Faculty of Resource Science and Technology

Cultivation and lipid extraction of *Scenedesmus* sp. from UNIMAS Lake and *Scenedesmus dimorphus* from Texas

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Bachelor of Science with Honors
Aquatic Resource Science and Management
2014

DECLARATION

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

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ACKNOWLEDGEMENT

Bismillahhirrahmanirrahim.

The work included in this research has been carried out at the department of Aquatic Science, Faculty of Resource Science and Technology, University Malaysia Sarawak (UNIMAS) from September 2013 to May 2014.

Firstly, I thank Allah S.W.T. for allowing me and giving me the opportunity to do this project and to finish it, and always giving me hope and bring me back up whenever I'm in lost or feeling down. I would like to thanks to my supervisor, Associate Professor Dr. Norhadi Ismail for his guidance, opinions, comments and encouragement he gave throughout finishing this project. Also, great appreciation to all lecturers, including my ex-examiner Dr. Siti Akmar whom when my supervisor was away she gave me opinions and motivations. Also thanks to my examiner Dr. Fazimah Azli who had encouraged me and giving me ideas.

I would like to dedicate precious acknowledgement to lab mates and lab assistant En. Zaidi, En. Azlan, En. Zul, and En. Richard that has been helping me and guiding me with the lab equipment throughout my project, thank you very much again.

Thanks to my three best friends, Diyana Azhari, Melissa D. Chong and Anis Shahira who contributed a lot during the outdoor culture of my project. Big thanks go to my roommates, Siti Maryam Mohamad and Siti Nurhirdayu Rahmat who have supported me emotionally and being an understanding friend whenever I'm busy and stressed out, also helping me in little things. I hope for the best in your FYP. I am very grateful to my friends, we walked down this path together while holding hands and giving strength to each other. I will miss you guys.

Finally big thanks, kisses and hugs to both of my parents Mrs. Sharifah Norkaya and Mr. Yamin Mahali, also my baby brother Wan Amier Ashraf for their support, prayers, motivation and nagging which had helped me go through the pains, sweats and tears throughout the days of finishing this project. I also thank Allah S.W.T. for that and everything.

TABLE OF CONTENT

	ACKNOWLEDGEMENT	I
	TABLE OF CONTENT	III
	LIST OF ABBREVIATIONS	V
	LIST OF TABLE	VI
	LIST OF FIGURES	VII
	ABSTRACT	VIII
1.0	INTRODUCTION	1
2.0	LITERATURE REVIEW	3
2.1	Microalgae vs. Macroalgae	3
2.1.1	Microalgae	3
2.1.2	*Scenedesmus* sp.	4
2.2	Lipid Content in Microalgae	5
2.3	Microalgae as Biofuels	6
2.4	Batch Culture Method	6
2.5	Outdoor Cultivation of Green Microalgae	8
2.6	Animal Manure as Fertilizers	9
2.7	Growth Curve of Microalgae	9
2.8	Microalgae Culture Harvesting	11
2.8.1	Chemical Flocculation	11
2.8.2	Centrifugation	11
3.0	MATERIAL & METHOD	12
3.1	Preparation of Stock Solution	12
3.2	Preparation of Microalgae Culture Medium, Modified Bristol's Medium (MBM)	13
3.3	Preparation of Animal Manure Stock Medium	13

3.4 Cell Growth Measurement 14
3.5 Outdoor Mass Cultivation 15
3.6 Harvesting 15
3.7 Biomass Dry Weight Measurement 15
3.8 Lipid Extraction 16
3.9 Statistical Analysis 17
4.0 RESULTS 18
4.1 Indoor Cultivation 18
4.1.1 Growth Rate of Microalgae Cultured Indoors. 19
4.2 Outdoor Cultivation 20
4.2.1 Growth Rate of Microalgae Cultured Outdoors 22
4.3 Light Intensities Range 23
4.4 Observation of Cell Shape and Structure 24
4.5 Water Content and Lipid Yield 25
5.0 DISCUSSION 26
5.1 Effect of Nitrogen towards cell densities 26
5.2 Effect Nitrogen and light towards lipid content in microalgae 26
5.3 Effect of light and temperature towards cell growth 27
5.4 Efficiency of harvesting and extraction method on lipid yield 28
5.5 Comparison of lipid yield (%dry weight) from other studies. 28
6.0 CONCLUSION 30
7.0 REFERENCES 31
8.0 Appendices 34

LIST OF ABBREVIATIONS

$CaCl_2 \cdot 2H_2O$	Calcium chloride dehydrate
CO2	Carbon dioxide
dH_2O	Distilled water
g	gram
h	hour
H_2O	Water
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
L	Litre
mg	milligram
ml	millilitre
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate
MBM	Modified Bristol's Medium
N	Nitrogen
NaCl	Sodium chloride
$NaNO_3$	Sodium Nitrate
NO_3	Nitrate
PGA	Polyglutamic acid
PUFA	Polyunsaturated fatty acid
UNIMAS	Universiti Malaysia Sarawak
μm	micrometer
℃	Degree Celsius

LIST OF TABLES

Table 1	Comparison of some sources biodiesel	6
Table 2	Chemical composition of Modified Bristol's Medium (MBM)	13
Table 3	Water content in biomass of *Scenedesmus* from different source	25
Table 4	The percentage of lipid yielded from *Scenedesmus* sp. of UNIMAS Lake	25
Table 5	Comparison of lipid yield (%dry weight) from other studies	29

LIST OF FIGURES

Figure 2.1	*Scenedesmus dimorphus* colony cells.	4
Figure 2.2	Transesterification of oil to biodiesel.	5
Figure 2.3	Batch Culture Scheme	7
Figure 2.4	Average Nutrient Analyses of Major Types of Manure in Oklahoma	9
Figure 2.5	Growth Curve of Microalgae	10
Figure 3.1	Soxhlet Lipid extraction method	16
Figure 4.1	Growth curves of *Scenedesmus* sp. from Lake UNIMAS and University of Texas during the indoor cultivation	18
Figure 4.2	Growth rate of *Scenedesmus* sp. from UNIMAS Lake and University of Texas	19
Figure 4.3	Growth Curves of S*cenedesmus* sp. cultivated in 3 outdoor tanks	21
Figure 4.4	Growth rate of *Scenedesmus* sp. from UNIMAS Lake and University of Texas cultivated in 3 outdoor tanks	22
Figure 4.5	Ranges of light intensities.	23
Figure 4.6	The photo on the left is a colony of *Scenedesmus* sp. from UNIMAS Lake, the photo on the right is a colony of *Scenedesmus dimorphus* from Texas during indoors cultivation.	24
Figure 4.7	The photo on the left is a colony of *Scenedesmus* sp. from UNIMAS Lake, the photo on the right is a colony of *Scenedesmus dimorphus* from Texas during outdoor cultivation.	24

Cultivation and Lipid Extraction of *Scenedesmus* sp. From UNIMAS Lake and *Scenedesmus dimorphus* From Texas

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ABSTARCT

The search for a more sustainable and renewable biofuels feed sources has drastically increased. One of the sources that have received much attention is microalgae as cultivation of this organism does not require much land and is less expensive. Their cultivation can also be carried out under natural condition such as tanks or open pond production systems. The current research aims to conduct a study on the mass production and lipid yield of green microalgae, *Scenedesmus* sp. which was previously isolated from a lake in UNIMAS East Campus and *Scenedesmus dimorphus* which was purchased from the University of Texas. The microalgae were cultured indoors in 2 L and 9 L carboys in Modified Bristol's Medium (MBM) and then were cultured outdoors in 3 tanks which volume was about 351.523 L. All of the cultured were enriched with chicken manure with concentration of 3.5%. The dry weight of *Scenedesmus* sp. and *Scenedesmus dimorphus* obtained from outdoor cultivation were 20.88 g and 36.98 g respectively. The lipid yield produced by *Scenedesmus* sp. of UNIMAS Lake was 38.17% of the dry weight. The lipid yield by *Scenedesmus dimorphus* could not be obtained.

Keywords: Scenedesmus sp., outdoor mass cultivation, lipid yield.

ABSTRAK

Usaha pencarian sumber biofuel yang lebih tahan dan boleh di perbaharui telah meningkat dengan drastik. Salah satu sumber yang menjadi perhatian adalah mikroalga, Penanaman organisma ini tidak memerlukan banyak ruang dan tidak mahal. Penanaman tersebut juga boleh dilakukan secara semula jadi seperti akuakultur dalam tangki atau sistem kolam terbuka. Penyelidikan ini bertujuan untuk mengkaji hasil pengeluaran secara besar-besaran dan lipid mikroalga hijau, iaitu Scenedesmus sp. *yang sebelum ini telah diasingkan daripada Tasik UNIMAS Kampus Timur dan Scenedesmus dimorphus yang telah dibeli daripada Universiti Texas. Mikroalga tersebut telah dikultur dalam ruang bilik di dalam carboy sebanyak 2 L dan 9 L dalam media Modified Bristol Medium (MBM) dan kemudian telah dikultur di kawasan luar di dalam 3 tangki berukuran kira-kira 351.523 L. Kesemua kultur dicampur dengan baja tahi ayam sebagai sumber nitrogen dengan kepekatan 3.5%. Berat kering Scenedesmus* sp. *dan Scenedesmus dimorphus diperolehi daripada kultur di kawasan luar adalah 20.88 g dan 36.98 g. Hasil lipid yang kering Scenedesmus sp. daripada Tasik UNIMAS adalah 38.17%. Hasil lipid Scenedesmus dimorphus tidak dapat diperolehi*

Kata kunci*: Scenedesmus* sp., *kultur besar-besaran luar, hasil lipid.*

1.0 INTRODUCTION

Fuel represents around 70% of the total global energy requirements, particularly in transportation, manufacturing and domestic heating (Mata *et al*., 2010). The seeking for a more sustainable and renewable fuel is becoming greatly important as a direct result of the rising of fossil fuel prices and climate change (Gulab Chand *et al*., 2012). Biofuel offers new opportunities to diversify fuel supply sources for long term replacement of fossil fuels along with carbon sequestration (Jena *et al*., 2012). The study of microalgae has revealed that the lipid content of this organism can be the substitute for the second generation biodiesel fuel at the same time fossil fuels (Jena *et al*., 2012).

Generally, many algal species are rich in oils (Huang *et al*., 2010). The lipid content analysis of microalgae showed potential properties of biodiesel. Mass production of microalgae species such as *Scenedesmus* sp. and their lipid extraction is aimed for their lipid.

In the past several years, there is an increase in production of biodiesel. It is because the petroleum reserves are to be depleted in less than 50 years at the present rate of consumption (Huang *et al*., 2010). The advantages of this third generation biodiesel is that it has several favorable environmental properties resulting in no net increased release of carbon dioxide and very low sulfur content (Antolin *et al*., 2002) and the gas generated during combustion could be reduced. For example, carbon monoxide will decrease is owing to the relatively high oxygen content in biodiesel (Huang *et al*., 2010).

Other than that, microalgae grow extremely rapidly and many algal species are rich in oils (Huang *et al*., 2010). For examples, heterotrophic growth of *Chlorella protothecoides* can accumulate lipids as high as 55% of the cell dry weight after 144 h of cultivation with

feeding of corn powder hydrolysate in fermenters (Xu *et al.*, 2006) and that oil levels of 20-50% are common in microalgae (Chisti, 2007). However, not much study is done on cultivation of green microalgae by using chicken manure as organic nitrogen source and at the same time growing under normal conditions.

This research attempts to determine whether cultivation under natural conditions and chicken manure as organic nitrogen source optimizes the lipid yield of this organism. The previously isolated green microalgae, *Scenedesmus* sp. from UNIMAS Lake and *Scenedesmus dimorphus* from the University of Texas through mass culture method will be establish. The main objective is to produce mass culture of this organism by outdoor cultivation and to extract their lipid content and finally quantify and compare the lipid yielded through this method.

2.0 LITERATURE REVIEW

2.1 Microalgae vs. Macroalgae

Microalgae are small microscopic aquatic photosynthetic plants that require the aid of a microscope to be seen. They do not have roots, stems or leaves and are able to perform photosynthesis which is vital to life on earth as they produce around half of the oxygen found in the atmosphere. It is easy to differentiate between microalgae and macroalgae because they possessed distinctive features. The macroalgae are large aquatic photosynthetic plants that can be seen without the aid of microscope. Macroalgae are not true plants but are actually large celled algae. They are photosynthetic and serve as the base for aquatic food web, providing oxygen and habitat for aquatic inhabitants (Shawn, 2012).

Both macro and microalgae contain lipid as their storage product. They also have high photosynthetic efficiency to produce biomass and have higher growth rates and productivity compared to the conventional crops (Carvalho *et al.*, 2011).

2.1.1 Microalgae

Microalgae are found all over the world and are distributed mainly in water bodies. They have different types of cell organization such as unicellular, filamentous and colonial. Microalgae which are unicellular are mostly immotile. Motility is only occurred in cells with flagella (Richmond, 2004). A few microalgae that have been reported to be used for culture are *Chlorella*, *Dunaleilla*, *Scenedesmus*, *Spirulina*, and *Prophyridium*. For other purpose, microalgae are also cultured for the use as live feed in aquaculture for the larval and juvenile stages of fish, shellfish and crustaceans (Borowitzka & Borowitzka, 1988).

2.1.2 *Scenesdesmus* sp.

Scenedesmus sp. is a freshwater green microalgae from the class Chlorophyceae. It is in bean shaped with the size of approximately 10 μm. The cells of *Scenedesmus* sp. usually are arranged in a row of 4 or 8 celled colonies. 2 to 16 celled colonies can occur and very rarely there are more than 16 cells per colony (Borowitzka & Borowitzka, 1988). The genus *Scenedesmus*, containing more than 200 species, appears to be somewhat heterogeneous. In order to distinguish the two genera, Komarek & Fott (1983) has differentiated the genera into two subgenera; spiny species (subgenus *Desmodesmus*) and spineless species (subgenus *Scenedesmus*). In 1978, Hegewald further divides the spineless species into those with spindle-shaped cells and acute poles (subgenus *Acutedesmus*) and those of a more or less ellipsoidal cell shape with obtuse or truncate cell poles, which are also characterized by mucilage production (subgenus *Scenedesmus*) (Borowitzka & Borowitzka, 1988).

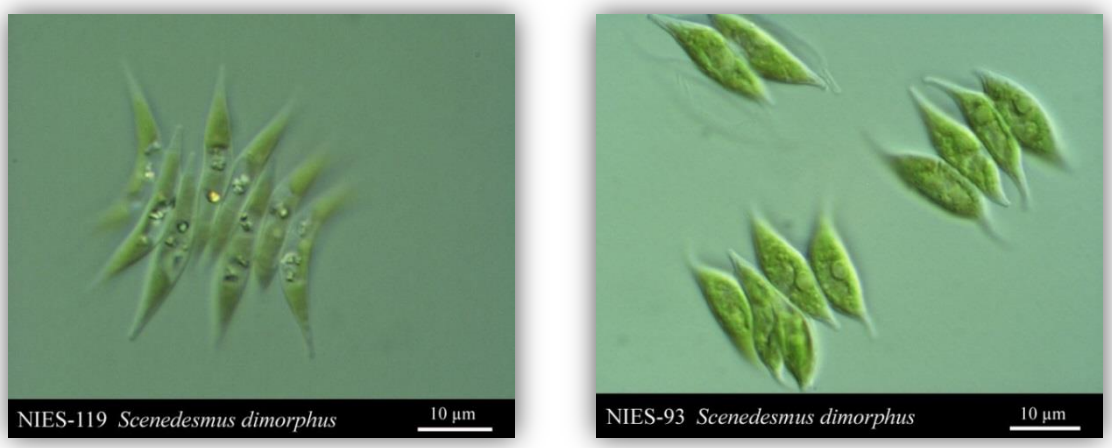

Figure 2.1: *Scenedesmus dimorphus* colony cells. (Source NBRP-Algae, 2009)

According to Jena *et al.* (2012), *Scenedesmus* sp. showed the highest lipid content among two other tested microalgae namely *Chlorococcum* sp. and *Chlorella* sp., *Scenedesmus* sp. had lipid of about 24% per dry weight at their early stationary phase of growth.

2.2 Lipid Content in Microalgae

The lipids are produced when both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate etc.) were utilized by microalgae (Huang *et al*., 2010). The contents and component of lipids in microalgae cells differs from species to species. The process in which lipids are produced into biodiesel in called Transesterification. Triglycerides in oil consist of three fatty acid molecules are esterified with a molecule of glycerol. In making biodiesel, triglycerides are reacted with methanol in a transesterification or alcoholysis. Transesterification produces methyl esters of fatty acids, which are biodiesel and glycerol. The reaction occurs in these steps: triglycerides are first converted to diglycerides, then to monoglycerides and finally to glycerol (Chisti, 2007).

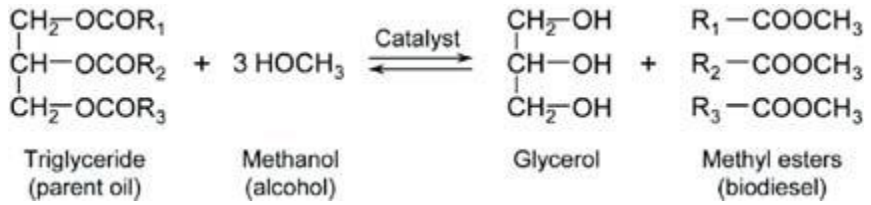

Figure 2.2: Transesterification of oil to biodiesel. $R_{1\text{-}3}$ is hydrocarbon groups (Chisti, 2007).

2.3 Microalgae as Biofuels

Biodiesel is an alternative fuel for conventional diesel that is made from natural plants oils, animal fats, and waste cooking oils (Gulab Chand *et al*., 2012). The third generation biodiesel which are derived from microalgae is the most suitable alternative to petroleum diesel fuel because it is renewable and ecofriendly. It has been said to be the most promising alternative sources of lipid for the use in biodiesel production. It burns in conventional diesel engines with or without any modification or can be used as a blend with petrodiesel, exhibiting lower exhaust than conventional diesel fuel (Mandal & Mallick, 2012).

Table 1: Comparison of some sources biodiesel (Chisti, 2007)

Crop	**Oil Yield (L/ha)**	**Land are needed (M ha)[a]**	**Percent of existing US cropping area**
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Micoalgae [a]	136,900	2	1.1
Microalgae [b]	58,700	4.5	2.5

[a] 70% oil (by wt) in biomass.
[b] 30% oil (by wt) in biomass.

Based on Table 1, microalgae have the least land needed for its cultivation and have the most oil yield. This is because microalgae are the fastest growing plants in the world and its mass production is not impossible.

2.4 Batch Culture Method

Batch culture method is the type of culture where the total culture is harvested and used (Laing, 1991). They consists of a single inoculation of cells into a container of fertilizes media followed by growing period of several days, and lastly harvesting the biomass as its

reaches maximum density. The algae are transferred to larger culture volumes prior to reaching the stationary phase then the cycle continues; the larger culture volumes are brought to a maximum density and harvested (Lavens & Sorgeloos, 1996).

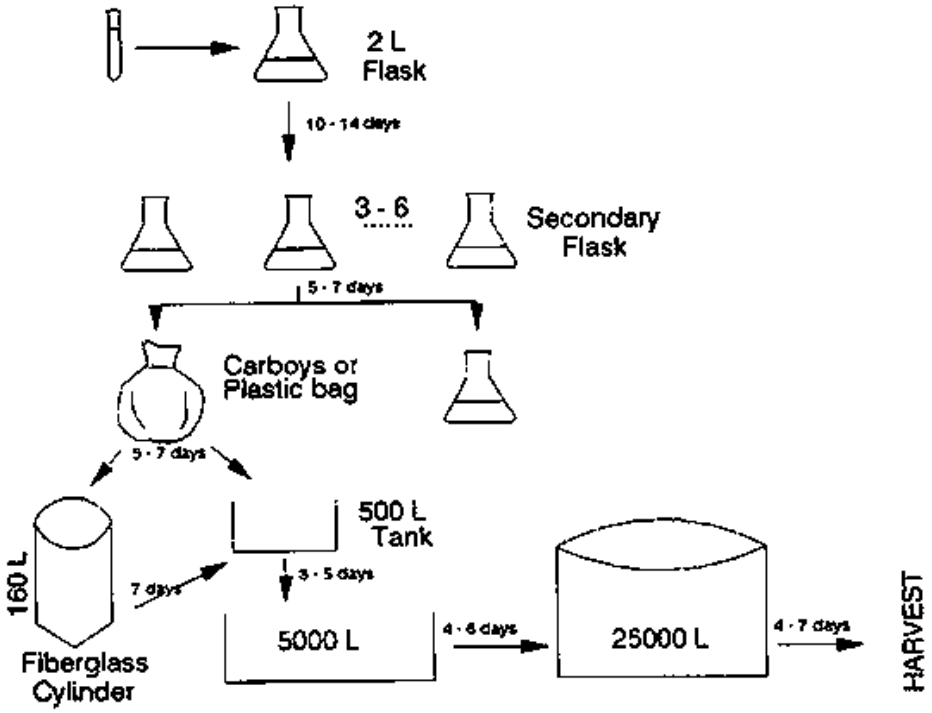

Figure 2.3: Batch Culture Scheme (Lavens & Sorgeloos, 1996)

Consecutive stages are utilized for the batch culturing of microalgae by starting in test tubes then into 2 L flask, 5-20 L carboys, 160 L cylinders, 500 L indoor tanks, and 5000 L to 25000 L outdoor tanks (Lavens & Sorgeloos, 1996).

2.5 Outdoor Cultivation of Green Microalgae

Outdoor cultivation allows algae to grow under natural conditions such as the algae absorb sunlight, and assimilate carbon dioxide from the air and nutrients from the aquatic habitats (Brennan & Owende, 2010). The use of natural conditions for commercial algae production has the advantage of using as a free natural resource (Janssen *et al*., 2003). Microalgae can fix and assimilate CO_2 from the atmosphere (Brennan & Owende, 2010). Other inorganic nutrients required for algae production include nitrogen, phosphorus and silicon (Suh & Lee, 2003). Some species can fix nitrogen from air in the form of $NO_{(x)}$ but most microalgae require it in a soluble form with urea being the best source (Hsieh & Wu, 2009).

There are a few mechanisms in which microalgae can grow and reproduce. They can grow as photoautotrophic, heterotrophic and mixotrophic, in which all follow the natural growth processes. Photoautotrophic production is autotrophic photosynthesis; whereas heterotrophic production requires organic substances (e.g. glucose) to stimulate growth, while some algae strains can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a mixotrophic process.

2.6 Animal Manure as Fertilizers

Animal manure is a by-product which contains many nutrients and organic matter for plants. Instead of being a problem, animal manure can be an advantage for producers if they are affectively managed and properly used (Zhang, n.d.).

Manure Type	Dry Matter %	Total N	P_2O_5	K_2O
		lbs./ton		
Feedlot Manure	62	24	21	25
Broiler Litter	77	63	61	50
		lbs./1000gal		
Lagoon Effluent	0.5	4.2	1.0	5.0
Lagoon Sludge	7	15	16	11
Dairy Slurry	3	13	11	11

*P_2O_5 and K_2O are commonly used for fertilizer ingredients instead of P and K. Some laboratories may still report in elemental P and K content. To convert, use the following equations: $K_2O = K \times 1.2$ or $P_2O_5 = P \times 2.29$

Figure 2.4: Average Nutrient Analyses of Major Types of Manure in Oklahoma (Zhang, n.d.)

2.7 Growth Curve of Microalgae

The growth of a microalgae culture is characterized by five phases, lag phase, exponential growth phase, declining relative growth, stationary phase, and lastly death phase (Figure 2.5).

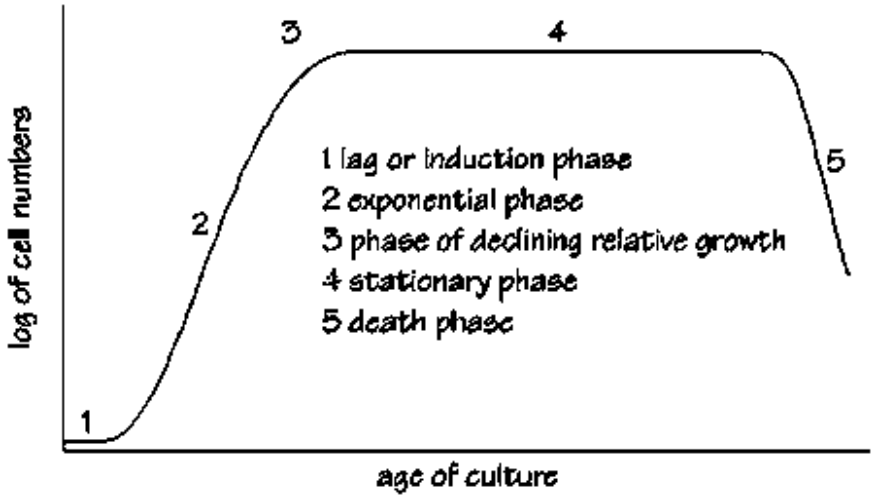

Figure 2.5: Growth Curve of Microalgae (Lavens & Sorgeloos, 1996)

In the lag phase the condition of the inoculum has a strong bearing. If an inoculum is taken from a healthy exponentially growing culture, when transferred to a fresh medium under similar conditions there might be no lag phase. However lag phase may occur if inoculum is transferred from one set of growth conditions to another (Australian National Algae Culture Collection, 2006).

During the exponential phase the cell density increases as a function of time (t) according to a logarithmic function (Lavens & Sorgeloos, 1996). The growth rate of the microalgae culture is a measure of the increase in biomass over time. Growth rate is an important way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed to it. At the third phase which is the phase of declining relative growth. The declining growth commonly happens when either any specific requirement for cell division is limiting or something else is inhibiting the reproduction of algae such as nutrients, pH, light, carbon dioxide. At stationary phase is where the net growth is zero (Australian National Algae Culture Collection, 2006). The limiting factor and the growth rate are balanced, and therefore results in a relatively

constant cell density. The last phase is the death phase or 'crash' phase where the water quality of medium deteriorates and nutrients are depleted to a level where it is incapable of sustaining growth. Finally causing the cell density to decrease rapidly and the culture eventually collapses (Lavens & Sorgeloos, 1996).

2.8 Microalgae Culture Harvesting

2.8.1 Chemical Flocculation

Flocculation using chemical is another way of harvesting microalgae. There are a few suggested chemicals to be used such as Poly Glutamic Acid (PGA) or Chitosan from shrimp or any other crustacean shells. The chitosan is a biopolymer obtained through chitin deacetylation using KOH reaction (Lubián, 1989). Chitosan has many carbonyl and amino acid distributed to them as cationic flocculants. Organic pollutants such as carbohydrate, protein and nucleic acid in water will be adsorbed and flocculating settling by adsorption, ion exchange, or subsidence (Zeng *et al*., 2013).

2.8.2 Centrifugation

In order to separate the algae present in the medium, centrifugation method is used which cause the algae component to be settled at the bottom of the flask. This centrifuge method is unique equipment that driven by a motor and rotated the specified objects around a fixed axis and fixed rotation rates. A centrifuge uses sedimentation principle in which the centripetal acceleration is used to distribute the substances in the centrifuge tube evenly either in greater or lesser density (Centrifugation, n.d.). However, this method is expensive as it requires relatively high energy.

3.0 MATERIALS AND METHODS

The study was conducted in Aquatic Botany Laboratory of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS). All glassware that was used in this study was washed and soaked with Deepol (a phosphate-free soap) and rinsed with tap water and distilled water at least 3 times before soaking them in diluted HCL (10% v/v) for 2 to 3 hours. Then all glassware was rinsed again with distilled water for 3 times and was dried in the oven.

The microalgae stock used for this study, *Scenedesmus* sp., was obtained from Lake UNIMAS and *Scenedesmus dimorphus* from the University of Texas.

3.1 Preparation of Stock Solution

The component $NaNO_3$ was weighed according to the measurement in Table 2. Then in a volumetric flask containing approximately 900 mL of dH_2O, the component $NaNO_3$ is added into the dH_2O. The mixture is swirled continuously until the component is dissolved completely. More dH_2O is added up to the 1 L mark of the volumetric flask. This stock solution is poured into a 1 L Schott bottle and labeled.

The steps were repeated with other components in Table 2. The 6 stock solutions were then stored in a refrigerator.

Table 2: Chemical composition of Modified Bristol's Medium (MBM)

No.	Component	Amount (ml/L)	Stock Solution Concentration
1.	$NaNO_3$	10	25 g/ 1 L dH_2O
2.	$CaCl_2 \cdot 2H_2O$	10	2.5 g/ 1 L dH_2O
3.	$MgSO_4 \cdot 7H_2O$	10	7.5 g/ 1 L dH_2O
4.	K_2HPO_4	10	7.5 g/ 1 L dH_2O
5.	KH_2PO_4	10	17.5 g/ 1 L dH_2O
6.	NaCl	10	2.5 g/ 1 L dH_2O

3.2 Preparation of Microalgae Culture Medium, Modified Bristol's Medium (MBM)

In a volumetric flask containing approximately 800 mL of dH_2O, each of the stock solution was added (10 mL each). The stock solution was measured using a clean measuring cylinder. The volumetric flask was swirled and dH_2O is added up to the 1 L mark. The mixture was poured into a 2 L Schott Bottle and another 1 L of dH_2O was added into the mixture.

The steps were repeated to prepare another two more MBM as replicates. The bottles were labeled MBM 1, MBM 2, and MBM 3. The medium was autoclaved at 121℃ for 20 minutes and cooled at room temperature and then kept in refrigerator.

3.3 Preparation of Animal Manure Stock Medium

Chicken manure was obtained and dried in the oven. The chicken manure was then pulverized and weighed. 500 g of dried chicken manure was dissolved in 2 L of dH_2O and filtered using 0.45 μm filter paper. The fresh stock medium of chicken manure was

autoclaved at 121 ℃ for 20 minutes and cooled to room temperature (24-27 ℃). The chicken manure stock solution was diluted with dH_2O at concentration of 3.5%.

3.4 Cell Growth Measurement

The cell numbers of *Scenedesmus* sp. was counted under light compound microscope (MOTIC BA210) with improved Neubauer Haemacytometer for every 2 days. Growth curve was constructed using the cell number data.

The density of cells (d) were calculated using formulae as below:

$$d(\text{cells/ml}) = \frac{total\ count}{No.of\ blocks} \times 10^4 \qquad \text{Equation (1)}$$

Or

$$d(\text{cells/ml}) = \frac{total\ number\ of\ cells\ counted}{10 \times 4 \times 10^{-6}} \qquad \text{Equation (2)}$$

Where 10 = the 10 squares of the 2 chambers

The growth rate of *Scenedesmus* sp. was determined from the growth curve by using the formulae below:

$$K' = \frac{\mathrm{Ln}\left(\frac{N_2}{N_1}\right)}{t_2 - t_1} \qquad \text{Equation (3)}$$

Where N_1 and N_2 = cell density at time 1 (t_1) and time 2 (t_2).