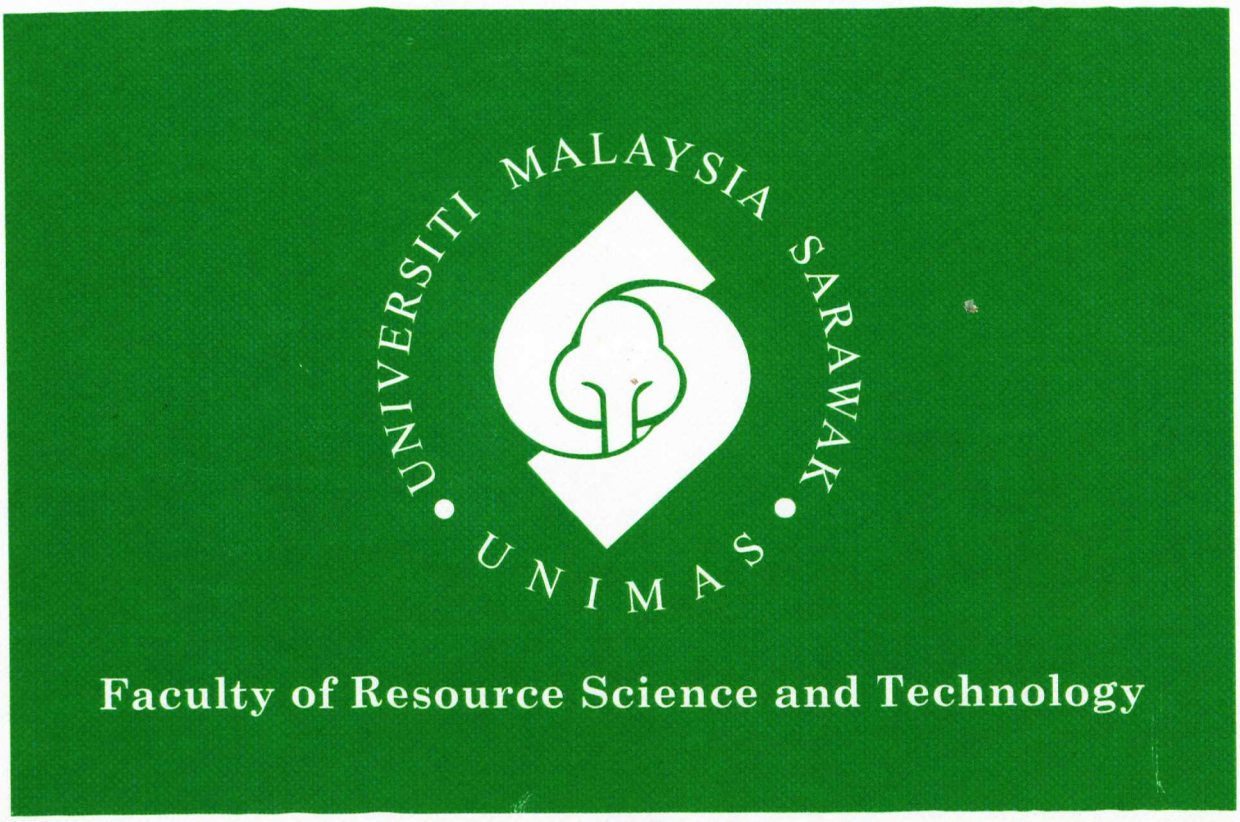

GROWTH KINETICS AND LIPID ACCUMULATION IN *Rhodotorula mucilaginosa* MV-5 CULTURED IN SAGO EFFLUENT

Reynold anak Ramsy
(53654)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)

Final Year Project Report ☑

Masters ☐

PhD ☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the ...14th.....day of...June..... year ...2018.....

Student's Declaration:

I REYNOLD ANAK RANSY, 53654, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY

(PLEASE INDICATE NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, Growth kinetics and lipid accumulation in R. mucilaginosa cultured in Sago effluent is my original work. I have not copied from any other students' work or from any other sources with the exception where due reference or acknowledgement is made explicitly in the text, nor has any part of the work been written for me by another person.

14/6/2018
Date submitted

REYNOLD ANAK RANSY 53654.
Name of the student (Matric No.)

Supervisor's Declaration:

I,---------- (SUPERVISOR'S NAME), hereby certify that the work entitled, ----------(TITLE) was prepared by the aforementioned or above mentioned student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of ----------
(PLEASE INDICATE THE DEGREE TITLE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: _____ Date: 14/6/2018

(Name of the supervisor)

Dr. Micky Vincent
Senior Lecturer
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
94300 Kota Samarahan

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

I declare this Project/Thesis is to be submitted to the Centre for Academic Information Services and uploaded into UNIMAS Institutional Repository (UNIMAS IR) (Please tick (√)):

☑ **YES**
☐ **NO**

Validation of Project/Thesis

I hereby duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic and research purposes only and not for other purposes.
- The Centre for Academic Information Services has the lawful right to digitize the content to be uploaded into Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis if required for use by other parties for academic purposes or by other Higher Learning Institutes.
- No dispute or any claim shall arise from the student himself / herself neither a third party on this Project/Thesis once it becomes the sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student himself/herself without first obtaining approval from UNIMAS.

Student's signature _____ 14/6/2018
(Date)

Supervisor's signature: _____
(Date) 14/6/2018

Dr. Michael Ident
Senior Lecturer
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
94300 Kota Samarahan

Current Address:

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the date of restriction indicated, and the reasons for the confidentiality and restriction.

[The instrument was prepared by The Centre for Academic Information Services]

Growth Kinetics and Lipid Accumulation in *Rhodotorula mucilaginosa* MV-5 Cultured in Sago Effluent

Reynold anak Ramsy
(53654)

A thesis submitted in partial fulfilment of the degree of Bachelor of Science with Honours (Resource Biotechnology)

Resource Biotechnology

Faculty of Resource Science and Technology

University Malaysia Sarawak

2018

DECLARATION

I hereby declare that this Final Year Project entitled **"Growth Kinetics and Lipid Accumulation in *Rhodotorula mucilaginosa* MV-5 Cultured in Sago Effluent"** is based on my original work except for the quotations and citations which have been fully acknowledged and this project has not been submitted for any other degree qualification in UNIMAS or in other institution of higher learning.

_____

(REYNOLD ANAK RAMSY)
Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ACKNOWLEDGEMENT

First, I would like to express my utmost gratitude to my supervisor, Dr. Micky Vincent for all his guidance and supervision in completing this Final Year Project. His constructive comments and suggestions are greatly appreciated and have contributed to the completion of this Final Year Project.

Next, I would like to thank Mr Azis and Mdm Sheela, for their assistance in handling various analytical machines, apparatus and equipment in the laboratory.

My special appreciation goes to Leslie Wong, Afizul, Queentety and Liyana, the postgraduate students for their patience and constant guidance throughout my learning process. Without them, I would not be able to complete my Final Year Project. Also, I would like to thank and my fellow lab mates who are there for me and for their assistances during my lab work.

Next, I would like to express my deepest appreciation towards my parents and family for their never-ending supports. I am truly blessed to have them as they continuously provided me with encouragement and fulfil my needs during my study in UNIMAS and in completing this Final Year Project.

Lastly, I would like to thank those who have involved directly or indirectly in the completion of this Final Year Project.

Growth Kinetics and Lipid Accumulation in *Rhodotorula mucilaginosa MV-5* Cultured in Sago Effluent

Reynold anak Ramsy

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

The increase in the world demand for diesel has led to the production of biodiesel from alternatives sources. One of the example of these alternative is from fungal lipids from oleaginous yeast such as *Rhodotorula sp.*. Fungal lipid is used as feedstock to replace plant and animal oils as the latter are competitive in terms of food and feeds. In this study, the growth kinetics of *Rhodotorula mucilaginosa* MV-5 shows that the culture is at log phase after 24 h. The highest biomass recorded during propagation stage was 1.63 g/L at 24 h. Next, *R. mucilaginosa* MV-5 was supplied with sago effluent as carbon source and the highest lipid extracted during lipid accumulation stage was 0.23 g/L. However, percentage of lipid by weight was not able to be determine due to presence of sago hampas particle which interfere with the actual weight of biomass. Phenol-sulphuric assay was used to determine the amount of starch reduction and the amount of starch reduced over the 168 h was 0.659 g/L. In conclusion, *Rhodotorula mucilaginosa* MV-5 can utilise sago effluent as carbon source to produce lipid.

Keywords: Oleaginous yeasts. *Rhodotorula mucilaginosa* MV-5, sago effluent, growth kinetics, lipid accumulation, phenol sulphuric assay

ABSTRAK

Peningkatan permintaan dunia untuk diesel telah menyebabkan pengeluaran biodiesel dari sumber alternatif. Salah satu contoh alternatif ini adalah dari lipid kulat dari yis oleaginous seperti Rhodotorula sp. Lipid kulat digunakan sebagai bahan mentah untuk menggantikan minyak tumbuhan dan minyak haiwan sebagai bahan mentah kerana bahan tersebut adalah kompetitif dari segi makanan dan makanan. Dalam kajian ini, kinetika pertumbuhan Rhodotorula mucilaginosa MV-5 *menunjukkan bahawa budaya berada pada fasa log selepas 24 jam. Biomas tertinggi yang dicatatkan semasa peringkat penyebaran ialah 1.63 g / L pada 24 jam. Seterusnya, R. mucilaginosa* MV-5 *dibekalkan dengan sagu efluen sebagai sumber karbon dan lipid tertinggi yang diekstrak semasa tahap pengumpulan lipid adalah 0.23 g / L. Walau bagaimanapun, peratusan lipid mengikut berat tidak dapat ditentukan kerana adanya zarah sagu hampas yang mengganggu berat sebenar biomas. Ujian phenol-sulfurik digunakan untuk menentukan jumlah pengurangan kanji dan jumlah kanji dikurangkan dalam tempoh 168 h adalah 0.659 g / L. Sebagai kesimpulan, Rhodotorula mucilaginosa* MV-5 *dapat menggunakan sagu efluen sebagai sumber karbon untuk menghasilkan lipid.*

Kata kunci: Yis berminyak, *Rhodotorula mucilaginosa* MV-5, efluen sagu, kinetic pertumbuhan, pengumpulan lipid, ujian asid fenol-sulfur

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

(v/v)	volume / volume
C : N	carbon : nitrogen
DCW	Dry cell weight
H_2SO_4	sulphuric acid
min	minute
nm	nanometer
RBDC	Rose Bengal Dichloran Chloramphenicol
rpm	revolution per minute
YMB	Yeast Malt Broth
t	tonne
sp.	species

CHAPTER 1

INTRODUCTION

Fossil fuels such as crude oils, coals and gas are known for their importance as the main resources for the world energy supply (Shafiee & Topal, 2009). However, those natural resources are depletable resources that cannot regenerate naturally (Dieren, 1995). One of the alternative in overcoming the problem is through the production of biodiesel. According to the National Biodiesel Board (2017), biodiesels are biodegradable, nontoxic, and essentially free of sulfur and aromatics compared to the conventional fuel used. Biodiesel can be produced by using animal oils and plant oils as feedstock however it is controversial as the production needs large land area, high cost and maintenance (Wang *et al.*, 2014).

To overcome these problems, many researchers suggested using microorganism which can produce the same lipid compositions with animal fat or plant oil as feedstock. These microorganisms are usually yeast, fungi, bacteria and microalgae, which can accumulate lipids of up to 20% of their biomass dry weight in a specific condition (Beopolus, Nicaud & Gaillardin, 2011). According to Signori *et al.* (2016), lipids produced by the oleaginous microorganism are also known as single cell oils or SCO. Among these oleaginous microorganisms, yeasts show the best characteristics as they have flexibility in term of culture conditions (Gohel *et al.*, 2013), unaffected by light and climatic variation (Ami *et* al., 2014) and have higher lipid content than oleaginous bacteria (Ochsenreither *et al.*, 2016).

One major obstacle to produce biodiesel from these microbials is high cost of media for cell culture and the low efficiency of oil production (Wang *et al.*, 2014). To overcome this problem, researchers have investigated the use of agro-industrial residue and waste as the carbon source in culture medium. In Sarawak, sago palm (*Metroxylon sagu*) is a type of commercial plant that has high economic value especially the production of sago starch. This industry generates abundant liquid and solid waste that are high in carbon content. Therefore, by utilizing sago effluent as the carbon source, this will overcome the high cost of culture media. In addition, this practise may provide alternative ways to dispose the industrial sago effluent.

In this study, *R, mucilaginosa* MV-5 was cultured in sago effluent to produce lipid. The lipid accumulation will involve two main stage. The first stage is the cell propagation of *R. mucilaginosa* MV-5 in Yeast Malt Broth (YMB). Next, the cell culture was cultured in sago effluent as carbon sources to produce biomass. Lipid accumulation and extraction was conducted and total carbohydrate test using phenol-sulphuric assays was done to quantify the amount of lipid produced from the dry biomass of *R. mucilaginosa* MV-5 cultured in sago effluent.

Objectives

The objectives of this project are to:

1. Analyze the growth kinetics of *R. mucilaginosa* MV-5 cultured in YMB
2. Measure the biomass produced from the yeast culture.
3. Quantify the amount of lipid during lipid accumulation stage.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid for Biodiesel Feedstock

Biodiesel is a mixture of mono-alkyl esters that are derived from triacylglycerides (TAG) with long fatty acids chain and can be produced from cheap raw materials such as fats and oil (Leesing *et al.*, 2011). Biodiesel commonly uses plant oil as feedstock where transesterification method (figure 2.1) is used to change the viscosity of the plant oil (Rao *et al.*, 2010). In addition, biodiesel can be easily produced from animal fat or plant oil as feedstock, but it is deemed controversial as this practice competes with food production (Wang *et al.*, 2014). Sheedlo (2008) reported that the absence of corrosive polycyclic hydrocarbon is what makes biodiesel a preferable choice for sources of fuel.

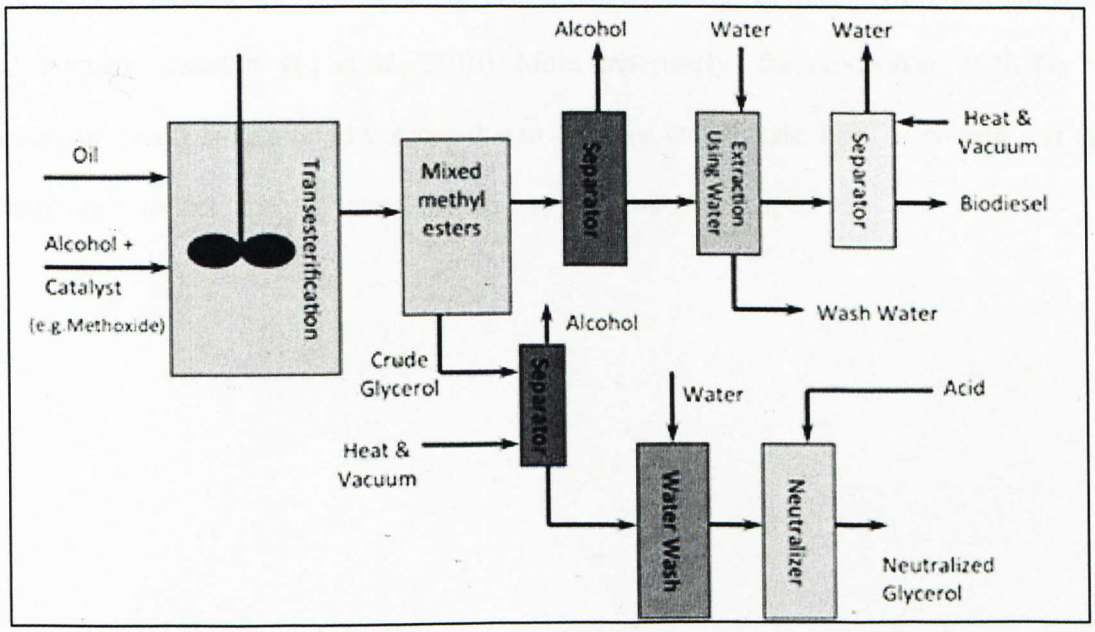

Figure 2.1 Schematic of biodiesel process using transesterification. Image retrieved from https://www.e-education.psu.edu/egee439/node/684

2.2 Oleaginous yeast

Yeasts are single-celled fungi. As fungi, they are closely related to other fungi which include edible mushrooms and the common baker's yeast. Oleaginous yeasts are those species of yeast that are rich in, covered with or produces oil. There are many species of oleaginous yeast such as *Yarrowia sp.*, *Candida sp.*, *Rhodotorula sp.*, *Rhodosporidium sp.*, *Cryptococcus sp.*, *Trichosporon sp.*, and *Lipomyces sp.* (Ageitos, 2010). The common yeast used oleaginous yeast used in research includes *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, and *Cryptococcus albidus* where they are reported to accumulate lipids up to 20% of their dry weight. In general, yeasts and molds can accumulate much more lipids than bacteria and microalgae (Helwani *et al.*, 2009).

Lipids produced by oleaginous fungi are also called single cell oils (SCOs). These SCOs can be transformed to FAME (fatty acid methyl esters) by means of both enzymatic and inorganic catalysis (Li *et al.*, 2010). More importantly, the production of SCOs by oleaginous yeasts has many advantages due to their fast growth rate, high oil content and the resemblance of their triacylglycerol fraction to plant oil (Ageitos, 2011).

2.3 *Rhodotorula mucilaginosa*

The genus Rhodotorula consists of a diverse group of pigmented basidiomycetous yeast. It is a common environmental yeast isolated from a diverse range of habitats and colonized plants and animals (Albertyn, Pohl & Viljoen, 2017). *Rhodotorula glutinis*, *Rhodotorula minuta*, and *Rhodotorula mucilaginosa* are the three well known strains that are pathogenic to humans. *Rhodotorula* species is commonly isolated from various foods and beverages and *R. mucilaginosa* is one of the common suspect that causes food spoilage (Albertyn, Pohl & Viljoen, 2017). *R. mucilaginosa* are known for its characteristic carotenoids production (Aksu & Eren, 2004) (figure 2.2) and for its ability to accumulate lipid for biodiesel feedstock production utilizing various carbon sources, agricultural wastes and industrial residues (Zhao et al., 2011).

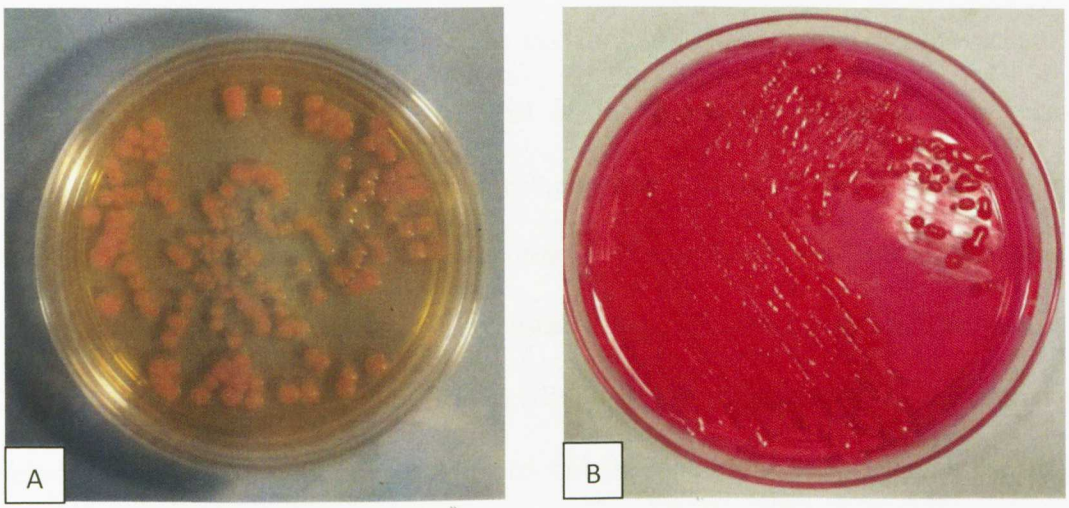

Figure 2.2 Pure colony of *R. mucilaginosa* MV-5 culture in Yeast Malt Agar (A) and Rose Bengal Dichloran Chloramphenicol Agar (B)

2.4 Lipid and Lipid Accumulation in Oleaginous Yeast

Lipid can be defined as a mixed clump of compound having several similar properties based on their morphology, mainly of non-polar group and they are partly water-soluble but readily soluble in organic solutions. (Campbell *et al.*, 2012). According to Wild *et al.* (2010), lipid production requires a medium with an excess of sugars or similar components and limited amount of other nutrients, usually nitrogen and phosphate. Leesing *et al.* (2011) reported that some species oleaginous fungi such as *Rhodosporidium* sp., *Lipomyces* sp., and *Rhodotorula* sp. are able to accumulate lipids exceeding 70% of their dry mass even with environment that have limited nutrients.

Throughout many years of study done by researchers on oleaginous yeast, it was found out that oleaginous yeast is able to utilize many substrates such as sewage sludge, sugar cane bagasse, industrial glycerol and industrial waste. By breaking down glycerol, soluble starch and other specific components exist in the medium, oleaginous yeasts are able accumulate lipid (Xia *et al.*, 2011). According to Wild *et al.*, (2010), the ratio of Carbon: Nitrogen supplied during lipid accumulation phase can affect the amount of lipid accumulated by the yeasts, where an environment of high carbon sources is preferred with limited concentration of certain nutrients. These nutrients are usually nitrogen and phosphate. Subhash *et al.* (2011) reported that the amount of lipid accumulated can be influenced by the condition of microorganisms, the variety of substrate, and various controllable parameters in the cultures. In addition, it is reported that lipid accumulation of oleaginous microorganism can be manipulated through genetic engineering and genetic modification.

2.5 Sago Palm, Sago Starch and Sago Effluent

Sago palm (*Metroxylon* spp.) or better known as 'Rumbia" is a type of plant that is native to Sarawak and is widely distributed throughout South East Asia (figure 2.5). An example of important species of this plant that is widely used by the local population is *Metroxylon Sagu*. Ahmad *et al.,* 1999) reported that sago palm has various uses and is crucial to the local population where the sago starch isolated from the sago palm is often used to produce either starch, sago flour or sago pearl. In South East Asia, sago starch also plays an important role within the food industry which usually involves in the production of vermicelli, bread, crackers, biscuits and many other traditional foods. In addition, sago starch is cheap and is reported to have important properties such as the ability to gelatinized, has high viscosity and can easily be moulded (Ahmad *et al.,* 1999).

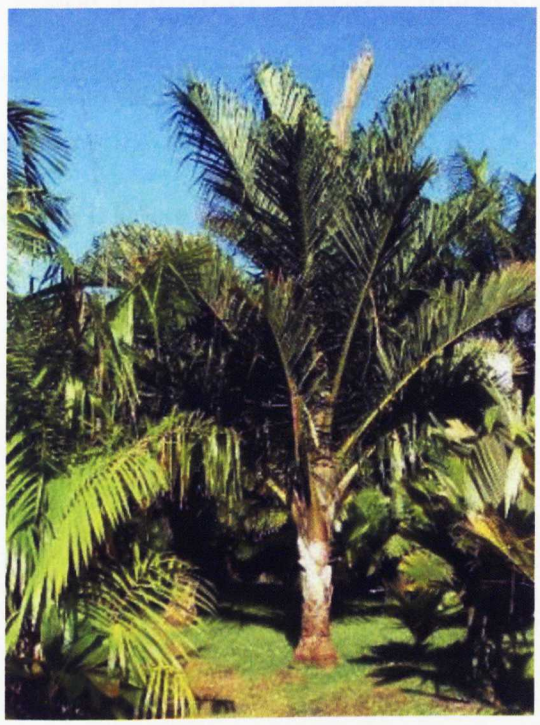

Figure 2.5 A matured sago palm tree (*M. sagu*). Image retrieved fromhttp://mydifferentperspectives.blogspot.my/2009/06/metroxylon-sagu-sago-palm_28.html

Sago effluent is the wastewater discharged by the agro-industry. The sago processed water being rich in carbohydrates is usually discharged to the river, near the lake and land. This untreated effluent caused a serious threat to the environment and affect the life of people around the industrial area (Elairayaju & Partha, 2012). In addition, a sago mill produced more than 500 t of effluent that consist of 25 t of fibres and 15 t starch. Also, solid residues that are found in the effluent usually contain around 3% starch and 5% dry matter (Bujang, 2008). These effluents can be hydrolyzed into fermentable sugars for ethanol production.

2.6 Total carbohydrate test

Total carbohydrate test or phenol-sulfuric acid assay are used to determine total carbohydrate in solution (Dubois *et al.*, 1956). The phenol–sulfuric acid method is an efficient colorimetric method to determine total carbohydrates in a sample. This method basically identifies all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides (Nielsen, 2010). The principle for this test is that the carbohydrate was dehydrated to hydroxymethyl furfural in hot acidic media and a yellow- brownish colored product was formed. Then, the absorbance test was conducted at 490 nm using UV spectrometer (Dubois *et al.*, 1956).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials that was used in this study are as follows:

1. Sago effluent (Pusa, Malaysia)
2. Yeast Malt Broth (HiMedia Laboratories, India)
3. Rose-Bengal Chloramphenicol (HiMedia Laboratories, India)
4. Hexane (Reagents, USA)
5. Isopropanol (Amresco, USA)
6. Sulphuric acid (AnalaR, England)
7. Phenol (EMD Millipore, Germany)
8. Glycerol (HmbG, Germany)
9. Hydrochloric acid (AnalaR, England)

3.1 Culture Revival and Stock Culture Preparation

Yeast Malt Broth (YMB) was prepared in 250 ml Schott bottle (SCHOTT, Duran, Germany). Then, the Schott bottle was sterilized using an autoclave (Foundryco Inc., USA) at 121°C for 15 minutes. After the Schott bottles have cooled down, one vial of 2 ml stock culture of *Rhodotorula mucilaginosa* (*R. mucilaginosa*) MV-5 obtained from the Microbiology Laboratory UNIMAS collection was poured into the fermentation media aseptically. Then, it was left for 3 days on a shaker at 150 rpm at room temperature to grow. At day 3, sample from the culture were streaked on Rose-Bengal Dichloran Chloramphenicol (RBDC) agar to obtain pure colony. A single colony was retrieved from the RBDC media culture aseptically and was inoculated in a new YMB media. The growth of the pure colony in the new YMB media was selected to be propagated for the propagation process and to be preserved as pure culture stock. For long term storage, glycerol stock was prepared. Stock culture was aliquoted in YMB supplemented with 20% (v/v) of glycerol and stored at - 80 °C in an ultralow-temperature freezer (Vincent *et al.*, 2011).

3.2 Propagation of *R. mucilaginosa* MV-5 in Fermentation Media

R. mucilaginosa MV-5 was propagated in sterile of 2 L Schott bottle, each consisted of 1.8 L of YMB media. Then, 18 ml (1%) (v/v) of *R. mucilaginosa* MV-5 innoculum was added into each replicate and was propagated for 7 days at ambient temperature. The setup of the experiment is as shown in figure 1.0 and it was placed on a clean sterile bench with no air movement and was static. Shaking of the bottles was done before any sampling was carried out.

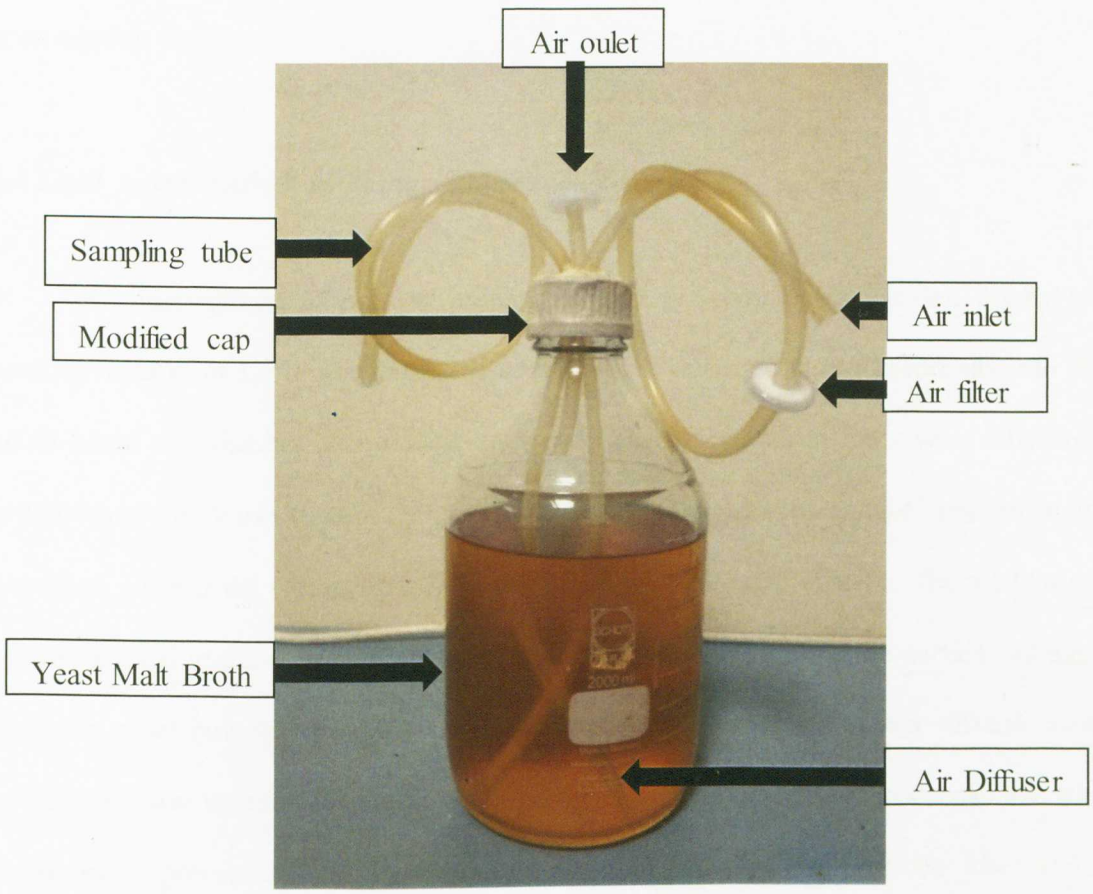

Figure 1.0 Experimental setup for propagation stage

During the sampling period, from day 1 to day 7, 15 ml of the initial sample was taken out through the sampling tube as waste. The next 100 ml of sample was then stored into two 50 ml pre-weighed Falcon tubes. This process was done using a peristaltic pump

(Masterflex Easyload L/S 7518-00, Cole-Parmer, Malaysia) attached to the bottles. The first 50 ml of the sample was centrifuged, and the supernatant was discarded. Then, the second 50 ml of the sample was poured into the first falcon tube containing the pellet and proceed to centrifuge at 4,000 rpm (T40385, Kubota Corporation, Japan). After removing the supernatant, the wet pellet was left to dry in the oven for 48 h at 70 °C. After the pellet have dried, it was weighed. Data of dry cell weight (DCW) against time (hours) was tabulated in the form of graph which marks the growth kinetics of the culture. From the graph that was tabulated, the day with the highest biomass was chosen to be the starting of lipid accumulation stage.

3.4 Lipid Accumulation in *R. mucilaginosa* MV-5

R. mucilaginosa MV-5 was propagated in 2 L Schott bottles at day 7 with total working volume of 1.5 L. Then, 200 mL of the sample was transferred aseptically into four 50 ml falcon tube that has been autoclaved earlier. Then, these falcon tubes were centrifuged at 4,000 rpm for 10 min to form the pellet. The pellet was retrieved, and the supernatant was discarded. Each pellet was resuspended with 25 ml of sterile sago effluent. The total volume of sample used in this experiment was 400 ml therefore this step will be carried out again. Then, the pellet collected from 400 ml sample was poured into 1.6 L of sago effluent Schott bottles. This was repeated for triplicates of bottles. Each day, 100 ml of the sample was taken for sampling process starting from day 0 to day 7 and divided into 2 falcon tubes (50 ml each) and was centrifuged. The wet pellet was obtained for lipid extraction while the supernatant left was taken for total carbohydrate test.

3.5 Acid Pretreatment and Lipid Extraction

After sampling, the first 50 ml of the sample was centrifuged, and the supernatant was discarded. Then, the second 50 ml of the sample was poured into the first falcon tube containing the pellet and proceed to centrifuge at 4,000 rpm. After discarding the supernatant, 10 ml of 4 M Hydrochloric acid was added then it was vortexed to mix the acid and pellet well. Then, the sample was incubated in a water bath for 2 hours at 75 to 80 °C. Next, the pellet was homogenized with Hexane:isopropanol (3:2) (v/v) added into the Falcon tube. The mixture was then homogenized (Superfine Homogenizer, Fluko, China) for 90 seconds with 30 seconds rest in between for every 45 seconds. The homogenized mixture was centrifuged at 4,000 rpm for 25 min and the supernatant was collected in pre-weighed test tubes. Next, the supernatant was heated to 100 °C until all hexane evaporated. Then, it was left in the oven overnight at 70 °C for a constant mass. The remaining lipid was weighed.

3.6 Phenol-Sulphuric Assay

0.2 ml of the supernatant from 1 ml sample was pipetted into triplicates of test tubes respectively. 0.2 ml of 5% (v/v) phenol was added and vortexed, and 1 ml of concentrated sulphuric acid was added rapidly and vortexed. Lastly, 5.6 ml of distilled water was added into the test tube and vortexed. 1 ml of each test tube was taken and was pipetted into each respective cuvette. A blank was prepared by using 0.2 ml of distilled water. Each cuvette was observed under UV spectrophotometer (SP-880, Metertech Inc., Taiwan) set at 490 nm. A graph of absorbance against carbohydrate concentration was plotted.