was done in order to determine retention time for both valencene and nootkatone compound as well as the standard curve for valencene concentration. According to Xie et al., (2009) the retention time for nootkatone appeared after the retention time of valencene (Figure 2.3) as Retention Indices (RI) and NIST 02 mass spectra library stated that the retention time for sesquiterpenes supposed to be in the range of 9.00-12.00 min meanwhile 12.31-24.00 min for oxygenous sesquiterpenes. From the result of analysis, the retention time for 1 uL sample of valencene is in the range of 11.5 to 11.9 min. For instance, a sample of 1 uL of 1.0 g/L valencene injected in GC shows the peak for valencene with the highest peak area at 11.765 min. However, the result of retention time from the GC analysis done on nootkatone sample cannot be detected as there was no peak response recorded at 20 to 22 minutes.

In order to obtain the peak of the nootkatone, some changes in GC setting were made. In GC analysis, the DB-5 ms 30 m × 0.25 mm × 0.25 µm capillary tube from Agilent Technologies with 1 ml min^{-1} helium as the carrier gas was used. The original setting of the oven temperature used initial temperature of 100°C that held for 2 min, the temperature was then raised by 10°C /min to 165°C. Next, it was raised by 1.5°C/min to 70°C and held constant

for 2 min and further raised to 183°C by 1.5°C/min. Finally, it was raised to 250 °C by increasing rate of 30°C/min and held for 2 min. The changes made for the last temperature increment of 250°C where the rate of increasing temperature was change from 30°C/min to 10°C/min and the hold time was increased from 2 min to 5 min which made the total run time extended from 24.5 min to 34.2 min. With the extended analysis period, the new run on nootkatone sample was done and the result shows the peak of nootkatone appeared at 30.714 min as seen in Figure 11.

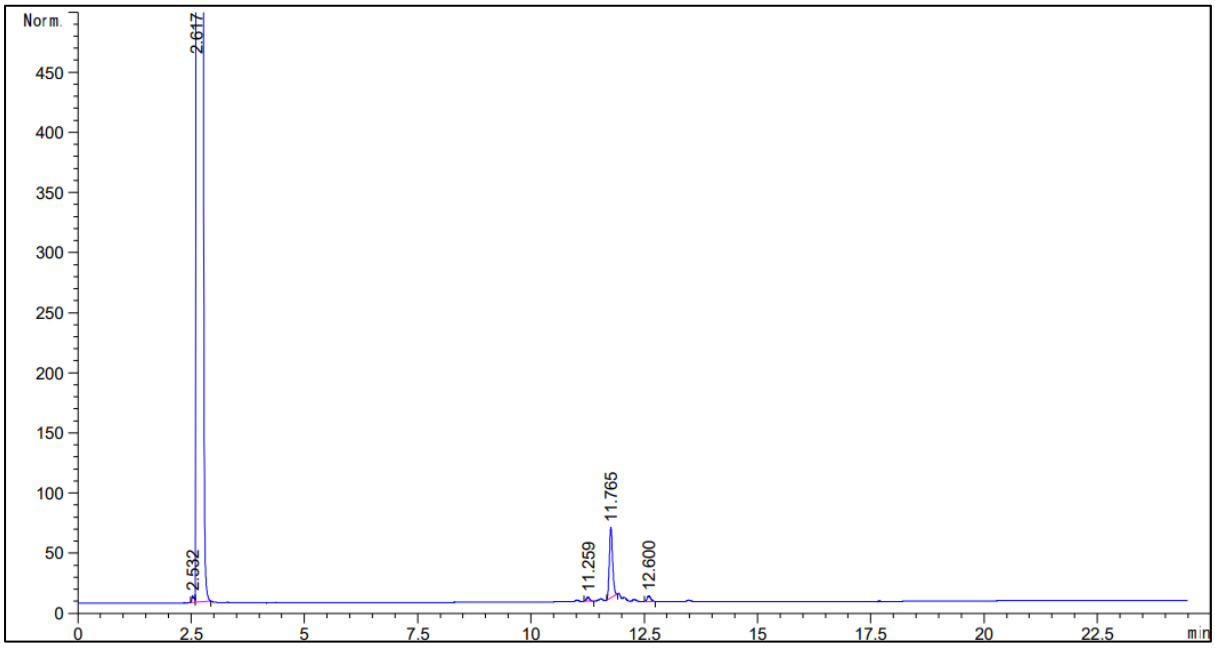

Figure 4.3 The retention time for valencene peak for 24.5 min of analysis

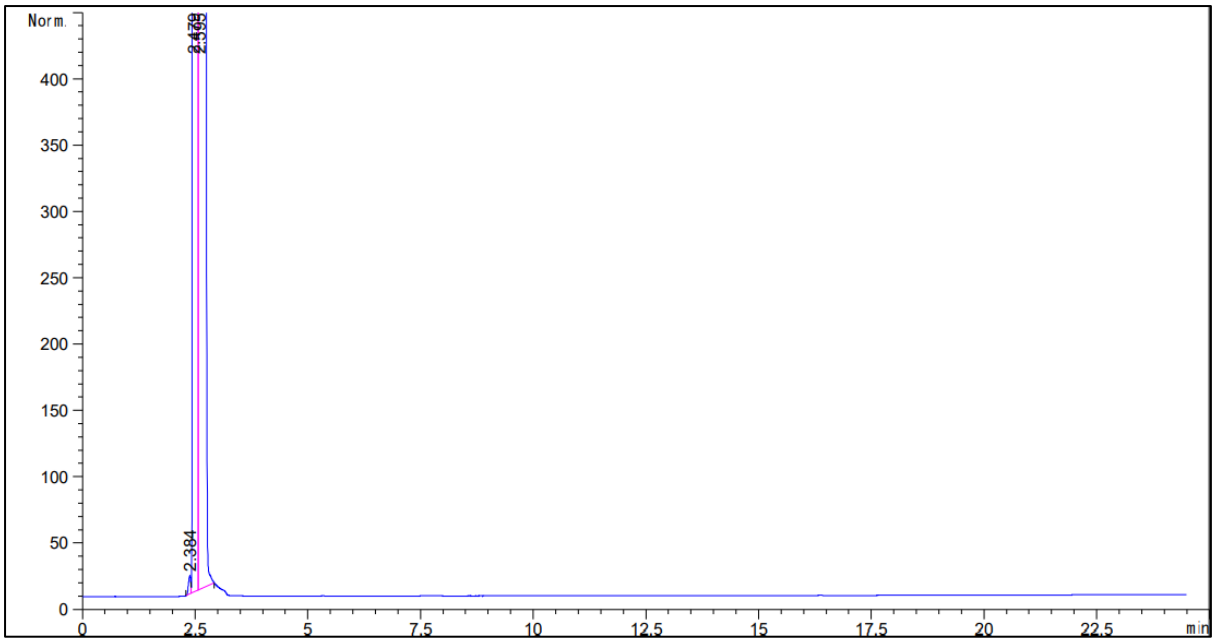

Figure 4.4 The retention time for nootkatone peak for 24.5 min of analysis

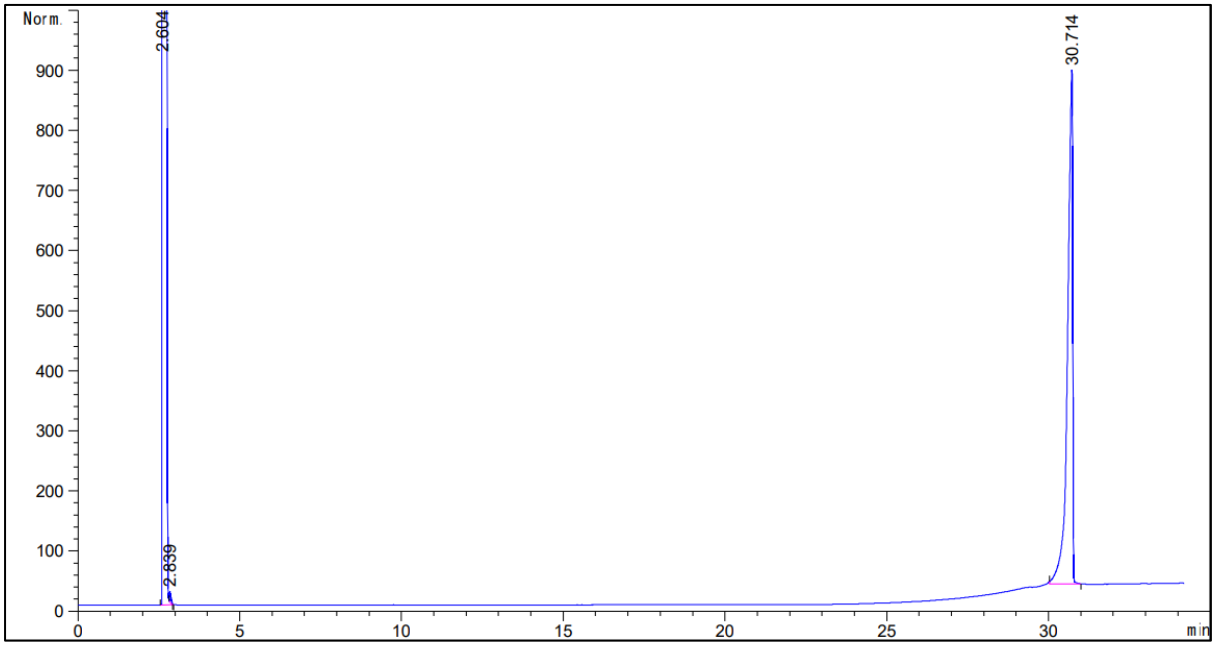

Figure 4.5 The retention time for nootkatone peak for 34.2 min of analysis

Then GC analysis is then proceeded to test the four different concentration of valencene to plot the standard curve of peak area against valencene concentration. The plot shows the directly

proportional relationship where, as the concentration of valencene increase, the peak area was also increase. The straight-line graph gives a linear equation of Y= 676.51 X which can be used to determine the concentration of the valencene that remained after the biotransformation process.

4.3 Effect of Biotransformation At Different Growth Phase of *Y.Lipolytica*

After obtaining the GC analysis result, it is observed that both exponential phase and stationary phase can only detect the peak for valencene but not for nootkatone. Therefore, only the data of valencene peak area and the dry cell will be taken into consideration for the discussion and determine the concentration. Table shows the summary results of valencene and dry cell weight in terms of concentration.

Table 4.1 Summary of biotransformation result

Time (hr)		Valencene Concentration (g/L)		Cell Concentration (g/L)	
Exponential phase	Stationary phase	Exponential phase	Stationary phase	Exponential phase	Stationary phase
8.40	56.92	1.77	0.65	0.60	1.60
9.53	58.30	1.64	1.03	0.40	1.40
11.12	59.63	2.27	0.60	0.60	1.80
12.67	60.87	1.73	0.80	0.80	1.20
31.08	-	1.86	-	1.20	-

Theoretically, the concentration of valencene should be decreased over time as it is converted to the nootkatone by *Y.lipolytica*. From Table 4.1, it can be seen that the valencene concentration for both exponential and stationary phase did not decrease significantly as the

time increase. However, the overall concentration of valencene for the exponential phase is observed to be higher than stationary phase although the same amount of valencene was used for both phases of biotransformation. This indicates that the most of the valencene added to the media during exponential phase did not convert to the nootkatone compound which is also the reason for the absence of nootkatone peak observed from GC analysis. The reason for the failure of valencene conversion might be due to the less amount of cell presence during the time for biotransformation. It is noticed that the concentration of the cell at exponential phase is less compare to stationary phase and only then start increase as time increase indicating the insufficient cells were unable to convert the valencene.

As for stationary phase, the amount of cell in the sample was higher than during the exponential. Meanwhile, the low concentration of valencene shows that there was some amount of valencene has been consumed to be converted into nootkatone yet the peak for nootkatone did not appear in GC analysis. The reason behind the absence of the nootkatone's peak is due the increase of the size of the organic compound. In this case, the valencene compound is transformed into nootkatone by using *Y.lipolytica*. In the yeast, CYP450 and ADH acts as the enzyme to assist the biotransformation. This process involved two important steps which is diffusion and reaction. Firstly, the valencene compound will diffuse into the *Y.lipolytica* cell and reacts with both CYP450 and ADH to produce nootkatone compound. However, the increase of the organic compound size from $C_{15}H_{24}$ to $C_{15}H_{22}O$ had caused the compound unable to diffuse out from the pore of the yeast and remain inside the cell.

The diffusivity of the valencene molecule into the yeast cell can be calculated by using Stoke-Einstein equation where diffusion coefficient equal to

$$D = \frac{k_B T}{6\pi\eta R}$$

Where;