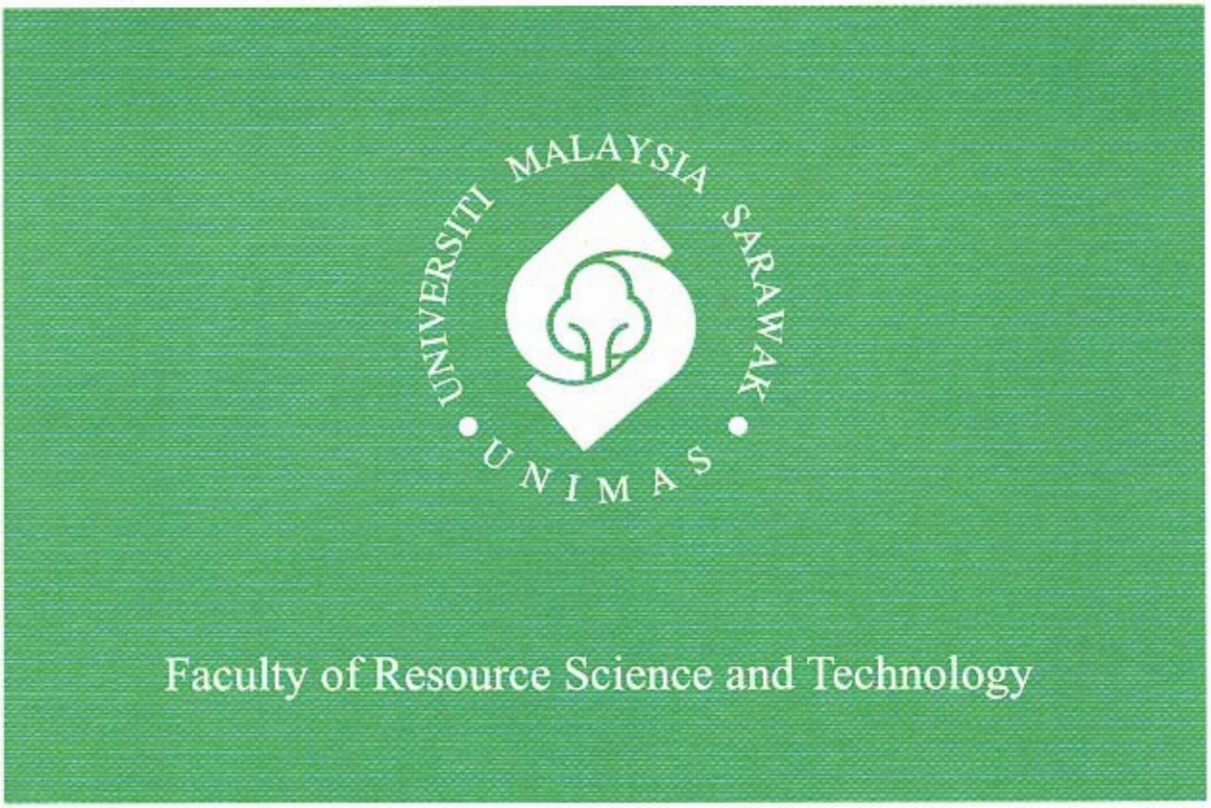

UNCONTROLLED BATCH CULTURE OF *Scenedesmus*

Wong Jing Jing

Bachelor of Science with Honours
(Resource Biotechnology)
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UNCONTROLLED BATCH CULTURE OF *Scenedesmus*

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DECLARATION

I hereby declare that no portion of the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

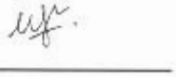

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

°C	Degree celcius
g	Gram
g/ L	Gram per liter
L	Liter
µm	Micrometre
mL	Mililitre
nm	Nanometre
%	Percentage
v/v	Volume over volume
w/v	Weight over volume
DCW	Dry cell weight
DNS	Dinitrosalicylic acid
FPW	Filtered pond water
FSE	Filtered sago effluent
OD	Optical density
PGA	Polyglutamic acid
TSS	Total suspended solids

LIST OF TABLES

LIST OF FIGURES

Uncontrolled Batch Culture of *Scenedesmus dimorphus*

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ABSTRACT

Biodiesel is an alternative to the fossil fuels. The microalgae are potentially used as non-food feedstock for biodiesel production because they have fast growth rate, sustainable in non-arable land, and high lipid content. The objective of this study is to determine feasibility of sago effluent as culture medium to grow *Scenedesmus dimorphus* for biomass and lipid production. In this project, *S. dimorphus* was grown in Proteose medium (control), filtered sago effluent (FSE), and filtered pond water (FPW) amended with 20 g/L $NaHCO_3$.The biomass was harvested after 20 days using polyglutamic acid flocculation method aided by centrifugation. Then, lipid was extracted from the dried biomass using Soxhlet extraction method. After 20 days cultivation, it was found that the biomass of *S. dimorphus* grown in stirred Proteose medium, FSE and FPW (amended with $NaHCO_3$) increased by 36.17%, 102.27%, and 106.45%, respectively. The lipid was 6.88%, 6.17%, and 5.94%, respectively. The results showed that FSE can be potentially utilized as the culture medium to grow *S. dimorphus* for biomass and lipid production. Although the increased of biomass in FSE was much higher (over 100%) compared to the standard medium (Proteose, 36%), the amount of lipid among the three tested was low and almost similar, at about 6%.

Keywords: Biodiesel; Polyglutamic acid flocculation; *Scenedesmus dimorphus*;

ABSTRAK

Biodiesel adalah sumber alternatif kepada bahan bakar fosil. Microalga berpotensi digunakan sebagai stok suapan bukan makanan untuk pengeluaran biodiesel kerana ia mempunyai kadar pertumbuhan yang cepat, mampu hidup dalam kawasan yang tak subur, dan kandungan lipid yang tinggi. Objektif kajian ini adalah untuk menentukan keberkesanan efluen sagu digunakan sebagai media pengkulturan untuk Scenedesmus dimorphus bagi penghasilan biojisim dan lipid. Dalam projek ini, S. dimorphus dikultur dalam media Proteose (kawalan), efluen sagu bertapis (FSE), dan air tasik bertapis (FPW) yang ditambah dengan 20 g/L $NaHCO_3$. Biojisim dituai dengan menggunakan kaedah flokulasi asid poliglutamik dengan bantuan emparan. Selepas itu, lipid diekstrak daripada biojisim yang kering dengan menggunakan kaedah pengekstrakan Soxhlet. Selepas pengkulturan selama 20 hari, kajian mendapati biojisim S. dimorphus yang dikultur dalam media Proteose, FSE, dan FPW telah meningkat sebanyak 36.17%, 102.27%, dan 106.45%, masing-masing. Kandungan lipid adalah sebanyak 6.88%, 6.17%, dan 5.95%, masing-masing. Ini menunjukkan FSE berpotensi digunakan sebagai media pengkulturan S. dimorphus bagi tujuan penghasilan biojisim dan lipid.Walaupun penambahan biojisim dalam FSE adalah lebih tinggi (melebihi 100%) berbanding media piawai (Proteose, 36%), kandungan lipid di antara ketiga-tiga media yang dianalisa itu rendah dan hampir sama, sekitar 6%.

Kata kunci: Biodiesel; Flokulasi asid poliglutamik; Scenedesmus dimorphus;

1.0 INTRODUCTION

The microalgae, *Scenedesmus dimorphus* have fast growth rate, high lipid content and sustainable in non-arable land (Gouveia & Oliveira, 2009). The *S. dimorphus* contains 16-40% lipid in its biomass as reported by Becker (1994). These microalgae are potentially utilized as non-food feedstock for the biodiesel production. Biodiesel are alternative to rapid depleting fossil fuels. Besides, it is more environmental friendly and renewable. The biodiesel production has expanded dramatically in developed and developing countries. Deng *et al.* (2009) pointed out that the production of biodiesel in European Union has increased from 1.9 million metric tons in year 2004 to 7.7 million metric tons in year 2008 based on data obtained from the National Biodiesel Board.

The biggest challenge of biodiesel production from microalgae is the production costs (Deng *et al.*, 2009). Cell harvesting cost contributes at least 20-30% of the total production cost (Gudin & Therpenier, 1986). Thus, the production cost could be reduced by utilizing sago effluent as culture media since sago effluent is abundantly available, especially in Sarawak. Besides, sago effluent contains sufficient nutrients for algal growth (Phang *et al.*, 2000). Utilization of sago effluent as culture medium would reduce environment impacts caused by sago effluent that discharged from sago mills as well.

The objectives of this study are as followed:

1. To determine the feasibility of filtered sago effluent (FSE) as culture medium for biomass production.
2. To compare the lipid productivity of the *S. dimorphus* grown in Proteose medium, FSE and filtered pond water (FPW). The FPW was amended with 20 g/L (best concentration) sodium bicarbonate (Manggi & Bujang, 2009).

2.0 LITERATURE REVIEW

2.1 The Microalgae, *Scenedesmus dimorphus*

2.1.1 Taxonomy

The biodiversity of microalgae is enormous. The microalga *Scenedesmus dimorphus* belongs to the Kingdom Plantae, Phylum Chlorophyta, Class Chlorophyceae, Order Chlorococcales, and Family Scenedesmaceae. In addition, it is belongs to Genus *Scenedesmus*.

2.1.2 Morphologies and Physiologies

The photoautotrophic microalgae, *S. dimorphus* appear in bean shaped with long axis between 10-20 μm (Shen *et al.*, 2009). They normally form colonies of 4, 8, or 16 cells and arranged in a row. Besides, they are green algae that non-motile.

Generally, the microalgae undergo photosynthesis to produce oxygen and biomass. According to Becker (1994), cited in Dermibas (2009), *S. dimorphus* contains 8-18% proteins, 21-52% carbohydrates, and 16-40% lipids. The lipid can be extracted from its biomass and converted into biodiesel through transesterification process.

2.1.3 Sustainability

Gouveia and Oliveira (2009) pointed out that the microalgae can be grown in non-arable land, non-potable water and their production is not seasonal, which can be harvested daily. This could avoid competition of arable lands used to grow food crops (Gouveia & Oliveira, 2009; Shen *et al.*, 2009). Besides, they have short life cycle, which is approximately 1-10

days (Verma *et al.*, 2010). In addition, the microalgae have fast growth rate (Gouveia & Oliveira, 2009). Furthermore, microalgae tolerate to salinity and able to grow in salty water (Shen *et al.*, 2009; Verma *et al.*, 2010). The *S. dimorphus* are freshwater microalgae.

The lipid content of algae usually increases under stress condition, typically nitrogen deficiency (Griffiths & Harrison, 2009; Shen *et al.*, 2009). Under nitrogen limited condition, the biomass growth is often inhibited because the algae stop its division and start to store energy in the form of lipid as a mechanism of survival.

2.1.4 Potential of Algae towards Biodiesel Production

S. dimorphus potentially developed as feedstock for biodiesel production. They have fast growth rate and high lipid content, which is 16-40% in their dry weight (Becker, 1994). The lipid can be extracted from its biomass and converted into biodiesel through transesterification process. The biodiesel is an alternative to fossil fuels because it is more environmental friendly, biodegradable, and renewable.

2.2 Sago

2.2.1 Sago Palm (*Metroxylon Sagu*)

Sago palm is a species belongs to Family Palmae and Genus *Metroxylon*. It is one of the socio-economically important crops in Sarawak, as well as in the regions of South East Asia. As claimed by Ishizaki (1997), cited in Bujang (2009), sago is the highest starch producer among the starch crops of the world, such as rice, corn, wheat, and tapioca.

According to Flach and Schuilling (1989), and Hisajima (1994) studies, cited in Adeni *et al.* (2010), the sago palms have ability to thrive in harsh environment such as swampy, acidic peat soils, submerged and saline soils. The sago palms have very thick stem and grow up to 25 m in height with 40cm in diameter (Adeni *et al.*, 2010).

2.2.2 Sago Starch

The sago starch mainly accumulates in the pith core of the sago palm stem (Bujang & Ahmad, 2000). Sago starch is extracted from the sago trunk using traditional or modern methods. Traditional method is practiced by the farmers at domestic and small scale processing plant level. The sago palm tree is first cut down using axe and its pith is rasped by means of chopper or a small hoe made from bamboo. Then, water is added to the mixture of fiber and pith. Later, the mixture is kneaded by hand or trampled by foot. After kneading, the wet starch is collected.

On the other hand, the modern method of sago starch extraction involves some modifications of small scale processing plant and integrated with technologies. The sago trunk is first debarked, followed by rasping. The sago pith is rasped using a hammer mill. Then, the resulting starch slurry is passed through a series of centrifugal sieves to separate the coarse fibers. Next, the sago starch is purified using nozzle separator. Later, the sago starch is dewatered using a rotary drum dryer, followed by hot air drying.

The processing of sago starch produces abundant amount of solid residues and liquid wastes. The solid residues are sago bark and fibrous pith residue, which is also known as '*hampas*'. In addition, huge amount of wastewater is discharged from sago processing mills. As stated by Bujang *et al.* (1996), a minimum of 400 tons of wastewater, containing 5% solids (20 tons) is generated daily from a typical sago mill.

According to Swinkels (1985), cited in Singhal *et al.* (2008), sago starch contains 27% amylase and 73% amylopectin. Sago starch is used in food and non-food industries. In biotechnology, sago starch potentially utilized in the production of ethanol, lactic acid, kojic acid, and fermentable sugars.

2.2.3 Sago Effluent

According to Phang *et al.* (2000), cited in Manggi and Bujang (2009), the sago effluent contains sufficient nutrients for algal growth. The sago effluent contains starch and glucose. Phang *et al.* (2000) reported that the sago effluent released from sago processing mill contains high carbon to nitrogen ratio (105: 0.12) and suitable for anaerobic fermentation.

In addition, Sivaraman and Thamizhiniyan (2010) reported that the sago effluent was acidic in nature and pale white color. Besides, they also reported that the sago effluent was rich in total suspended solids (TSS) and with high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values. The Extended Aeration (EA) system has a potential to be used as sago effluent treatment unit. The EA system, which consists of physical and biological treatment units, could reduce BOD, COD, and TSS up to 84%, 87.80%, and 73% respectively (Rashid *et al.*, 2010).

2.3 Cultivation Method

2.3.1 Batch Culture

According to Lee and Shen (2004), batch culture involves incubation of algal with limited

amount of complete culture medium under favorable environment in the culture vessel, such as conical flask or fermentor. Batch culture is a closed system. The culture medium is only added at the initial of the experiment. The culture is agitated to ensure nutrient and gaseous exchanged occur at the cell-water interface (Lee & Shen, 2004). The pattern of growth curve for batch culture method is sigmoid shape.

2.3.2 Advantages and Limitation of Batch Culture

There is no wastage of culture medium since the substrates are completely utilized. Besides, it requires low maintenance. The risk of contamination is also very low since it is closed system. However, high amount of labor required in order to start, end, and restart the cultivation.

2.4 Cell Harvesting Methods

2.4.1 Flocculation

Grima *et al.* (2004) states that flocculation is the collection of cells into aggregate mass by addition of polymers. Polyglutamic acid (PGA) is a polymer of glutamic acid, which can act as flocculant agent. Aggregated microalgae cells ease the sedimentation process.

2.4.2 Centrifugation

Centrifugation can separate microalgae biomass from the culture medium (Grima *et al.*, 2004). However, the costs and energy demands to harvest biomass by this method is high. This method is more problematic at a large scale as the capitals costs increase with scale.

2.5 Oil Extraction Methods

2.5.1 Physical method

The physical method involves mechanical crushing to disrupt the cells. The oil is extracted out from algae using expeller or press, homogenizer, bead mills, and ultrasound. According to Demirbas (2009), 70-75% of oil is extracted out from algae using a press.

2.5.2 Chemical method

The cell disruption is required to extract primary metabolite, which is lipid. Cell disruption can be accomplished using Solvent extraction, Soxhlet extraction or supercritical fluid extraction. For Solvent extraction, hexane is usually used because it is relatively inexpensive (Demirbas, 2009). Shen *et al.* (2009) claimed that the most effective method of lipid extraction for *S. dimorphus* was wet milling followed by hexane extraction. This method is more effective compared to bead-beater, French press, and sonication in their studies. Besides, Shen *et al.* (2009) reported that the lipid discovery from *S. dimorphus* using hexane solvent was higher than hexane/ethanol solvent in Solvent system.

2.6 Biodiesel

2.6.1 Advantages of Biodiesel

Biodiesel is also known as methyl ester. It is an alternative to fossil fuels as source of energy due to the dwindling of fossil fuel reserves. It is renewable, biodegradable, and environmental friendly.

2.6.2 Biodiesel Feedstocks

The feedstocks for biodiesel production include waste cooking oil, animal fats, and oleaginous crops, such as rapeseed, soybean, sunflower, and palm. Besides, some microalgae such as *S. dimorphus* potentially utilized as non-food biodiesel feedstocks.

2.6.3 Biodiesel Production

Biodiesel is produced through transesterification process of triglyceride with short chain alcohols such as methanol and in the presence of a catalyst to produce methyl ester and glycerol as a byproduct. Generally, transesterification involves three reversible steps, where triglyceride is converted to diglyceride, followed by the conversion of diglycerides to monoglycerides, and the conversion of monoglyceride to methyl ester or biodiesel. Transesterification process was shown in **Figure 1**.

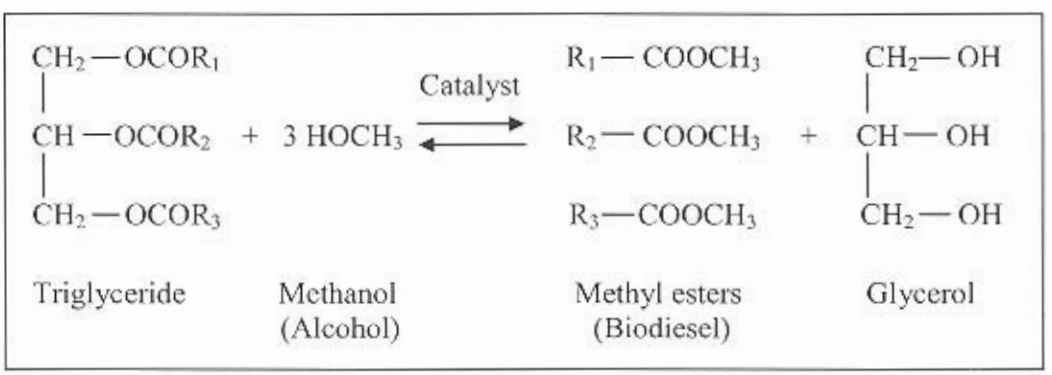

Figure 1: Transesterification of triglyceride into biodiesel.

3.0 MATERIALS AND METHODS

3.1 Stock Culture of Microalgae, *S. dimorphus*

The stock culture of *S. dimorphus* was obtained from University of Texas at Austin, USA. The microscopic examination was performed prior to use to ensure the stock culture was free from contamination. The optical density (OD) of the stock culture was measured using Biochrom Libra S12 spectrophotometer at the wavelength of 560 nm. One mL of the stock culture was pipetted into the cuvette and placed in the holder of spectrophotometer to obtain the reading. The reading was recorded.

Ten percent (v/v) inoculum of stock culture was grown in 90% (v/v) culture media. The culture media used were Proteose medium, filtered sago effluent (FSE), and filtered pond water (FPW).

3.2 Culture Media

3.2.1 Proteose Medium

Ten percent (v/v) stock culture of *S. dimorphus* was grown in 90% (v/v) Proteose medium as a control. The Proteose medium was prepared by adding 1 g/L of protease peptone into Bristol medium and then autoclaved. The components of Bristol medium were stated in **Appendix A**. The pH of the Proteose medium was adjusted to approximately pH 9 prior to use.

3.2.2 Filtered Sago Effluent (FSE)

The sago effluent was obtained from Herdsen Sago Mill at Pusa, Sarawak. The samples were stored at 4°C prior to use. The sago effluent was allowed to sediment first. Then, it was filtered using U. S. Standard Sieve Series with a final mesh size of 63 µm filter as shown in **Figure 2**, to obtain filtered sago effluent (FSE). Next, the pH of FSE was adjusted using 1 M sodium hydroxide, NaOH to approximately pH 9.

Figure 2: The sago effluent filtration process.

3.2.3 Filtered Pond Water (FPW)

The pond water was collected from the lake at west campus of University Malaysia Sarawak (UNIMAS), Kota Samarahan, Sarawak. The pond water was allowed to sediment. Then, the pond water was filtered as before, to obtain filtered pond water (FPW). Next, the FPW was amended with 20 g/L sodium bicarbonate, $NaHCO_3$. After that, the pH of the FPW was adjusted using 1 M sodium hydroxide, NaOH to approximately pH 9.

3.3 Cultivation Parameters

The *S. dimorphus* was grown in three different culture media, which were Proteose medium (control), FSE, and FPW. Ten percent (v/v) inoculum of stock culture was added into 90% (v/v) of each culture medium. The cultures were grown for 20 days continuously using batch culture method.

Besides, the cultures were grown in stirred and unstirred or static condition throughout the cultivation period. Three sets of the cultures grown in stirred condition were stirred constantly at 550 rpm using magnetic stirrer, as shown in **Figure 3**. These cultures were placed near to a fluorescent light. The fluorescent light was set up with a timer and adjusted to be 12 hours ON/OFF. Meanwhile, another three sets of cultures were unstirred throughout an experiment, as shown in **Figure 4**. These cultures were placed at sunlight coverage area.

Furthermore, the initial pH of culture media for all replicates was adjusted to approximately pH 9. Then, the stock culture was added into pH adjusted culture media. The pH and temperature of the cultures were uncontrolled throughout an experiment.

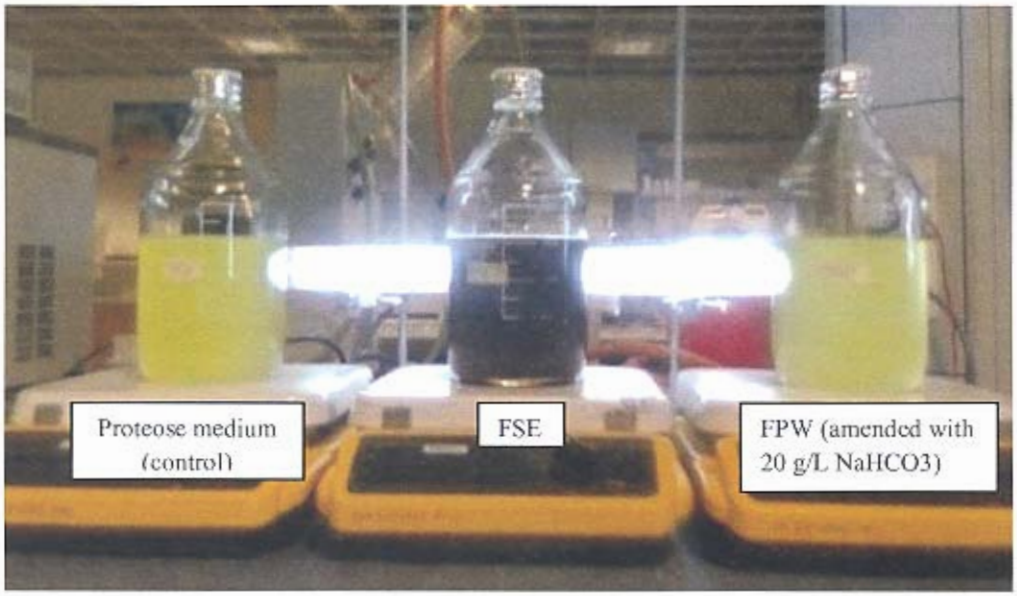

Figure 3: *S. dimorphus* grown in stirred condition.

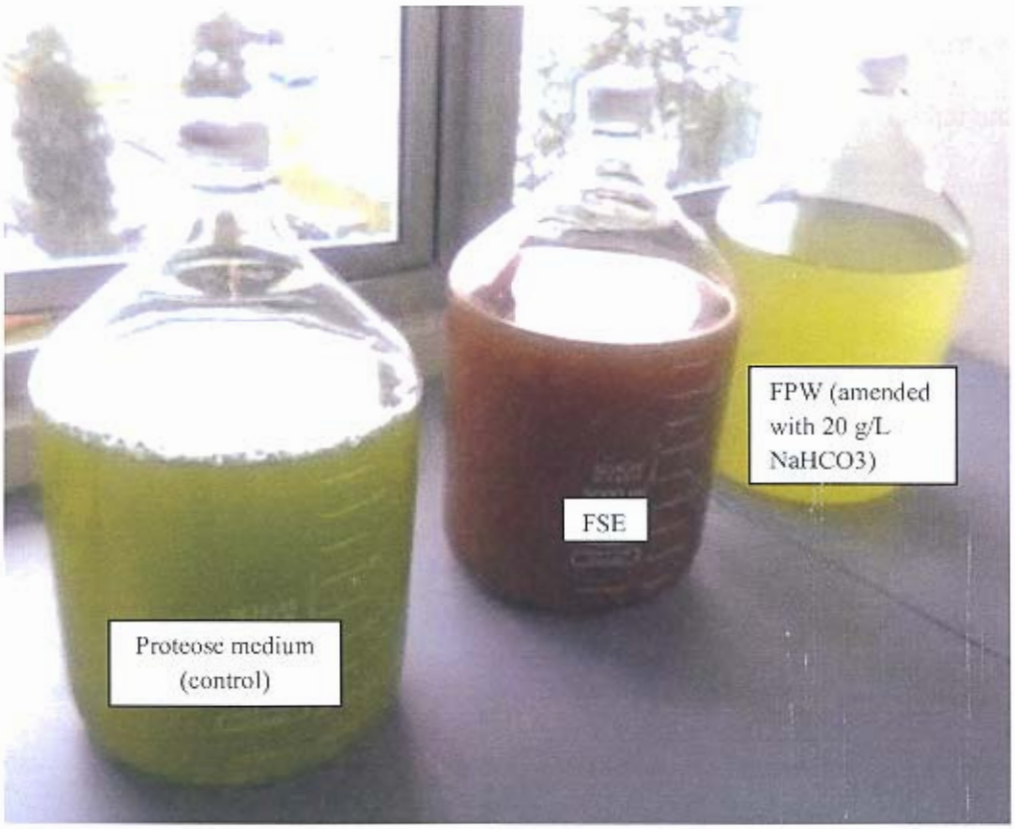

Figure 4: *S. dimorphus* grown in unstirred or static condition.

3.4 Sampling

Sampling was performed on days 0, 5, 10, 15, and 20. About 50 mL sample was extracted from each experiment for all analyses.

3.5 Harvesting

Biomass of each treatment was harvested on day 20. The biomass was separated from its culture medium using polyglutamic acid (PGA) flocculation method with an aid of centrifugation. 0.5 g/L of PGA was added into each culture medium and shook vigorously to mix them. Then, the mixtures were filled into 250 mL centrifuge bottles and centrifuged using high speed refrigerated centrifuge, Himac CR 21G. The samples were centrifuged at 8000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discharged and the pellet which comprised of biomass was collected. The biomass harvested from each treatment was transferred into crucible separately and labelled. Next, all of the biomass harvested dried in the oven at 60°C for 2-3 days.

3.6 Lipid Extraction

Lipid extraction was performed using dried biomass of each treatment. Firstly, the dried biomass was weighed. Then, the dried biomass was grinded into powder form using mortar and pestle. The lipid extraction was performed using Soxhlet extraction method, as shown in **Figure 5**. The glass Soxhlet extractor was set up with a condenser and 500 mL round bottom flask. The round bottom flask was filled with 350 mL of hexane solvent. Meanwhile, the dried biomass was filled into the thimble and covered with cotton on the top. The thimble was placed in the Soxhlet extractor. Then, the hexane solvent in the round

bottom flask was heated to reflux for 24 hours. After that, the solvent was vaporized from the lipid extracted using Heidolph rotary evaporator at 40°C under reduced pressure, as shown in **Figure 6a** and **Figure 6b**.

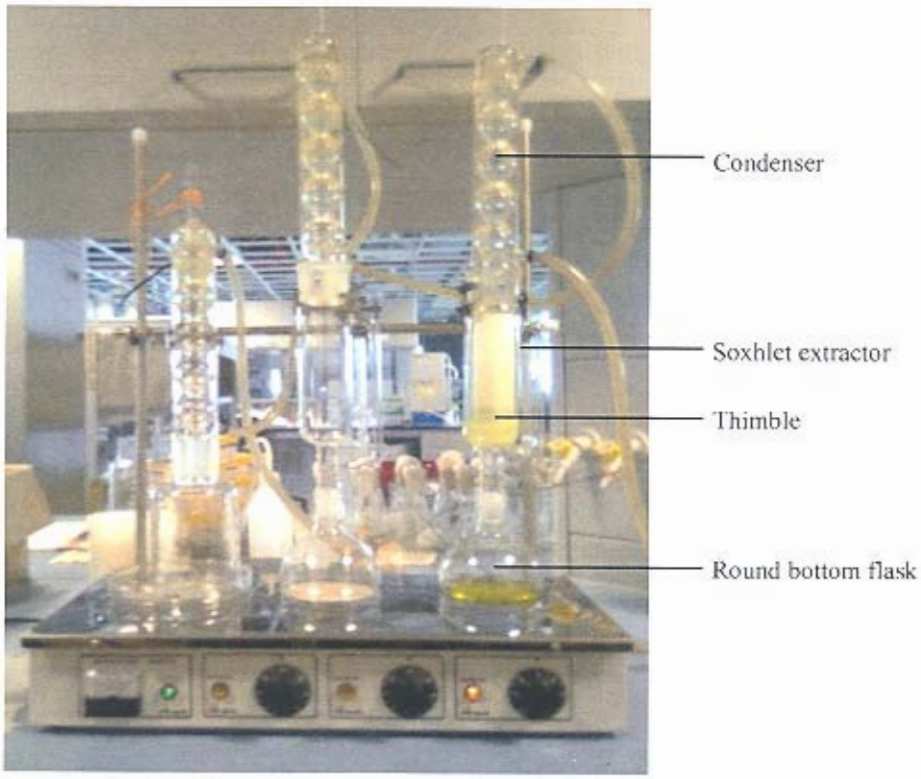

Figure 5: Lipid extraction using Soxhlet extraction method.

Figure 6a & 6b: Vaporization of hexane from the lipid extracted using rotary evaporator.