BIOTRANSFORMATION OF VALENCENE UTILIZING *YARROWIA LIPOLYTICA* IN BUFFER SYSTEMS

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by

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LIST OF ABBREVIATIONS

Symbol	Description
$C_{15}H_{22}O$	(+)- nootkatone
pH	Potential of Hydrogen
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
$C_{15}H_{24}$	(+)- valencene
Y. lipolytica	*Yarrowia lipolytica*
CAGR	Compound Annual Growth Rate
USD	United States Dollar
Co (II)	Cobalt (II)
Cu (II)	Copper (II)
Mn (II)	Manganese (II)
H+	Hydrogen ions
OH-	Hydroxide ions
LB	Luria-Bertani
GC	Gas Chromatograph
rpm	Revolutions per minutes
dt	Doubling time

LIST OF SYMBOLS

Symbol	Description	Unit
γ	gamma	-
μ	Specific growth rate	h^{-1}
μmax	Maximum specific growth rate	h^{-1}
pA*s	picoAmpere-second	-
Wdcw	Dry cell weight	g/L
M	molarity	M
Wo	Initial cell weight	g
Wc	Cell weight	g
t_e	Exponential phase	-
t_s	Stationary phase	-
k_B	Boltzmann's constant	J/K
Ks	Substrate concentration constant	g/L
[S]	Glucose concentration	g/L
T	Absolute temperature	℃
r	radius of the spherical particle	cm
n	Dynamic viscosity	Pa.s
V_V	Volume of (+)- valencene	μL
V_m	Volume of media used in shake flask	mL
ρ_V	Density of (+)- valencene	mg/L

BIOTRANSFORMASI VALENCENE MENGGUNAKAN *YARROWIA LIPOLYTICA* DALAM SISTEM PENAMPAN

ABSTRAK

Biotransformasi ialah proses di mana kumpulan berfungsi sebatian organik diubah suai oleh sel hidup kepada produk yang berbeza secara kimia. Proses ini meneroka sifat khusus pemangkin biologi, yang merangkumi kekhususan stereo dan kekhususan rantau serta keupayaannya untuk menahan tindak balas tanpa suhu dan nilai pH yang melampau. Yis, *Y. lipolytica* digunakan sebagai biomangkin untuk pengoksidaan steroid hidrofobik yang sukar larut dalam biotransformasi (+)-valencene untuk menghasilkan (+)-nootkatone. Parameter seperti dalam sistem penampan disodium fosfat dan monosodium fosfat dimanipulasi untuk menentukan keadaan optimum untuk sintesis (+)-nootkatone. Analisis GC-MS digunakan untuk mengesan keputusan atau kawasan puncak (+)-nootkatone dan (+)- valencene. Keluk pertumbuhan *Y. lipolytica* telah ditentukan dan mendapati bahawa fasa pertumbuhan terbaik untuk biotransformasi adalah dalam fasa eksponen 19 jam. Kepekatan dcw maksimum ialah 5.70 g/L dan diperhatikan pada 55 jam dalam fasa pegun. Biotransformasi (+)-valencene menggunakan *Y. lipolytica* dalam sistem penampan telah dilakukan dengan jayanya. Kawasan maksimum (+)- nootkatone diperhatikan pada pH 6 dengan menambahkan 250 µL (+)- valencene dan beroperasi pada 30 ℃ dan 150 rpm. Kajian ini menyimpulkan bahawa pH sistem penampan mempunyai kesan yang signifikan terhadap penggunaan yis semasa biotransformasi. Pada pH yang berbeza, kepekatan (+)-valencene dan (+)- nootkatone yang berbeza diperhatikan. PH yang ideal untuk kadar tindak balas terpantas dan pengeluaran tertinggi untuk kultur yang dibangunkan telah ditemui. Pengeluaran (+)-nootkatone telah dikaji pada pH 6, dan ia mempunyai puncak terbesar berbanding yang lain.

ABSTRACT

Biotransformation is the process through which the functional groups of organic compounds are modified by living cells to a chemically different product. The procedure investigates the unique qualities of biological catalysts, such as stereospecificity and area specificity, as well as their capacity to endure reactions at no severe temperatures or pH values. In the biotransformation of (+)-valencene to produce (+)-nootkatone, *Y. lipolytica* is used as a biocatalyst for the oxidation of barely soluble hydrophobic steroids. The parameters such as in disodium phosphate and monosodium phosphate buffer system are manipulated to determine the optimum condition for (+)-nootkatone synthesis. GC-MS analysis was used to detect the results or the peak area of (+)- nootkatone and (+)-valencene. The growth curve of *Y. lipolytica* was determined, and the best growth phase for biotransformation was in the exponential phase at 22 h. The maximum Wdcw concentration is 5.70 g/L was observed at 55 h in the stationary phase. Biotransformation of (+)-valencene utilizing *Y. lipolytica* in buffer systems is done successfully. The maximum (+)- nootkatone area was observed at pH 6 by adding 250 µL (+)- valencene and cultivating at 30 ℃, 150 rpm. This study concludes that the buffer system's pH significantly impacts the utilization of yeast, *Y. lipolytica*, during biotransformation. The optimal pH for the fastest reaction rate and maximum production for the created cultures were discovered. The (+)- nootkatone production has been studied at pH 6, and it has the most significant peak compared to the others.

CHAPTER 1

INTRODUCTION

Chapter 1 introduces the overview of this research and the significance of biotransformation of (+)- valencene utilizing *Y. lipolytica* in a buffer system. In general, this chapter summarizes the research background of (+)-nootkatone, (+)-valencene, biotransformation, and *Y. lipolytica*, the problem statement, and the objectives of this final year project.

1.1 Research background

1.1.1 (+)-Nootkatone

(+)-Nootkatone as shown in **Figure 1.1**, is a ketone derivative of valence and has the molecular formula $C_{15}H_{22}O$. It is one of the key chemical components of grapefruit's scent and flavour and is commonly found as crystals in its solid state. In liquid, it is thick and yellow. (+)-Nootkatone is usually derived from grapefruit, although it can also be made with genetically engineered organisms or by oxidizing (+)-valencene chemically or biochemically (Furusawa et.al., 2005).

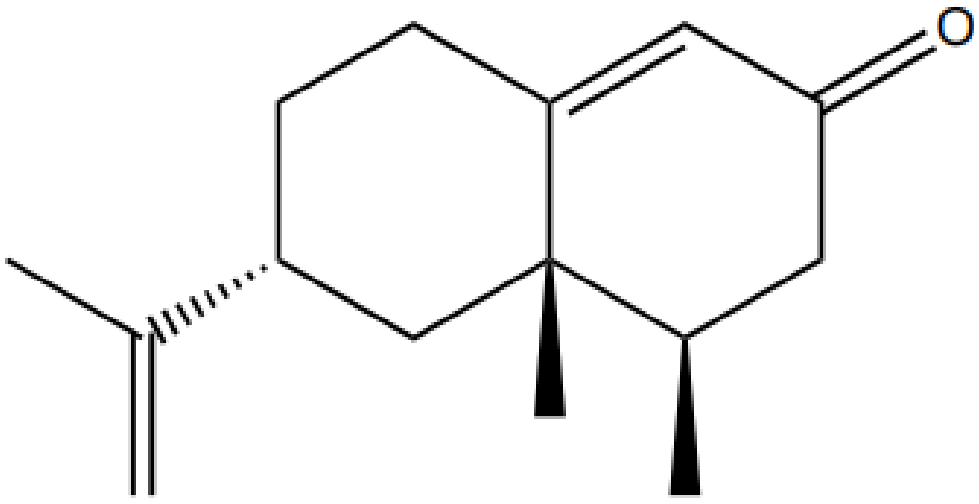

Figure 1. 1: Chemical structure of (+)- Nootkatone

It is a sesquiterpenoid with a grapefruit-like odour and a slightly bitter taste that's found in nature. It was first discovered in the heartwood of Cupressus nootkatensis (Alaska cedar). Since (+)-nootkatone is distinctive organoleptic qualities, its demand for food and fragrance applications is growing. (+)-Nootkatone's pharmacological significance has also been noted for its use as a flavouring ingredient (Gupta and Phulara, 2021). (+)-Nootkatone is frequently utilised in the fragrance business to manufacture perfumes and colognes, with benefits such as lasting on skin and clothing for several hours and being used in citrus-scented shampoos, conditioners, and lotions (CDC, 2020).

(+)-Nootkatones appear to be safe for humans and other mammals since the FDA (Food and Drug Administration) considers (+)-nootkatone to be a GRAS (Generally Recognized as Safe) substance. (+)-Nootkatone also is an antitumor drug that inhibits the anticancer growth of retinoblastoma cells and has antiproliferative, proapoptotic, and protective effects. Furthermore, (+)-nootkatone has the potential to be used as a treatment for neuroinflammation and Alzheimer's disease, as well as having neuroprotective properties (Li et al., 2021).

However, the amount of (+)-nootkatone in plants is low and cannot meet the demand because to extract one kilogram of (+)-nootkatone, around 400,000 kg of grapefruit are required. Disease, hurricanes, and a drop in demand for fresh grapefruit and juice have all had an impact on grapefruit supply. (+)-Nootkatone can be difficult to come by during storm seasons.

There is another option to produce (+)-nootkatone, which is using compounds responsible for the flavour of orange, (+)-valencene, and chemically converting it. (+)-Valencene can be made from a genetically modified yeast and then chemically oxidised. Currently, the synthesis of (+)-nootkatone is from the fermentation of bacteria to produce

an enzyme which is then used to make (+)-nootkatone. The enzyme is then combined with an orange flavouring ingredient in the next step. It is converted by the enzyme into a chemical present in grapefruit.

1.1.2 (+)- Valencene

(+)-Valencene as shown in **Figure 1.2**, is a carbocyclic compound and has a molecular formula $C_{15}H_{24}$. It is colourless to pale yellow oily liquid and insoluble in water, and soluble in oils. (+)-Valencene, a sesquiterpene, has a wide spectrum of citrus scents, with overtones of orange, tangerine, mango, and grapefruit being particularly prominent. The fragrant terpene has a scent that some people associate with freshly cut wood or herbs. (+)-Valencene is a synthetic conversion of (+)-nootkatone, the terpene that gives grapefruit its fragrance. (+)-Valencene is used in commercial mosquito and tick repellents since it is known to repel these insects (Weedmaps, 2020). According to Statista (2020), the major regions for the production of oranges are Brazil (35.98%) followed by China (16.82%). In the production of (+)-nootkatone, (+)-valencene behaves as a substrate that combines with the *Y. lipolytica* and releases products.

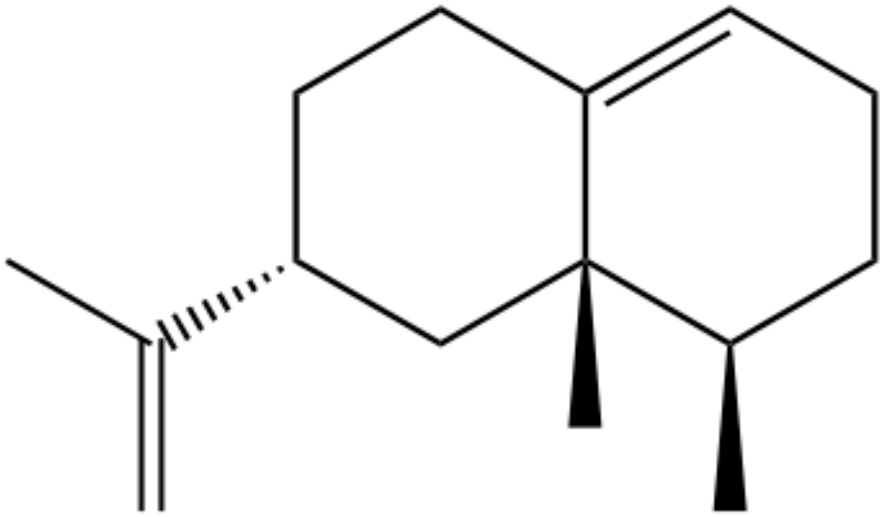

Figure 1. 2: Chemical structure of (+)-valencene

1.1.3 *Y.lipolytica*

Yarrowia lipolytica or *Candida Lipolytica* is an ascomycetous yeast known for its ability to break down lipids and proteins while growing solely on n-paraffins (Heard and Fleet, 1999). It is also a nonpathogenic dimorphic aerobic yeast that is harmless to other organisms and can stand out due to its ability to grow in hydrophobic environments. The characteristic of yeast is that it is able to develop the ability to metabolise triglycerides and fatty acids as carbon sources (Gonçalves et. al, 2014). This yeast can be found in a range of meats and dairy items, particularly sausages and cheeses, where it tolerates low pH, gastric juice, and bile salts. It can also be isolated from the mouth, lungs, and intestinal system, as well as dirt, seawater, and hypersaline lakes (Sutherland et. al., 2014).

1.2 (+)- Nootkatone Market

(+)-Nootkatone is a fine chemical derived from grapefruit that is responsible for the fruit's distinct flavour and scent. (+)-Nootkatone is a member of the terpene family, which are volatile chemicals that are easy to smell and taste. In the perfume industry, there is a higher demand for (+)-nootkatone, and Europe and North America account for the majority of production and consumption in the global market. In addition, the cosmetics and food and beverage industries are predicted to increase sales of (+)-nootkatone over the projection period due to increased demand (TMR, 2019).

There are two (2) products of (+)-nootkatone; (+)-nootkatone crystals and (+)-nootkatone liquid. The demand can be seen in the flavours and fragrances, personal care, and pest control industries. The global market for (+)-nootkatone was estimated at US$ 6353 million in 2019 and is predicted to reach US$ 12070 million by the end of 2026, rising at a compound annual growth rate, CAGR of 14.7 % between 2021 and 2026 (Erik,

2021). **Figure 1.3** depicts the market size of (+)-nootkatone production from 2013 to 2024, showing that it continues to grow and is predicted to reach its peak in 2024.

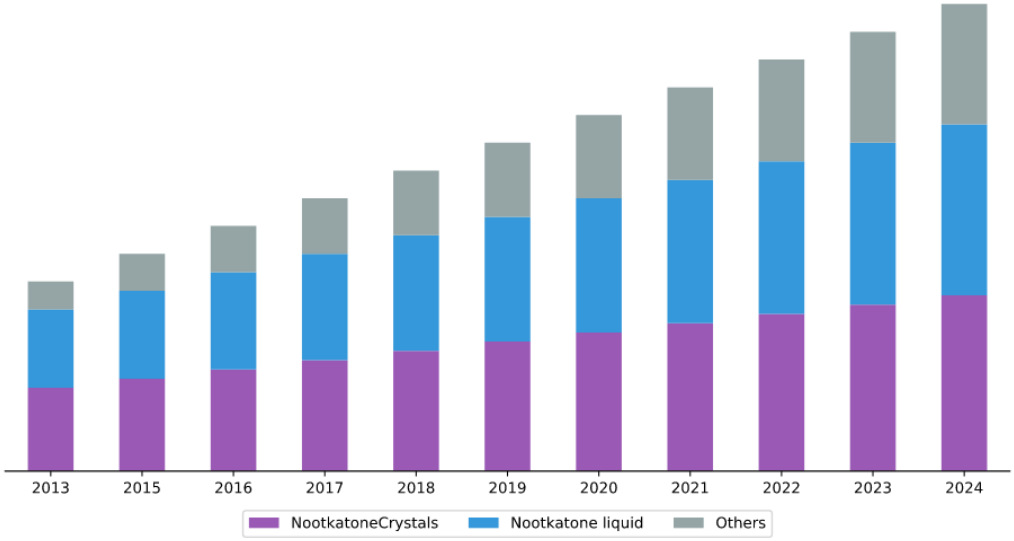

Figure 1. 3: Global (+)-nootkatone market size, by product, 2013-2024 (USD million) (Market Intellica, 2019)

1.3 Problem statement

(+)- Nootkatone is a highly sought-after speciality chemical due to its pleasant grapefruit-like scent and a variety of other intriguing molecular properties. (+)-Nootkatone is only collected in trace levels in its producer plants. Thus, the supply of the (+)- nootkatone for the perfume, food, cosmetics, and pharmaceutical industry has not reached the demand. Therefore, some of the (+)- nootkatone are produced from the chemical syntheses method to meet the demand. These usually necessitate the use of environmentally hazardous chemicals, catalysts, and solvents, and the result cannot be advertised as a "natural" compound. Hence, it represents a marketing disadvantage. Biotechnologists have thus been encouraged by both the market pull and the technological push to create more appealing paths to natural (+)-nootkatone. The new approach is the biotransformation of (+)- valencene utilising whole-cell systems of

microorganisms to produce (+)- nootkatone. This biotechnology utilizing microorganisms is more sustainable and environmentally friendly. *Y. lipolytica* is a type of microbe that has been employed extensively in scientific studies for ecologically benign biotransformation processes. Throughout this study, *Y. lipolytica* will be utilized as a whole cell biocatalyst in the biotransformation of (+)- valencene. This process will be optimized in order to obtain the optimum pH for the highest rate of reaction of (+)- nootkatone.

Normal biotransformation typically occurs in the LB medium. According to (Chackoshian, 2017), however, the pH in LB medium decreases during fermentation and then rises after a while. This is because microbes absorbed the nutrients and released organic acids into the medium, resulting in a fall in pH. After the interval, microbes encounter a dearth of nutrition and begin consuming organic acids as a source of nutrients, increasing pH. Since pH variation is uncontrollable, it may affect the behaviour of the cell. Since potassium phosphate has a strong buffering capacity and is highly soluble in water, it is employed as a buffer at several pH levels (pH 6, pH 7, and pH8).

1.4 Research objectives

The objective of this research:

i. To determine the growth curve of *Y. lipolytica* cell.
ii. To identify the performance of the biotransformation of (+)- valencene utilizing *Y. lipolytica* in a buffer system.
iii. To determine the optimum pH for the highest rate of reaction for the cultures grown.

CHAPTER 2

LITERATURE REVIEW

(+)- Nootkatone is a component of the oil in grapefruit and has been in commercial use for years as a flavoring for foods and beverages and as a fragrance ingredient in perfumes (American Chemical Society, 2013). It is extracted from grapefruit through the chemical or biochemical oxidation of (+)-valencene. It is found in Alaska yellow cedar trees, as well as in vetiver grass. However, only trace levels of (+)-nootkatone are found in the plants that produce it and it takes three (3) years to produce quality grapefruit for consumption. There is high demand for the (+)-nootkatone in the food, cosmetics, and pharmaceutical industry. Therefore, the chemical synthesis and biotechnology to produce (+)-nootkatone is created to make sure the (+)-nootkatone can be supplied based on the demand.

2.1 Chemical synthesis method

Based on the literature, the chemical synthesis method often relies on reagents, catalysts, and solvents to produce (+)-nootkatone and is not labeled as a “natural” substance which increasingly represents a marketing disadvantage. For example, employed tert-butyl peracetate and used tert-butyl hydroperoxide in combination with surface-functionalised silica-supported metal catalysts based on Co(II), Cu(II), and Mn(II) for the allylic oxidation of (+)-valencene (Fraatz, Berger and Zorn, 2009). Hence, this method handles the strong oxidants and required specific safety precautions in the industry to prevent fire hazards. **Table 2.1** depicts an overview of the various chemical used to produce (+)-nootkatone.

Table 2. 1: Overview of different chemical used for producing (+)- nootkatone (Fraatz, Berger and Zorn, 2009)

Chemical used	Via	Product
4-acetyl-1-ethoxycylohexene	- Cyclohexane derivatives - dimethyl γ-ketopimelate - (+)- nopinone	Racemic (+)-nootkatone
Carcinogenic tert-butyl chromate	Convert the presumed metabolic precursor, the sesquiterpene hydrocarbon (+)-valencene	(+)- Nootkatone
Sodium dichromate (carcinogenic or acetic acid)	-	(+)- Nootkatone
Photooxygenation of (+)- valencene	Stereo-selelctive in the presence of catalytic amounts of rose Bengal	(+)- Nootkatone

2.2 Biotechnology utilizing microorganism

Biotechnology is a method for producing products that are both ecologically friendly and commercially desirable. Generally, biotechnology utilizes a microorganism called industrial microbiology which can be the commercial or large-scale use of microbes to obtain a commercially significant product or activity. In the industrial processes, the microorganism used can be a natural, laboratory-selected mutant, or genetically modified strain (Harzevili and Chen, 2018). *Y. lipolytica* is the type of yeast in the microorganism and has huge advantages such as its excellent transformation efficiency, gene integration stability, and ability to produce high-quality proteins. **Table 2.2** depicts the yield of (+)- nootkatone produced by utilizing original *Y. lipolytica* is high compared to other microorganisms (Li et al., 2021).

Table 2. 2: Overview of different organism used for producing (+)- nootkatone.

Organism	Type of microorganism used	Production of (+)- nootkatone	References
BMD_2094	-	44 mg/L	(Milhim et al., 2019)
Y.lipolytica ATCC 201249	Yeast	45.6 mg/L	(Li et al., 2021).
Y.lipolytica 2.2ab	Yeast	420.9 ug/L	(Li et al., 2021).
Rhodococcus	Bacteria	2.5 mg/L	(Wriessnegger et al., 2014)
B. subtilis CYP109B1/expression in *E. coli*	Bacteria	16 mg/L	(Wriessnegger et al., 2014)
P. sapidus/fungi *lyophilisate*	Fungi	225 mg/L	(Wriessnegger et al., 2014)
Saccharomyces cerevisiae	Yeast	0.04 mg/L	(Wriessnegger et al., 2014)
Y. lipolytica	Yeast	628.41 ± 18.60 mg/L	(Li et al., 2021)

2.3 Effect of pH

The pH is a measurement of how acidic or basic the medium it is. The range is 0 to 14, with 7 being the neutral value. Acidity is indicated by a pH less than 7, while a pH greater than 7 indicates a base. According to Bouchedja et al. (2018), the pH value in the culture medium controls various cellular processes, hence it's crucial to investigate the impact of this parameter on the microbial cell population. For instance, it was reported in the studies of Bouchedja et al. (2018) that the *Y. lipolytica* growth at a pH of 6 was faster, in contrast with the cultivation at pH 5. Adjusting the acid-to-base ratio required to form a buffer allows for manipulating the value of pH.

2.4 Effect of buffer system

Buffer systems play a vital role where the system is utilized in the laboratories to maintain a constant pH. It can be defined as a solution that resists pH changes when acid or bases are added. Generally, the buffer basics can be made of a weak acid or a weak base. Phosphate buffer is an example of an effective buffer, as in the following reaction (Christie et. Al., 2020):

$$HPO_4^{2-} + (H^+) \leftrightarrow H_2PO_4^-$$

A buffer is most efficient when the concentrations of acid and conjugate base are nearly equal. The calculation of the buffer system is using the Henderson-Hasselbalch equation. Buffer systems are important to live organisms because most metabolic reactions can only take place when the pH remains within a restricted range. Many biomolecules, particularly proteins, can have their structure and activity disrupted by an excess of H+ or OH-. As a consequence, buffers are frequently utilised in living creatures to assist maintain a pH that is relatively constant.

CHAPTER 3

METHODOLOGY

This chapter discloses the information on the methods applied in this final year project. It includes the chemical and equipment used, a general research flow diagram, and the experiment preparation.

3.1 Chemical and equipment

There are some equipment and chemical were used for this research. The usage of these types of equipment is crucial to completing this study. The chemicals, organisms, and equipment required for the biotransformation of (+)- valencene are shown in **Table 3.1**. LB media were prepared using glucose, yeast extract, and peptone while buffer solutions were prepared using monopotassium phosphate, and dipotassium phosphate, (+)-Valencene was used as the substrate during the biotransformation process with *Y. lipolytica*. The buffer solution, which is a pH solution, was measured using a pH meter. The vortex equipment was provided to have a perfect mixing of the solution. An autoclave was implemented to sterilize the LB media and the pH solution before being used in the experiment. The gas chromatography machine detected the concentration of (+)- valencene and (+)-nootkatone present.

Table 3. 1: The chemicals and organism required for the biotransformation of (+)-valencene

Type	Material	Manufacturer
Chemical	(+)- Valencene	Sigma-Aldrich USA
	Monopotassium phosphate, KH_2PO_4	Sigma-Aldrich USA
	Dipotassium phosphate, K_2HPO_4	Sigma-Aldrich USA
	Glucose	Sigma-Aldrich USA
	Yeast extract	Sigma-Aldrich USA
	Ethyl acetate	Sigma-Aldrich USA
	Peptone	Sigma-Aldrich USA
Organism	*Y. lipolytica*	Sigma-Aldrich USA

3.2 Experimental procedures

The flowchart for this research was shown in **Figure 3.1**, the overview of the overall experimental processes.

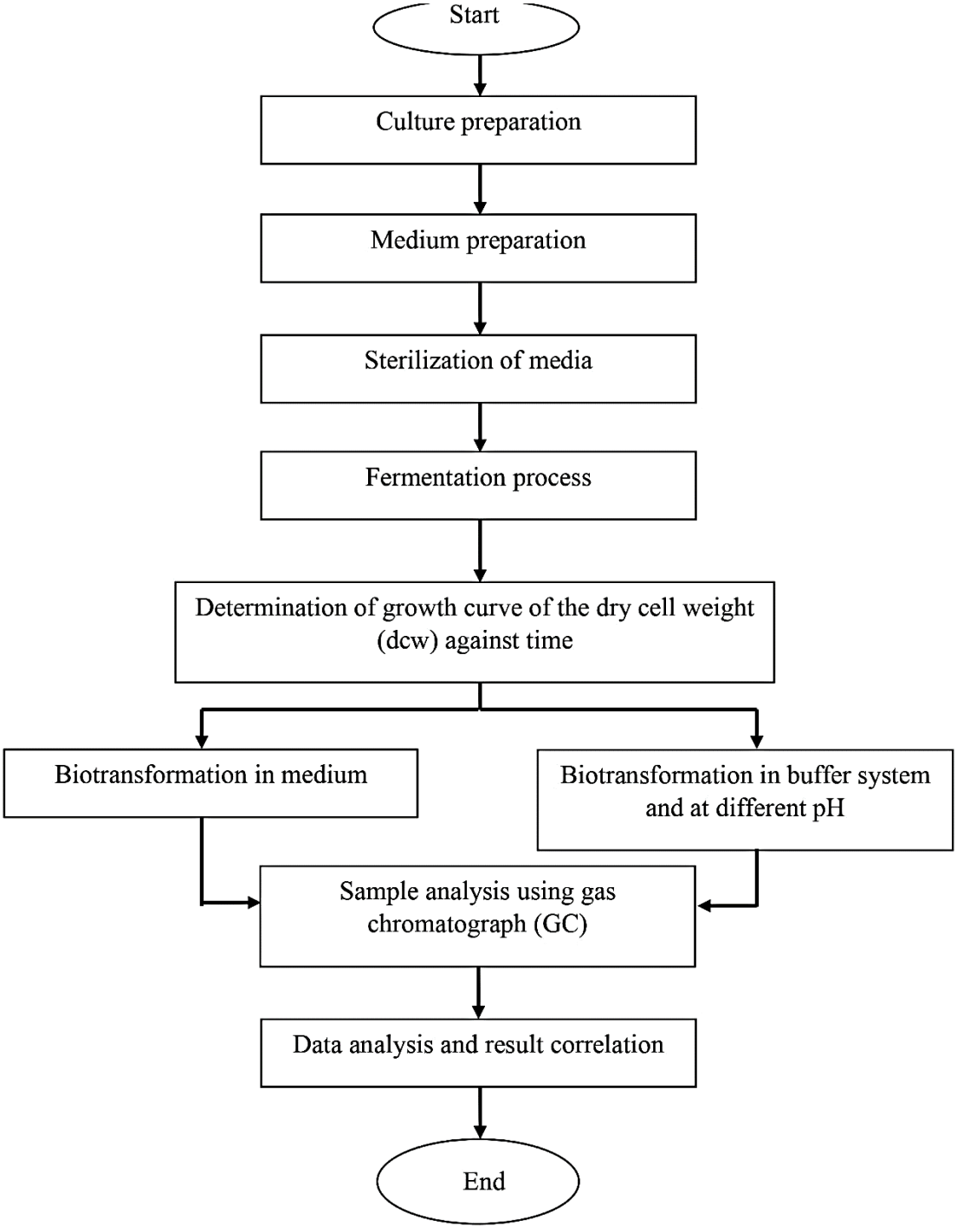

Figure 3. 1: Flowchart for overall research activities

3.2.1 Preparation of culture

Colonies of yeast were prepared on agar of a petri dish. The agar contained 18 g/L agar, 20 g/L glucose, 20g/L peptone, and 10 g/L yeast extract dissolved in 1L deionized water. The yeast grew on the surface of the plate on agar at a condition of 30 ℃ for 1 and a half days in the oven. The yeast used in this method is the wild *Y. lipolytica*. Then, a colony of yeast was taken from the petri dish and put into the sterilized media. This process is only allowed to happen in a clean room to make sure there is no contamination in the media during the process.

3.2.2 Preparation of media

The medium for liquid fermentation is Luria-Bertani (LB) media which contained 20 g. L^{-1} peptone, 10 g. L^{-1} yeast extract, and 20 g. L^{-1} glucose. The LB media prepared in a 1L bottle and being transferred in a shake flask. There are 2 types of baffled shake flask, a 200 mL baffled shake flask with 100 mL reaction volume and a 300 mL baffled shake flask with 250 mL reaction volume. The 300 mL shake flask with 250 mL reaction volume is chosen.

3.2.3 Sterilization of media

The sterilization process was conducted in a 250 mL covered shake flask. The shake flasks were covered with cotton plugs and aluminium foil after the media was added inside. The covered shake flask containing media was autoclaved at conditions of 121˚C and 1.5 bar for 20 min. The sterilization process or the whole process took 2 h before the media is ready to use.

3.2.4 Fermentation process

The sterilized media contained a colony of yeast being cultured inside the incubator shaker. The flask was incubated at 30 ˚C and shaking speed of 150 rpm. After every 1 h, 1mL of the sample was taken out from the flask using a pipette. The 1 mL sample was put into the Eppendorf tube. Before using the Eppendorf tube, the weight of the empty tube is needed as W_o. After that, the tube was closed and centrifuged for 5 min at 5,000 rpm. There are 2 layers of cell and media (supernatant) performed after the centrifugation process. It is required to take out the supernatant from the tube. The tube contained only cells and was dried in the oven at 70˚C for at least 15 to 20 min. The dried cell and tube weighted as W_c.

Generally, in the early stages of fermentation, the yeast activates genetic processes that allow it to import sugar and other components necessary for cell growth and nutrition absorption (Brown, 2021). Fermentation kinetics can be described by a number of models that show the time dynamics of change in cell number X during time t (dX/dt), specific growth rate (μ), and dry cell weight (X). The generation time is the amount of time required for a cell to double in size, whereas the specific growth rate is the fastest rate of growth possible under the current environmental circumstances (Maier, 2015). The kinetic of fermentation commonly employed during the exponential phase and the mathematical terms are as below:

$$\frac{dX}{dt} \propto t \qquad \text{(Eq. 3.1)}$$

The cell growth of yeast can be described by:

$$\frac{dX}{dt} = \mu t$$

By integration:

$$\ln X = \ln X_o + \mu t \quad \text{(Eq. 3.2)}$$

Let $\mu t = \mu dt$ and $X = 2X_o$, substituting in Eq. 3.2:

$$\ln 2X_o = \ln X_o + \mu td$$

Upon rearrangement:

$$\ln 2X_o - \ln X_o = \mu dt$$

$$\ln \frac{2X_o}{X_o} = \mu dt$$

$$\ln 2 = \mu dt$$

Thus, doubling time (dt):

$$dt = \frac{\ln 2}{\mu} \quad \text{(Eq. 3.3)}$$

Where μ:

$$\mu = \frac{\mu_{max}\,[S]}{K_s + [S]} \quad \text{(Eq. 3.4)}$$

The doubling time of a species is the amount of time it takes for its size or number to double relative to its initial value.

3.2.5 Determination of growth curve of *Y. lipoytica*

The weight of the cell, W (W_{dcw}) were recorded at time the sample taken. The experiment was performed in triplicate. The weight of the cell was calculated using formula below:

$$W\ (W_{dcw}) = W_c - W_o \quad \text{(Eq. 3.5)}$$

Where:

Wdcw = Dry cell weight

W_c= Cell weight

W_o= Initial cell weight

The growth curve of dry cell weight against time were plotted and the time where there is the exponential phase, t_e and stationary phase, t_s were determined.

3.2.6 Preparation of phosphate buffer

1 L of 0.1 M phosphate buffer at pH 6 was prepared. 0.01381 M concentration of potassium phosphate dibasic with an amount of 0.60126 g were mixed with 0.08619 M of potassium phosphate monobasic with an amount of 2.932 g in a 250 mL bottle (Aatbio, 2019). 200 mL of deionized water are prepared in a suitable container. The distilled water was added to the solution until the volume reached 250 mL. Then, the phosphate buffer was transferred directly into a new shake flask and sterilized as the same parameter as the sterilization of the media. The same method employed for pH 7 and 8. The masses of monobasic and dibasic compounds are as follows:

Table 3. 2: The buffer preparation at different pH

pH	Potassium phosphate dibasic (g)	Potassium phosphate monobasic (g)
6	0.60126	2.932
7	2.336	1.577
8	4.07	0.2219

3.2.7 Biotransformation of (+)-valencene utilized *Y. lipolytica*

a) Biotransformation of (+)-valencene in a standard media

Biotransformation was carried out using the same process starting from the culture preparation until the fermentation process using a new shake flask. Once the growth curve

was determined and the time at the exponential phase, t_e was recorded, 250 µL of (+)-valencene will be added to the new fermented shake flask at a time, t_e.

Then, 0.5 µL of the sample was taken out from the flask and transferred into the Eppendorf tube using a pipette. 0.5 µL of ethyl acetate will be added into the tube as a solvent. The closed tube was vortex to make sure perfect mixing before centrifuge. After the centrifugation process, there are 3 layers performed, the first layer (above) is ethyl acetate and followed by a layer of supernatant and cell. The layer of ethyl acetate was taken out carefully using a pipette and was transferred into a sample bottle of the gas chromatograph. The supernatant will be taken out from the tube, and the remaining cell in the tube will be dried together in the oven at 70 ˚C for 15 to 20 min. The weight of the dry cell in the biotransformation process was measured.

b) Biotransformation of (+)-valencene in a buffer system

Biotransformation of (+)-valencene was carried out using the same process starting from the culture preparation until the fermentation process using a new shake flask. After the standard fermentation process was done, all the samples in the flask were filtered using filter paper. The filtered cell was put directly inside the sterilized flask of buffer and was going the same process of biotransformation in a standard media.

3.2.8 Effect of pH

To study the effect of pH by manipulating the pH from 8.0 to 6.0 with the decrease of 1.0 pH. The calculation or the measurement of the pH using the Henderson-Hasselbalch equation. The result of the optimum pH was based on the highest rate of reaction for the cultures grown. The calculation of ratio to get a value of pH is calculated using the Henderson-Hasselbalch equation as below:

$$pH = pKa + \log\left(\frac{[Base]}{[Acid]}\right) \quad \text{(Eq. 3.6)}$$

$$M = [Base] + [Acid] \quad \text{(Eq. 3.7)}$$

Where the pH is the pH value of the solution, Ka is the dissociation constant of the acid, [Base] is the concentration of the conjugate base, [Acid] is the concentration of the acid and M is the desired molarity of the buffer.

3.2.9 Effect of buffer system

The effect of the buffer system is studied based on the comparison of the performance of biotransformation of (+)-valencene utilized *Y. lipolytica* to produce (+)-nootkatone with the basis.

3.2.10 Analytical analysis

The standard curve of (+)-valencene was plotted at 5 concentration values which are 0.5 $g.L^{-1}$, 1.0 $g.L^{-1}$, 1.8 $g.L^{-1}$, 3.76 $g.L^{-1}$. The concentration of (+)-valencene was injected into the gas chromatograph to get the calibration curve. In addition, the ethyl acetate content will be analysed by using a gas chromatograph equipped with a capillary column. The curves were plotted from the sample performed 1st peak from (+)- nootkatone and 2nd peak from (+)- valencene

CHAPTER 4

RESULT AND DISCUSSIONS

This chapter presents the results obtained from the work as described in **Chapter 3**. All the data and results are thoroughly discussed to meet the outlined research objectives.

4.1 Determination of growth curve of *Y. lipolytica*

The growth curve of the strain was determined, and the dry cell weight values were measured every 1 h in the following time. The growth curve of *Y. lipolytica* was plotted with the time the sample was taken, as illustrated in **Figures 4.1 and 4.2.**

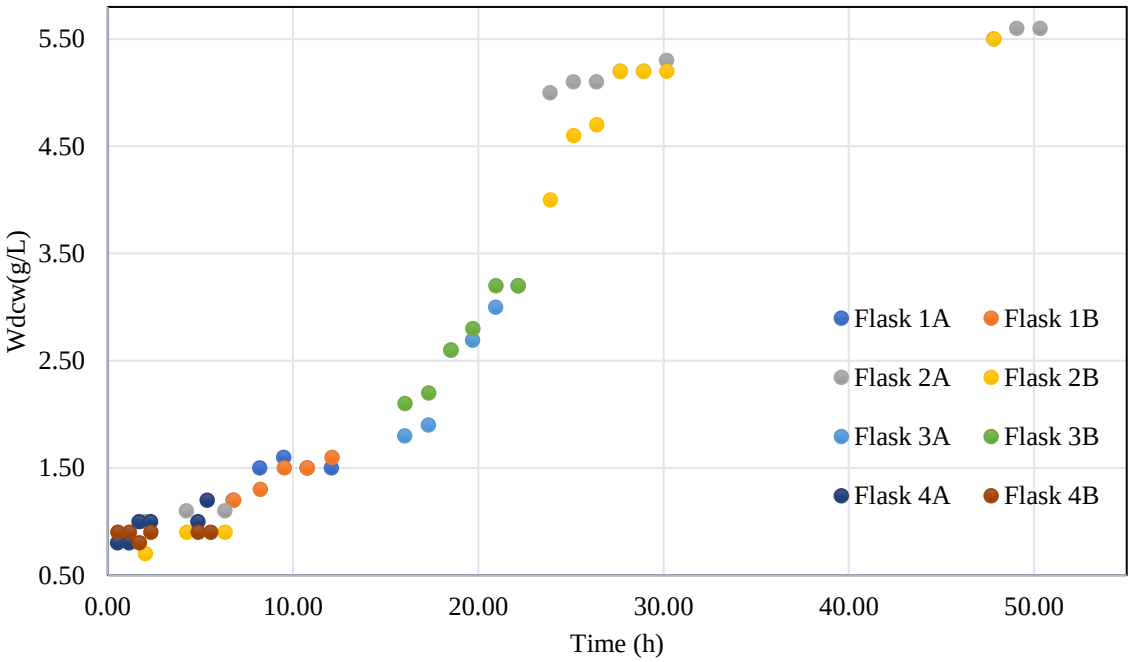

Figure 4. 1: Growth curve of *Y. lipolytica* including all the data obtained

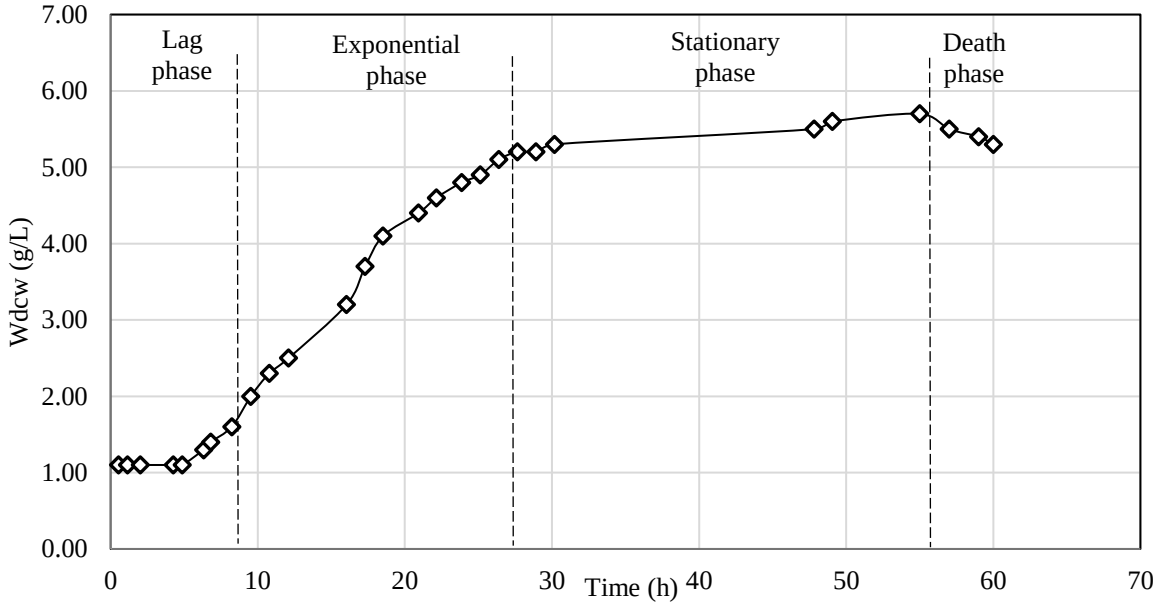

Figure 4. 2: Growth curve of *Y. lipolytica*

As shown in the figure, bacterial growth can be categorized into four phases: lag, exponential, stationary, and death. The lag phase is defined as the first phase to be observed under batch conditions in which the growth rate is nearly zero. It occurs when inoculum is added to a new medium and the process of growth begins. The exponential phase is defined by a period of exponential growth—the highest rapid growth conceivable under the parameters inherent in the batch system. A cell's rate of growth during exponential growth is directly proportional to the total number of cells in the culture. In the stationary phase, there is no net growth. However, cells continue to divide and expand, despite the fact that there is no net growth during the stationary phase. Death is the final phase of the growth curve, which is marked by a decrease in culturable cell numbers. Since individual cells may be metabolising and dividing during the death phase, there is a net loss of viable cells because more viable cells are lost than are gained (Maier, 2015).

All the data from **Appendix A** are displayed in **Figure 4.1**, which displays the growth curve's general shape. Depending on the colour of the labels, there are four (4) separate runs and two shake flasks (A and B) used for each run. Combining all of the data illustrates the four (4) stages of bacterial development. To make it clearer and more understandable, the growth curve depicted in **Figure 4.2** was constructed.

As illustrated in **Figure 4.2**, *Y. lipolytica*'s logarithmic growth time ranged from 8 to 55 h. At this point, the yeast was rapidly growing. Within 0 to 8 h, *Y. lipolytica* grew slowly, and this belonged to the initial stage or phase of growth. During the initial stage, the yeast activates genetic mechanisms that enable it to import sugar and other components required for cell reproduction and nutrition absorption which is fermentation. In the next 19 h, the strain had a significant growth effect which refers to the exponential phase. The dry cell weight content of 5.70 g/L is the highest at 55 h in the stationary phase, referring to the maximal dry cell weight of *Y. lipolytica*. From 55 to 60 h, the strain has a brief period of stability as it enters the decline of the dying phase due to a lack of nutrients or the presence of harmful byproducts from the reaction, the cells begin to die and the growth rate becomes negative (Brown, 2021).

According to Li et al. (2022), the substrate concentration constant (K_s) is 0.1818 g/L, the maximum specific growth rate (μ_{max}) is 0.19 h^{-1}, and glucose concentration [S] is 20 g/L. Therefore, using **Eq. 3.4**., the specific growth rate (μ):

$$\mu = \frac{0.19\ h^{-1} \times 20\ g/L}{0.818 \frac{g}{L} + 20\ g/L}$$

$$\mu = 0.1825\ h^{-1}$$

By substituting the value of μ into **Eq. 3.3**:

$$\mathrm{dt} = \frac{\ln 2}{0.1883\ \mathrm{h}^{-1}}$$

$$\mathrm{dt} = 3.80\ \mathrm{h}$$

The dt refers to the duration of the cell's production. The estimate above indicates that the doubling time for *Y.lipolytica* is 3.80 h. *Y. lipolytica* ferments more slowly than ordinary yeast, with each phase lasting longer. Consequently, as depicted in **Figure 4.2**, each phase of *Y. lipolytica* is longer, particularly the logarithmic phase, because of its lengthy doubling time.

In addition, the (+)- valencene diffusivity is investigated in greater depth to explain how the cell and substrate function. However, evidence on *Y. lipolytica* is scarce. To describe the research, the porosity of the cell wall and membrane of *Saccharomyces cerevisiae* is utilised in this study (Scherrer et. Al., 1974). The diffusivity of (+)-valencene is explained using the mathematical term below which is the Stokes–Einstein equation:

$$\mathrm{D} = \frac{k_B T}{6\pi n r} \qquad \text{(Eq. 4.1)}$$

Where:

k_B = Boltzmann's constant

T = absolute temperature

n = dynamic viscosity

r = radius of the spherical particle

The Stokes-Einstein equation is typically applied to the diffusion of spherical particles in a liquid with a low Reynolds number. According to Scherrer et. Al. (1974), the r is 0.81 nm and k_B is 1.3807 x 10 -23 J/K. Cell diffusion is a type of passive cell movement. In diffusion, molecules travel from locations of high concentration to areas of low concentration to lessen the concentration gradient. From an energy standpoint, diffusion from areas of low concentration to areas of high concentration is unfavourable (Brightstorm, 2022). As in this study, (+)- valencene act as a substrate, and the yeast used is *Y. lipolytica.* During biotransformation, (+)- nootkatone will diffuse out of the yeast cell, and GC will detect the presence of (+)-nootkatone.

4.2 Analysis of the (+)-Nootkatone

Y. lipolytica is the type of yeast in the microorganism and has huge advantages such as its excellent transformation efficiency, gene integration stability, and ability to produce high-quality proteins. Based on **Figure 4.3 (a)(b)(c)(d)**, the table depicts the yield of (+)-nootkatone produced by utilizing original *Y. lipolytica* at a certain retention time.

The peak area at each retention time of the presence of (+)-nootkatone from the biotransformation of (+)-valencene using *Y. lipolytica* in GS-MS analysis was shown. The majority of the peaks were seen to be dispersed in the retention region over 27 to 30 min. According to an estimate based on the GC's operating temperature, the retention period for (+)-nootkatone is approximately 32 min. The retention time of (+)-nootkatone in the experiment has a minor flaw. However, it is determined by the time the sample is injected into the GC equipment's hole.