THE EFFECT OF GLUCOSE CONCENTRATION ON THE BIOTRANSFORMATION OF VALENCENE TO NOOTKATONE

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THE EFFECT OF GLUCOSE CONCENTRATION ON THE BIOTRANSFORMATION OF VALENCENE TO NOOTKATONE

by

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Heah Joo Yee

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Symbol	Description
C_{10}	monoterpenes
C_{15}	sesquiterpenes
C_{20}	diterpenes
C_{30}	tetraterpenes
$C_{15}H_{24}$	valencene
$C_{15}H_{22}O$	nootkatone
Y. lipolytica	*Yarrowia lipolytica*
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
CAGR	compound annual growth rate
GRAS	generally regarded as safe
FDA	Food and Drug Administration
EPA	Environmental Protection Agency
SC	synthetic complex
UV	ultraviolet
3D	three-dimensional
LB	Luria-Bertani
GC	gas chromatography
YPD	yeast extract peptone dextrose

List of Symbols

Symbol	Description	Unit
W_0	weight of empty Eppendorf tube	g
W_c	weight of Eppendorf tube with cell after dried	g
dcw	dry cell weight	g/L
t_e	exponential phase	-
μ	micro	-
pA·s	picroAmpere-second	-

Abstrak

Nootkatone ialah sebatian organic yang mempunyai aroma yang unik dengan menggunakan valencene sebagai substrat untuk menghasilkan nootkatone melalui sintesis kimia. Walau bagaimanapun, proses sintesis kimia melibatkan penggunaan logam berat sebagai pemangkin untuk mengoksidakan ikatan ganda dua antara valencene. Oleh itu, yis yang bernama *Yarrowia lipolytica* dicadangkan untuk menggantikan logam berat. Penggunaan yis membolehkan produk nootkatone diiktirafkan sebagai produk semula jadi. Ia lebih kekalan kerana logam berat biasanya jarang ditemui secara semula jadi di Bumi, jadi dengan mengurangkan penggunaanya, kita boleh memastikan generasi akan datang boleh memenuhi keperluan mereka atas logam berat. Selain itu, jika logam berat tidak dilupuskan dengan cara yang betul, pencemaran kepada alam sekitar akan berlaku. Dengan menggunakan *Y. lipolytica* semasa biotransformasi, nootkatone boleh dihasilkan secara optimum dengan kepekatan glukosa dalam LB media sebagai 15 g/L. Jumlah nootkatone mencapai tertinggi semasa valencene pada kepekatan tertinggi. Namun begitu, ada sample tertentu yang puncak nootkatone tidak keluar. Ini mungkin disebabkan oleh beberapa lantaran, seperti saiz liang membrane sel yis terlalu kecil untuk nootkatone meresap keluar, tahap nootkatone terlalu rendah, sesetengah yis mati, dan kromatografi gas tidak dikendalikan secara ideal. Oleh itu, sonikasi dicadangkan untuk memecahkan sel bagi membolehkan nootkatone untuk meresap keluar dari sel.

Abstract

Nootkatone is an organic compound which has a unique aroma with valencene as the substrate for nootkatone production that is commonly synthesised through a chemical reaction. However, the chemical synthesis process involved the usage of heavy metals as catalysts to oxidize the double bond in valencene. Therefore, a non-pathogenic yeast named *Yarrowia lipolytica* is suggested to replace the heavy metals catalyst. The usage of yeast allows the nootkatone products to meet their natural properties. In addition, it is more sustainable as heavy metals are usually rarely found naturally on the Earth, so by reducing their usage, we can ensure that future generations meet their own needs. Plus, if the heavy metals do not dispose of properly, they will cause contamination to the Earth, which will harm the environment. Using *Y. lipolytica* in the biotransformation step, nootkatone can be produced from valencene with the optimum glucose concentration in LB media as 15 g/L. The nootkatone amount reaches the highest when the valencene is at the highest concentration. But the nootkatone peak does not come out for certain samples. This might be due to several reasons, such as the pore size of the yeast's cell membrane is too small for nootkatone to diffuse out from the cell, the nootkatone level is too low to be diffused out, some of the yeasts might die, and gas chromatography is not ideally operated. Therefore, sonication is recommended to break the cells to allow nootkatone to release from the cells.

1. Introduction

1.1. Research background

In 2020, the demand for flavors and fragrances, with a market size of $27,850 million, is estimated to grow and reach $37,286.3 million by 2026, at a compound annual growth rate (CAGR) of 5.12% between the years 2021 and 2026 (Markets, 2021). This situation is due to the growing population in emerging economies and increasing awareness about these flavors' advantages in the food and beverage industries. Moreover, due to the increasing concern about protecting our mother nature, a more sustainable method to produce those products with few or no toxicological effects becomes preferable. Thus, a biotechnological method to produce a natural aroma has recently raised interest.

1.1.1. Terpenes

Terpenes are the dominant class of natural aromatic compounds found widely in various organisms such as plants, insects, fungi, and bacteria. In general, terpenes could be classified into several classes according to the number of carbons form in the core skeleton structure, which are monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and also tetraterpenes (C_{40}) with the examples of monoterpenoids are limonene and linalool; sesquiterpenoids are (+)-nootkatone and valencene; triterpenoids are oleanolic acid and squalene; tetraterpenoids are lycopene and astaxanthin (Zhang, et al., 2021). Among all the classes of terpenes, sesquiterpenes are the largest class, with more than 7,000 known compounds, mainly in the form of volatile plant oils.

1.1.2. Valencene

Valencene is one of the examples of sesquiterpenoids that are usually found in plants or fruits, such as the peels of Valencia oranges. It is an aroma ingredient responsible for the smell of oranges. The chemical structure of valencene is shown in Figure 1.1, and its molecular formula is $C_{15}H_{24}$. It could be separated into different classes or purity, such as valencene 94%, valencene 80%, valencene D with a minimum purity of 70%, valencene T with a minimum purity of 65%, and valencene N with a minimum purity of 60%. Valencene is widely used in several industries to produce various products such as food and beverage, fragrances, personal care, and home care products. Evolva is a fermentation process that produces valencene in large amounts in the conventional industry. This process is introduced due to the extraction of valencene from the orange peel oil to produce valencene is restricted by the low supply and high cost (EVOLVA, 2021). Plus, the Evolva process is not the only method to produce valencene, but also some other methods, such as isolating it from citrus oils through steam distillation and introducing (+)-valencene synthases in *Saccharomyces cerevisiae* (Li, et al., 2021).

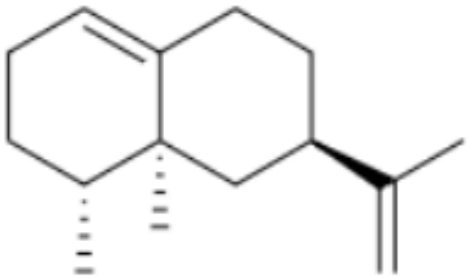


Figure 1.1 Chemical structure of valencene

1.1.3. Nootkatone

(+)-Nootkatone, one of the examples of sesquiterpenoids is valuable mainly in fragrance industries due to its unique and natural grapefruit odor. In 2020, the nootkatone market worldwide achieved $331.3 million, and it is predicted to reach $772 million by the end of 2026, with a growing CAGR of 12.7% from the year 2022 to 2026 (TV, 2021). This increment causes the market price of nootkatone to be comparatively high in future years. (+)-Nootkatone could be produced through either chemical or biological oxidation of (+)-valencene as a substrate. However, the biological oxidation method is favorable due to the public's preference for natural products instead of chemical products. Nootkatone has a structure similar to valencene, but it is a ketone, not a ring-type alkene. The chemical structure of nootkatone is shown in Figure 1.2, and its molecular formula is $C_{15}H_{22}O$. Moreover, microorganisms such as yeast, bacteria and microalgae could be used in the biotransformation process, which makes the process meet the Europe and U.S. legal requirements for natural properties. Therefore, several types of microorganisms, such as *Yarrowia lipolytica* which is a non-pathogenic dimorphic aerobic yeast, attract researchers' attention to be implemented industrially in producing nootkatone.

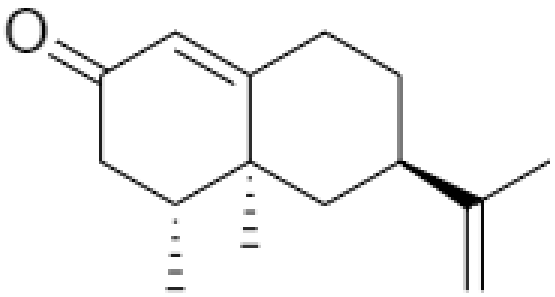


Figure 1.2 Chemical structure of nootkatone

1.1.4. *Yarrowia lipolytica*

Yarrowia lipolytica is a non-pathogenic yeast that replaces the heavy metal catalyst to oxidize the double bond in (+)-valencene to form (+)-nootkatone. This oxidation is through a biotransformation process that makes the products form to meet the natural requirement in Europe and U.S. legal. When comparing *Y. lipolytica* with the common use bacteria, *E. coli*, the former has a stronger intrinsic flux of the acetyl-CoA as the precursor into the mevalonate pathway that is responsible for the biosynthesis of the sesquiterpenes (Mai, et al., 2021). Additionally, it is labelled as generally regarded as safe (GRAS) by the USA Food and Drug Administration (FDA), which means it is safe for humans. Furthermore, this yeast relies on glucose as a carbon source in the auxotrophic synthetic complex plate and Luria-Bertani medium. Therefore, glucose concentration is expected to affect the biotransformation of valencene to nootkatone. However, it is assumed that a higher glucose concentration will not necessarily lead to a better transformation, but beyond the optimum glucose concentration may lead to less production of (+)-nootkatone. Thus, controlling the glucose concentration for *Y. lipolytica* synthetic plate or media is dominant.

1.2. Problem statement

Nootkatone is not a new ingredient since nootkatone was discussed at the American Chemical Society meeting in 2013 due to its pleasant grapefruit flavor. This special characteristic causes high demand in the market, and the demand is predicted to rise in the future years. Although the chemical synthesis method is widely used in the commercial industries to produce grapefruit odor using non-natural components, this is not the best option as it involves high operating costs and has the high possibility of fire hazards due to the presence of strong oxidizing agents. Therefore, to overcome these limitations and produce a (+)-nootkatone with qualified natural properties, a non-pathogenic yeast named *Y. lipolytica* is introduced to replace the heavy metal catalyst to oxidize the double bond in (+)-valencene. Also, glucose as the most common carbon source for the (+)-nootkatone production is expected to affect the biotransformation of valencene to nootkatone. For instance, if the glucose in the yeast culture medium exceeds an amount, the cell growth will lead to more citric acid production instead of the pathway of (+)-nootkatone synthesis. However, to my knowledge, no previous studies have specifically evaluated the effect of glucose concentration on the biotransformation of valencene to nootkatone in the culture medium. Hence, this study will focus on the effect of glucose concentration on the production of (+)-nootkatone from (+)-valencene.

1.3. Research objectives

The objectives of this research are:

i. To determine the maximum cell concentration in the growth curve of *Y. lipolytica.*

ii. To identify the effect of glucose concentration in LB medium for yeast cell growth on the biotransformation of valencene to nootkatone.

iii. To determine the optimum glucose concentration for the highest reaction rate.

2. Literature Review

2.1. (+)-Valencene

(+)-Valencene is a substrate of (+)-nootkatone production through biotransformation, and it has the common terpene class with (+)-nootkatone. The main difference is (+)-nootkatone is a ketone, whereas (+)-valencene is a carbobicyclic compound. (+)-Valencene is also an organic compound with lipid-like molecules. The biological roles of valencene are as an energy source, nutrient, and membrane stabilizer in the organism. In contrast, the industrial applications of valencene act as a powerful insecticide, especially for mosquito repellent, personal care, and cleaning products. Apart from that, research shows that it is useful in pharmaceutical industries as it is beneficial as an anti-inflammatory, to improve the efficiency of Doxorubicin, which is the medicine used in chemotherapy, and as an adjuvant to improve the immune response (Trulieve, 2020).

(+)-Valencene is usually found in fruit, such as Valencia orange and Chinese Bayberry. The most common method of synthesizing the valencene will be (+)-valencene is isolated from citrus oil by steam distillation, and another example is through fermentation as an Evolva process, but recently, it was found out that valencene could be biosynthesized by introducing synthases in *S. cerevisiae* (Li, et al., 2021). Hence, to produce nootkatone by using valencene as a substrate, this biotransformation process is truly natural, sustainable, and environmentally friendly as the substrate's synthesis is also a biological method.

2.2. (+)-Nootkatone

(+)-Nootkatone belongs to bicyclic sesquiterpenes ketone of the *Yashilane* series and could be found in peel oils or skin from pomelo and grapefruit. It is valuable due to the low content, as 1 kg of nootkatone production requires several tons of grapefruit. It is also broadly used in fragrance, food, and beverages due to its pleasant grapefruit flavor. On the other hand, a synthetic (-)-nootkatone has less commercial interest due to its higher odor threshold with woody and spicy flavor and less bioactivity.

The unique aroma of (+)-nootkatone causes it to have high demand in the market, but the supply fails to meet the requirement due to its low concentration in plants or fruit peels, low extraction efficiency, and long growth cycle of the plants. Therefore, chemical synthesis is another option for producing greater amounts of synthetically derived (+)-nootkatone. The application of (+)-nootkatone is not restricted to flavoring products but could be used as an insecticide or insect repellent approved by the Environmental Protection Agency (EPA). Research shows that (+)-nootkatone from *Alpinia Oxyphy*lla gives insecticidal activity against the larvae of *Drosophila melanogaster* (Fraatz, et al., 2009). Plus, a low dose of nootkatone has proven to kill ticks, including *Ixodes scapularis,* which transmit bacteria that cause Lyme disease in humans and animals. This research is beneficial as Lyme disease caused 29,959 confirmed cases in 2009 (Magazine, 2011). Apart from that, nootkatone is also a possible useful compound in the pharmaceutical field because it has an antiplatelet aggregation effect, anti-proliferative effect on cancer cells, and protective effects on the neurons.

2.3. Chemical synthesis of (+)-nootkatone

Due to the high demand for (+)-nootkatone in the market, especially in the food, cosmetics, and pharmaceutical industries, a chemical synthesis method is commonly applied as this could produce a greater amount of (+)-nootkatone as compared to a biosynthesis method that limit by the low concentration in plant or fruit sources. However, this method is not ideal due to its high cost, involvement of hazardous components, and difficulty in separating the chiral isoforms to obtain pure synthesis as their complex structures. Furthermore, the production method is unsafe due to the presence of strong oxidizing agents such as tert-butyl peracetate and tert-butyl hydroperoxide when catalyzing the production of (+)-nootkatone from (+)-valencene that may cause fire hazards. But these oxidizing agents do not pose environmental issues. Besides, a nootkatone will have three chiral centers. Therefore, there is a possibility of forming eight stereoisomers at every stage of nootkatone synthesis. But out of these stereoisomers, only one of them is useful, which expands the difficulty of the chemical synthesis process (Bioscience, 2017).

Another example of the chemical synthesis method is by using the carcinogenic tert-butyl chromate or sodium dichromate to convert (+)-valencene into (+)-nootkatone, but this method causes health problems in humans (Fraatz, et al., 2009). These chemical processes might not be a direct conversion but involve a few steps, as shown in Figure 2.1 below, in which (+)-valencene oxidize to nootkatol, and the nootkatol will be transformed into (+)-nootkatone in the presence of hydrogen. Nevertheless, the products from these chemical syntheses of (+)-nootkatone are not natural flavoring, making them less preferred by the public. Therefore, several organisms, such as yeast, fungi, bacteria and plant cells, are introduced for the biotransformation of (+)-

nootkatone from (+)-valencene.

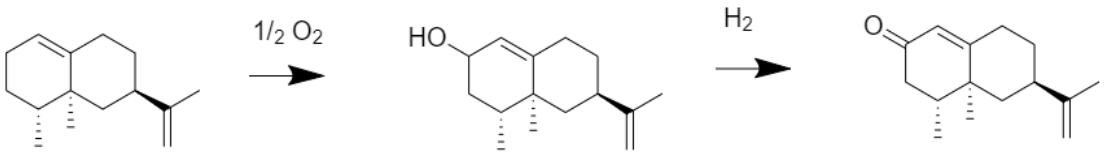


Figure 2.1 From (+)-valencene (left) to nootkatol (middle) and lastly to (+)-nootkatone (right)

2.4. Biosynthesis of (+)-nootkatone

A biotransformation process is a convenient replacement for chemical processes, and it encounters the Europe and U.S. legal requirements for natural properties. *Y. lipolytica* is a non-pathogenic and safe dimorphic aerobic yeast with diverse biotechnological applications such as producing citric acid, enzymes including lipases, acid and alkaline proteases, and bio-surfactants. The species name "*lipolytica*" shows that it can hydrolyze lipids. *Y. lipolytica* commonly grows in a hydrophobic environment, with a maximum growth temperature is 34℃ (Gonçalves, et al., 2014). It is also classified as generally regarded as safe (GRAS) by the USA Food and Drug Administration (FDA), making it a good yeast in conventional industries as it is not a possible human pathogen. Generally, the biosynthesis of (+)-nootkatone from (+)-valencene could be seen in Figure 2.2, which indicates the decrease of the substrate (valencene) and increase of the product (nootkatone) that reaches the maximum point at 326.3 mg/L in the tri-phasic system using *Y. lipolytica* at 96th hours, with bioconversion of 39.1% (Palmerín-Carreño, et al., 2016).

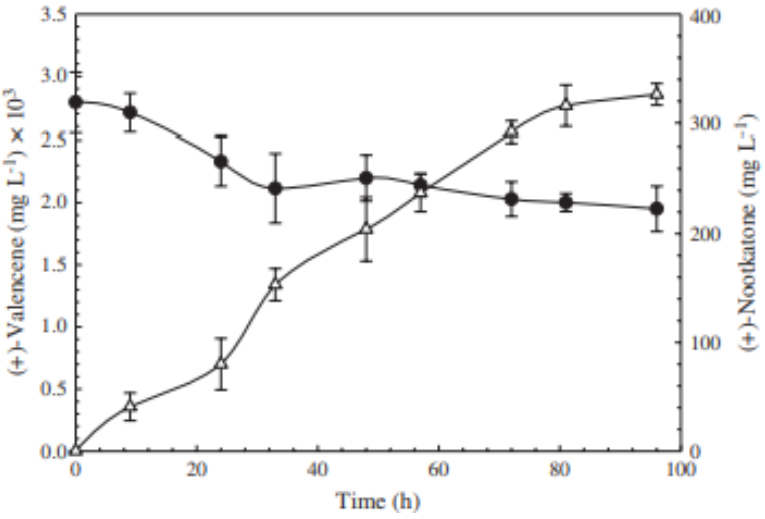


Figure 2.2 Biotransformation of (+)-valencene to (+)-nootkatone in a tri-phasic system using Y. lipolytica (Palmerín-Carreño, et al., 2016)

Through research and study, *Y. lipolytica* 2.2ab is suggested to oxidize (+)-valencene to (+)-nootkatone effectively, in which this biotransformation process can produce 852.3 mg/L of (+)-nootkatone in a partitioning bioreactor after 4 days. This conversion means the use of catalysts differs from the conventional method that uses a heavy metal catalyst. Still, it uses *Y. lipolytica* to oxidize the double bond in (+)-valencene. However, if the concentrations of (+)-valencene and (+)-nootkatone in baffled conical flasks are above 1.5 g/L and 400 mg/L, respectively, it will lead to inhibitory effects on *Y. lipolytica* 2.2ab (Castillo-Araiza, et al., 2017). Additionally, not only the *Y. lipolytica* is suggested to undergo biotransformation of (+)-valencene to (+)-nootkatone, but there are also other types of microorganisms, such as fungi, bacteria, and microalgae, shown in Table 2.1.

Table 2.1 Microbial biotransformation of (+)-valencene to (+)-nootkatone (Li, et al., 2021)

Microorganism	**Strain**	**Time**	**Nootkatone maximum yield (mg/L)**
Fungi	*Laccases* from *Botrytis cinerea*	2 days	1296
	Mucor species	7 days	328
	Botryosphaeria dothidea	7 days	336
	Botryodiplodia theobromae	7 days	168
	Chaetomium globosum	3 days	25
	Lyophilisates of *Pleurotus sapidus*	13 hrs	250
	Pleurotus sapidus	24 hrs	320
	Homogenized fresh mycelium of *Pleurotus sapidus*	42 hrs	600
	Botryodiplodia theobromae 1368	12 days	239.7 +/- 2.1

	Phanerochaete chrysosporium	12 days	110.3 +/- 11.8
	Kluyveromyces marxianus NCYC1429	12 days	14.51 +/- 0.83
	Aspergillus tamarii V12307	12 days	4.7 +/- 0.56
	Rhyzomucor species	12 days	0.315 +/- 0.23
	Yarrowia lipolytica 2.2ab	4 days	825.3
Bacteria	*Rhodococcus species*	5 days	50
Microalgae	*Chlorella fusca var. vacuolata IAMC-28*	18 days	252
	Chlorella fusca	14 days	252
	Chlorella pyrenoidos	14 days	320
	Chlorella vulgaris	14 days	360

Furthermore, Figure 2.3 (a) shows the heterologous synthesis of (+)-nootkatone in yeast, and *Escherichia coli* (Figure 2.3 (b)), which produce (+)-nootkatone with natural properties (Li, et al., 2021). Compared to *E. coli*, *Y. lipolytica* has a stronger intrinsic flux of the acetyl-CoA as the precursor into the mevalonate (MVA) pathway, which is responsible for the biosynthesis of the sesquiterpenes (Mai, et al., 2021). Glucose is the simple sugar that usually acts as the carbon source of yeast. Thus, the culture medium of a *Y. lipolytica* usually consists of glucose with a concentration of 20 g/L or 30 g/L.

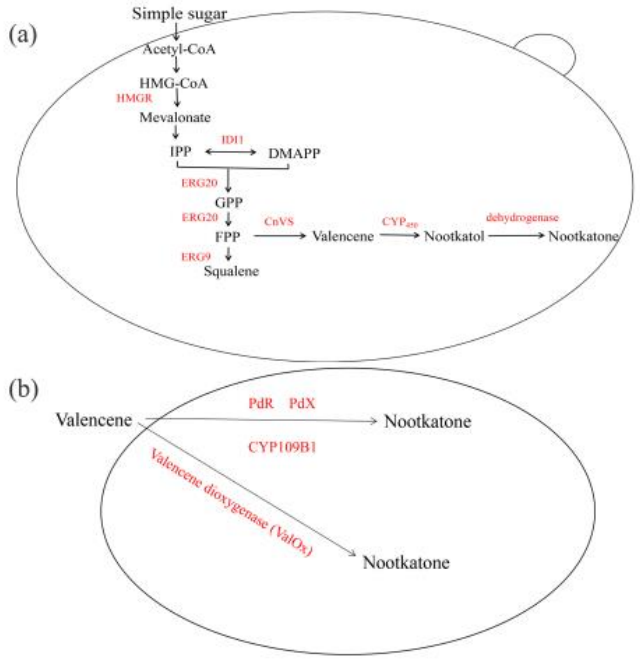


Figure 2.3 Heterologous synthesis of (+)-nootkatone in yeast (a) and E. coli (b) (Li, et al., 2021)

Furthermore, since *Y. lipolytica* is suggested to be used as the replacement for heavy metal catalyst in the biotransformation of valencene to nootkatone, the growth curve of the yeast should be identified. Based on the optimal growth phase of the yeast through literature and study, the time for the substrate to be injected into the media for the biotransformation step could be estimated. Based on the literature, the optimal growth phase is in the exponential phase, with the peak hour as the 36th hour (Li, et al., 2020), as shown in Figure 2.4. It means the yeast activity is the highest at that peak hour, so the biotransformation of valencene to nootkatone is expected to reach the best performance during that hour. Figure 2.4 shows the growth curve of *Y. lipolytica* with the cultivation conditions as 30℃ and 100 rpm shaking speed.

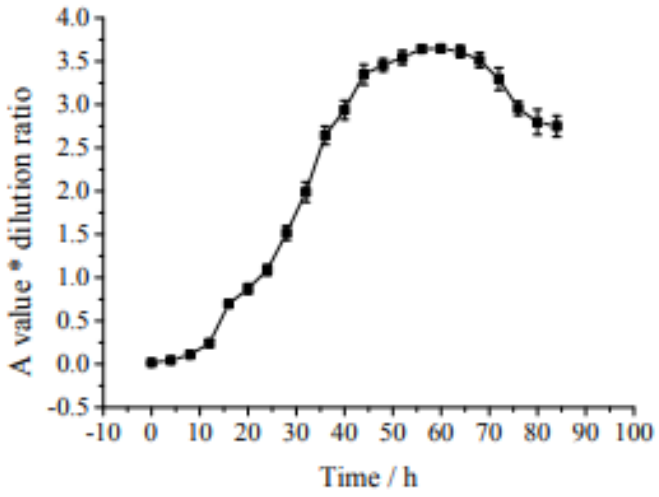


Figure 2.4 Growth curve of Yarrowia lipolytica with cultivation conditions of 30℃ and 100 rpm

2.5. Glucose concentration

Y. lipolytica can use different carbon sources for the MVA pathway, with either hydrophilic or hydrophobic sources, such as glucose, fructose, glycerol, and xylose, as shown in Figure 2.5. Glucose is commonly selected as the carbon source in the culture medium of the yeast. The screening of yeast transformants in an auxotrophic synthetic complex (SC) plate was performed at 10.0 g/L glucose, 6.7 g/L yeast nitrogen base, 16.0 g/L agar, and 2.0 g/L amino acid mixture under insufficient nutrients. In contrast, the growth and fermentation of yeast in yeast extract peptone dextrose (YPD) medium was performed at 20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone (Guo, et al., 2018). However, if the glucose in this culture medium exceeds the required amount, the cell growth might lead to more citric acid production and less lipid accumulation (Carsanba, et al., 2020).

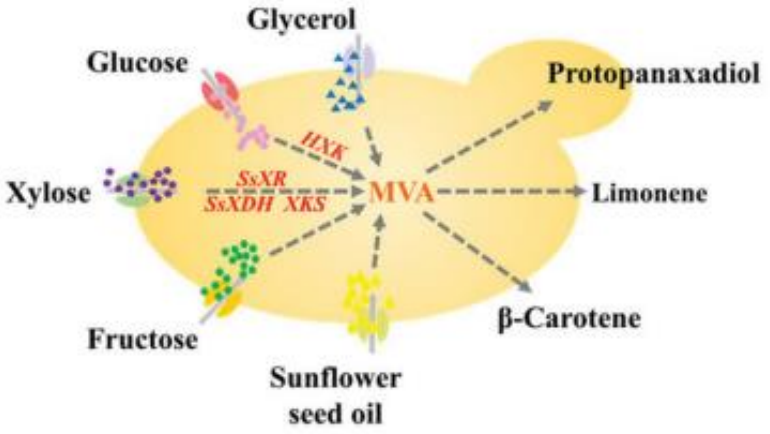


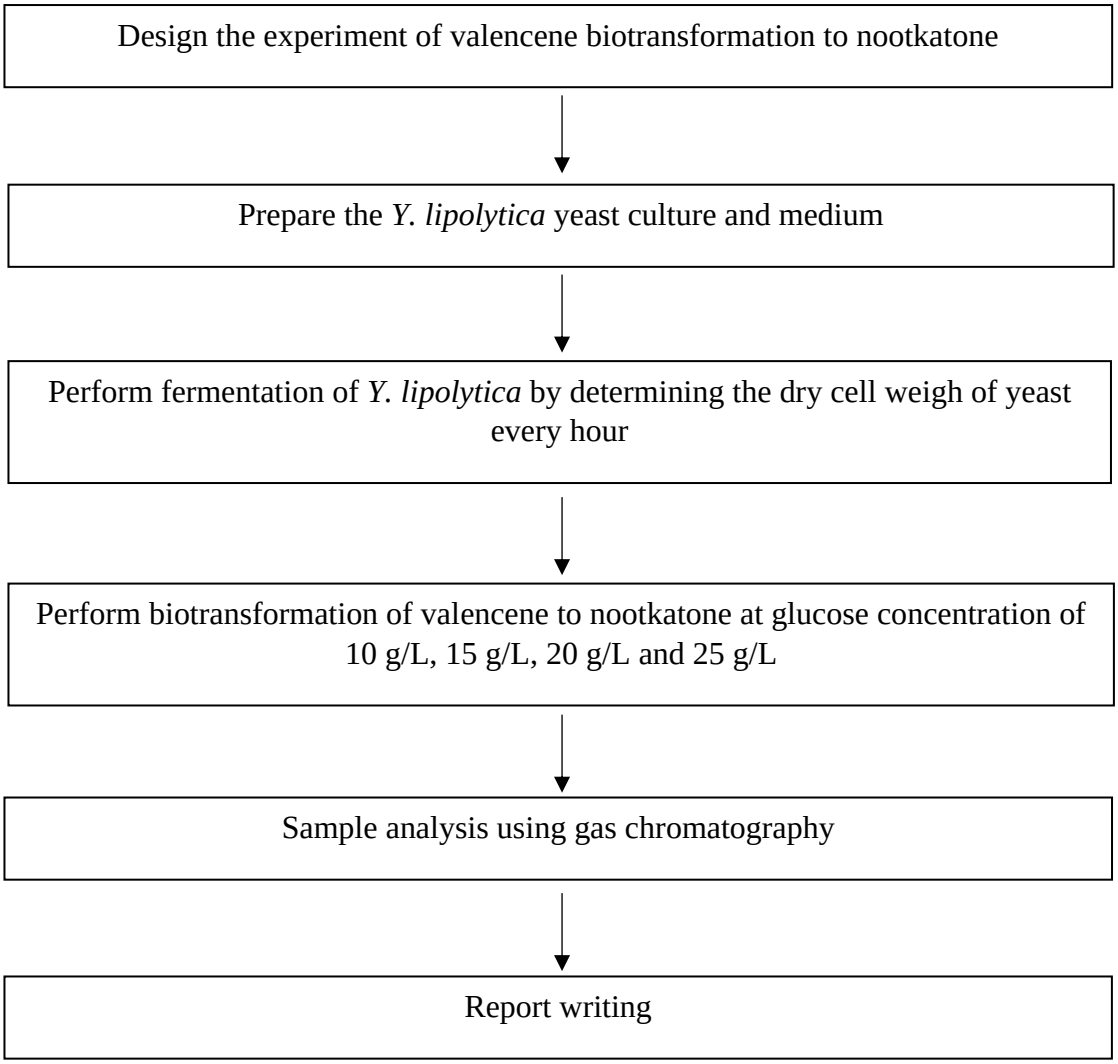
Figure 2.5 Utilization of different carbon sources (Zhang, et al., 2021)

Other than that, the carbon source glucose consumption in the medium throughout the process could be determined using the dinitrosalicylic acid method and an ultraviolet (UV) spectrophotometer with a wavelength of 550 nm (Palmerín-Carreño, et al., 2016). This method is highly sensitive, roughly 0.4 mg/L of the glucose

detection limit. Plus, it has higher specificity than a mass spectrometer to differentiate the compounds with identical chemical formulas, such as glucose and fructose (Caudy, 2017). Therefore, the presence of glucose as the carbon source of yeast could be identified and differentiated.

3. Research Methodology

The flowchart for this research with overall experimental processes is shown below:



3.1. Materials and Equipment

The following tables list the materials and equipment involved in the project.

Table 3.1 List of materials needed for the project

Materials	Manufacturer	Purpose
Peptone	Sigma-Aldrich, USA	To prepare the LB medium and agar for medium and yeast culture
Yeast extract	Sigma-Aldrich, USA	
Glucose	Sigma-Aldrich, USA	
Deionized water	-	To prepare the LB medium and agar
Agar	Sigma-Aldrich, USA	To prepare the agar for the yeast culture
Cotton plug	-	To cover the shake flask and ensure no contamination
Aluminum foil	-	
Ethyl acetate	Sigma-Aldrich, USA	To act as a solvent for the yeast culture

Table 3.2 List of equipment needed for the project

Equipment	Manufacturer	Purpose
Analytical balance	Shimadzu ATX224, Japan	To weight the Eppendorf tube and cells
Magnetic stirrer	Heidolph, Germany	To allow perfect mixing of peptone, yeast extract, and glucose in deionized water as LB medium
Autoclave	Tuttnauer 5075 MLV	To sterile the media
Incubator shaker	IKA, KS4000i Control, Korea	To allow shaking for introducing oxygen, other gases and nourishing compounds to metabolize the cell cultures actively
Centrifuge unit	Eppendorf 5702R, Germany	To centrifuge the sample so that supernatant and cell layers will be formed
Drying oven	Memmert UFB 500, Germany	To dry the cell by removing the moisture
Vortex mixer	Heidolph, Germany	To allow perfect mixing of sample and ethyl acetate
Gas chromatography	Agilent Technologies, USA	To observe the graph with peaks of valencene and nootkatone

3.2. Method of Procedure

3.2.1. Culture preparation

Yarrowia lipolytica colonies are prepared by growing the colonies on an agar surface in a petri dish. First, the agar solution is prepared in the medium bottle using 18 g/L agar, 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. Deionized water is added based on the required concentration. Next, the agar solution is sterilized in an autoclave at temperature 121℃, pressure 1.5 bar, and 20 min, with the complete process taking around 2 hrs including cool down step. The sterilized agar solution is poured and covers the bottom half of the petri dish. Once the agar is cooled and hardened, one loop of yeast is taken using a sterilized loop, and squiggly lines were drawn on the agar surface. The petri dish is then sealed tightly, allowing the yeast to grow in the oven at 30℃. The yeast can only be taken from the petri dish in a cleanroom or sterilized media to ensure no contamination.

3.2.2. Medium preparation

Luria-Bertani (LB) medium is prepared using peptone at 20 g/L, yeast extract at 10 g/L, and glucose at 20 g/L. Deionized water is added based on the required concentration. The medium bottle with a volume of 1L LB medium is transferred to the shake flask. A total of 2 shake flasks with a volume of 500 mL are chosen to pour 250 mL of reaction volume into each shake flask. The medium level must be below the buffer level of the shake flask. A magnetic stirrer is used to allow the perfect mixing of the solids in the deionized water before transferring from the medium bottle to the shake flasks.

3.2.3. Sterilization of media

All the shake flasks are firstly covered with a cotton plug, and the shake flask, together with the cotton plug, is then covered with aluminium foil. In this condition, the shake flasks are ready to be autoclaved. The condition of sterilization is at temperature 121℃, pressure 1.5 bar, and 20 min, with the complete process taking around 2 hrs including cool down step.

3.2.4. Fermentation

A loop is sterilized using flame before using it. Next, one colony of yeast from the culture medium is picked up by using the sterilized loop. After that, the aluminium foil and cotton plug that is used to cover the shake flask is opened in the cleanroom to insert the yeast colony into it. Once the yeast is put in, the cotton plug and aluminium foil are closed. Next, the shake flash is removed from the cleanroom and put inside an incubator shaker at a temperature of 30℃ and a shaking speed of 150 rpm.

Following this, an empty Eppendorf tube and the labelling or marker on it are weighed and recorded as W_o before using it. Then, a sample of 1 mL is taken from the shake flask every hour using a micropipette and put the sample into the Eppendorf tube. The tube with media is closed and centrifuged for 5 min at 4,400 rpm. After that, two layers are formed in the Eppendorf tube, with the upper layer being media or supernatant, whereas the lower layer is cell. After that, the supernatant is thrown, and the tube is left with only the cell. After that, the tube with the cell is dried in the oven at a temperature of 70℃ for 15 to 20 minutes. Subsequently, the tube together with the labelling and cell are weighed and recorded as W_c. Next, the dry cell weight is

determined by subtracting W_o from W_c. By using the dry cell weight, the concentration of cells could be calculated with equation (1), as shown below. Then, by taking the concentration of cells and time, a growth curve of yeast is plotted with the y-axis as cell concentration in g/L and the x-axis as time in hr. These steps are repeated twice to get similar growth curves with similar phases, including lag, exponential, stationary, and death phases.

$$C_{cell} = \frac{W_c - W_0}{V} \quad (1)$$

C_{cell} = concentration of cells (g/L)

W_c = weight of Eppendorf tube with cell after dried (g)

W_0 = weight of empty Eppendorf tube (g)

V = volume of the sample (L)

Furthermore, Monod's model is used to determine the relationship between specific growth rate and substrate concentration. The Monod equation, as shown below as equation (2), calculates the doubling time of *Y. lipolytica* using equation (9).

$$\mu = \frac{\mu_{max}[S]}{K_s + [S]} \quad (2)$$

μ = the specific growth rate (hr^{-1})

μ_{max} = the maximum specific growth rate (hr^{-1})

$[S]$ = the substrate concentration (g/L)

K_s = the substrate saturation constant (g/L)

$$\frac{dx}{dt} \propto x \tag{3}$$

$$\frac{dx}{dt} = \mu x \tag{4}$$

Integrate the equation above and gives,

$$\ln x = \ln x_0 + \mu t \tag{5}$$

Let,

$\mu t = \mu t_d$

$x = 2x_0$

Substitute in the previous equation, and gives,

$$ln\, 2x_0 = ln\, x_0 + \mu t_d \tag{6}$$

$$ln\, 2x_0 - ln\, x_0 = \mu t_d \tag{7}$$

$$ln \frac{2x_0}{x_0} = \mu t_d \tag{8}$$

$$ln2 = \mu t_d \tag{9}$$

t_d = doubling time (hr)