Extraction Technique for Lipid from Microalgae for Biodiesel

by

Siti Humairah Mohd. Aris (8280)

Supervised by AP Dr Mohd. Azmuddin Abdullah

A project dissertation submitted to the

Chemical Engineering Programme

Universiti Teknologi PETRONAS

in partial fulfillment of the requirement for the

BACHELOR OF ENGINEERING (Hons)

(CHEMICAL ENGINEERING)

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Universiti Teknologi Petronas,

Bandar Seri Iskandar,

31750 Tronoh,

Perak Darul Ridzuan.

CERTIFICATION OF APPROVAL

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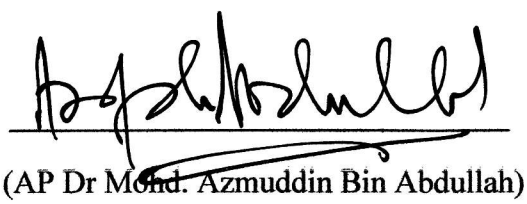

(AP Dr Mohd. Azmuddin Bin Abdullah)

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH, PERAK

JUNE 2010

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or person

SITI HUMAIRAH MOHD. ARIS

ABSTRACT

A study on the technique of lipid extraction from microalgae for the biodiesel production is performed, in which the author has narrowed down the research to the microalgae cultivation method, harvesting technique, lipid extraction of microalgae and also transesterification. At the early stage of the research, the author has batch-cultivated the *Nannochloropsis* sp and *Chlorella* sp to conduct the growth evaluation by using the cell count method. The microalgae are then harvested by centrifugal separation. The project work continued with the sample collection of the mixed-culture of microalgae from the pond for the extraction of lipid. For this project, the author has selected the solvent extraction method continued with the rotary evaporator to extract the lipid from microalgae. The solvent systems used for the extraction are n-hexane, methanol and the mixture of chloroform/methanol. The result from the extraction has shown that methanol is the best solvent to extract lipid from the mixed-culture of microalgae. The selection of this technique is made based on the ease of application and low cost consumption. Transesterification is also performed in order to convert the oil which is in the form of lipid to methyl ester. The alkali catalyzed process is applied in transeterification which is by using sodium hydroxide. Lastly, the pH measurement of the esterified product is conducted and analysis by using Thin Layer Chromatography is performed.

ACKNOWLEDGEMENT

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Apart from these, the author would like to express her sincere thanks to the Fisheries Research Institute (FRI), Batu Maung, Penang, for their great cooperation in helping the author to conduct the experiment at the Biotoxin Lab. Besides, the author would like to take this opportunity to thank the postgraduate students, Siti Syuhada Yusof and Uthman for their contribution for this project. Deepest gratitude also goes to the UTP Lab Technicians for their respective professionalism to this research.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Algae was initially examined as a potential replacement fuel source for fossil fuels in the 1970s amidst the gas scare (Barkley et al., 1987) but prohibitive production costs and limitations discouraged the commercial development of algae-based fuel production. Subsequent studies, continued through the 1980s and heightened in the last 15 years, illustrate that research developments are enabling the commercial potential of microalgae to shift from aquaculture, fine chemicals, and health food (Noue J et al., 1988) to fuel production.

Most studies have so far focused on the use of conventional biomasses from forestry and agricultural sources (Demirbas A, 2001). It was estimated that in year 2000, the majority of biomass energy was produced from wood and wood wastes (64%), followed by municipal solid waste (MSW) (24%), agricultural waste (5%) and landfill gases (5%) (Demirbas A, 2000). Energy from biomass would contribute to a stable energy supply and to local society due to an increase in commercial activities. When biomass is processed under high temperature at the absence of oxygen, products are produced as three phases which are the vapor phase, the liquid phase, and the solid phase. The liquid phase is a complex mixture called bio-oil (Yanqun Li et al., 2008). The compositions of bio-oils vary significantly with the types of feedstock and processing conditions.

Recently, a few investigations have been carried out regarding the suitability of microalgal biomass for bio-oil production. It was shown that, in general, microalgae bio-oils are of higher quality than biooil from wood. Microalgae have been suggested as potential candidates for fuel production because of a number of advantages including higher photosynthetic efficiency, higher biomass production and higher growth rate compared to other energy crops (Milne TA et al., 1990). Moreover, according to biodiesel standard published by the American Society for Testing Materials (ASTM), biodiesel from microalgal oil is similar in properties to the standard biodiesel, and is also more stable according to their flash point values.

1.2 Problem Statement

Recently, there are two kinds of important issues arising which are environmental and energy crisis issue (Amin S, 2009). From the environmental aspect, global warming which induced by increases of greenhouse effect gases concentrations in the atmosphere, has become an important environmental concern. The build up of atmospheric CO_2 is caused by the emission of fossil fuel combustion.

As for energy crisis, the increasing of petroleum crude oil prices has impacts on domestic energy scenario as well as on social life. The lack of stability of future energy supplies has motivated the development of alternative energy sources in order to eliminate the possibility of a future energy shortage. Microalgae, as biomass, are a potential source of renewable energy, and they can be converted into energy such as bio oil and gas (Amin S, 2009). So, it is a need to further develop the most efficient technique for extracting the lipid from microalgae as the algae is the most green biofuel compared to other biomass.

1.3 Objectives and Scope of Study

The main objective of this research project is to study on the extraction technique of lipid from microalgae with ease of application. The scope of study for this project will be narrowed down to the:

a) microalgae cultivation

b) microalgae harvesting technique

c) extraction method of oil from microalgae

d) transesterification of microalgal oil to biodiesel

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgae

Microalgae are unicellular, microscopic aquatic plants which exist individually, or in chains or groups. Depending on the species, their sizes can range from a few micrometers (μm) to a few hundreds of micrometers. Unlike higher plants, microalgae do not have roots, stems and leaves.

Microalgae are categorised in a variety of classes, which are primarily distinguished by their pigmentation, life cycle and basic cellular structure. The four most important classes are diatoms (Bacillariophyciae), green algae (Chlorophyceae), blue-green algae (Cynophyceae), and golden algae (Chrysophyceae). Microalgae, capable to perform photosynthesis by converting sunlight, water and carbon dioxide into biomass and oxygen as the following reaction (Ruan, 2007):

$$H_2O + CO_2 + NH_3 + P_2O5 + \text{Photons} \rightarrow \text{Biomass } (CNxHyOz) + O_2$$

They produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically. It has been estimated that about 200,000-800,000 species exist of which about 35,000 species are described (John Benemann, 2009).

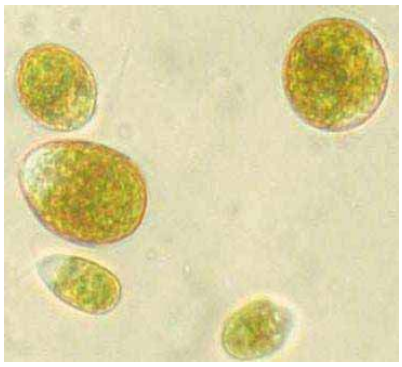


Figure 1a: Dunaliella Salina

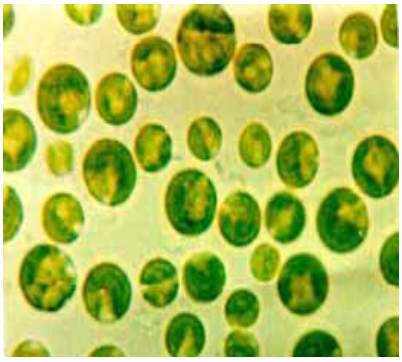


Figure 1b: Chlorella vulgaris

Table 1 below shows some specieses of microalgae with their oil content (in % dry weight).

Microalgae	Oil Content (% dry weight)
Botryococcus Braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Dunaliella primolecia	23
Isochrysis sp.	25-33
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Tetraselmis sueica	15-23

Table 1: The spieceses of microalgae and the oil content (Ruan, 2007)

2.2 Energy Conversion from Biomass

Microalgae, as biomass, are a potential source of renewable energy, and they can be converted into energy such as biofuel oil and gas. The properties of the microalgae product are almost similar to those of offish and vegetable oils, and therefore, it can be considered as a substitute of fossil oil (Amin S, 2009). There are many advantages of culturing microalgae as a resource of biomass which are:

i. Algae are considered to be a very efficient biological system for harvesting solar energy for the production of organic compounds.
ii. Algae are non-vascular plants, lacking complex reproductiveorgans.
iii. Many species of algae can be induced to produce particularly high concentrations of chosen, commercially valuable compounds, such as proteins, carbohydrates, lipids and pigments.
iv. Algae are microorganisms that undergo a simple cell division cycle.
v. The farming of microalgae can be grown using sea or brackishwater.
vi. Algal biomass production systems can easily be adapted to various levels of operational or technological skills. (Amin S, 2009).

The energy conversion reaction of biomass can be classified into biochemical, thermochemical, and direct combustion (Tsukahara K et al., 2005). Biochemical conversion can be further devided into fermentation, anaerobic digestion, bioelectrochemical fuel cells and other fuel producing processes utilizing the metabolism of organisms. Thermochemical conversion can be subdivided into gasification, pyrolisis and liquefaction. **Figure 2** shows the energy conversion processes from microalgae. Biomass can also be converted into three main products: two of them related to energy and one as chemical feedstock (Mckendry P, 2003).

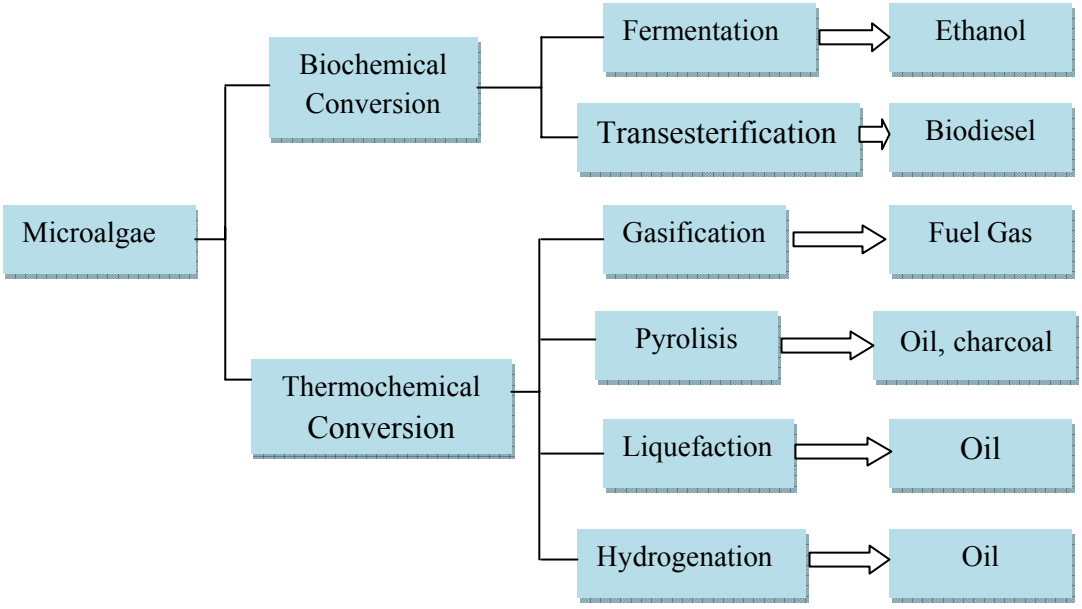


Figure 2: Energy conversion processes from microalgae

2.3 Microalgal Biosynthesis of Lipid / Fatty Acid

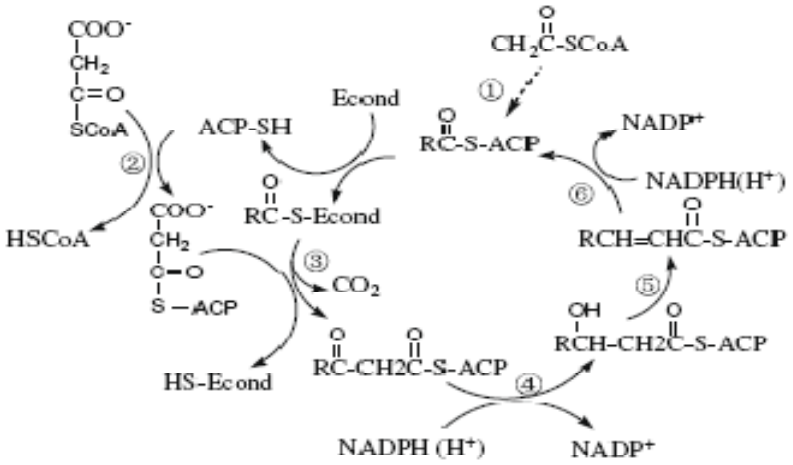
It is known that both inorganic carbon (CO_2) and organic carbon sources (glucose, acetate, etc.) can be utilized by microalgae for lipids production. The components and contents of lipids in microalgal cells vary from species to species. The lipid classes basically are divided into neutral lipids (e.g., triglycerides, cholesterol) and polar lipids (e.g., phospholipids, galactolipids). Triglycerides as neutral lipids are the main materials in the production of biodiesel. The synthesis routes of triglycerides in microalgae may consist of the following three steps: the formation of acetyl coenzyme A (acetyl-coA) in the cytoplasm; the elongation and desaturation of carbon chain of fatty acids; and the biosynthesis of triglycerides in microalgae.

2.3.1 The formation of acetyl coenzyme A (acetyl-coA) in cytoplasm

Glyceraldehyde phosphate (GAP) is a key intermediate both for the two metabolism systems; utilization of carbon dioxide and glucose for the formation of acetyl-coA in microalgae (Yang et al.). The formation of acetyl-coA in photosynthetic reactions, including the light reactions, Calvin cycle and synthesis, is located in chloroplast. GAP is withdrawn from Calvin cycle and exported to cytoplasm for consumption. After the export of GAP from chloroplast to cytoplasm, the flow of carbon is directed to the synthesis of sugars or oxidation through the glycolytic pathway to pyruvate. Sugars including sucrose are the major storage products in the cytoplasm of plant cells. Glucose was easy to be stored as starch without prior conversion to GAP and then uptake by the chloroplast which suggested starch is the main storage formation for carbon source in Chlorella sp. (Akazawa et al., 1980). Therefore, one part of the exogenous glucose was directly converted to starch, and the remainder was oxidized through glycolytic pathway.

2.3.2 The elongation and desaturation of carbon chain of fatty acids

The elongation of carbon chain of fatty acids is mainly dependent on the reaction of two enzyme systems including acetyl-coA carboxylic enzyme (ACCE) and fatty acid synthase (FAS) in most organisms. In the process of synthesis of fatty acids (**Figure 3**), acetyl-coA is the primer. The process of carbon chain elongation needs the cooperation with malonyl-coA, the substrate on which enzyme act are acetyl-ACP and malonyl-ACP. The C16–C18 fatty acid thioester can be formed after several reaction steps. The formation of short carbon chain fatty acids is similar in the cells of advanced plants, animals, fungi, bacteria, and algae.



① Acetyl-CoA-ACP acyl transferase

② malonyl CoA-ACP acyl transferase

③ β-ketoacyl-ACP Condensing enzyme)

④ β-ketoacyl-ACP reductase

⑤ β-hydroxyacyl-ACP dehydrase

⑥ enoyl-ACP reductase

Figure 3: Reaction process of the FFA biological synthesis system (Shen T et al., 1989)

For example, in the cell of green algae, the reaction routes of primer such as palmitoleic acid, oleic acid, linoleic acid, linolenic acid in fatty acid synthesis are similar to that in plant cells and yeast cells (Stumpf PK, 1984). The desaturation of carbon chain of fatty acid occurs from C18 and further elongation of carbon chain takes place to produce long-chain fatty acids which are unusual in normal plant oils (**Figure 4**). Long-chain fatty acids (C20–C22) often exist in microalgae and the content varies from species to species (Miereles LA et al., 2003). Normally, short-chain fatty acids (C14–C18) which are the main components of biodiesel are majority of fatty acids in Chlorella sp., but high content of long-chain fatty acid and hydrocarbons exist in some specific species of microalgae. So, it is vital to choose proper microalgae species as materials of biodiesel production.

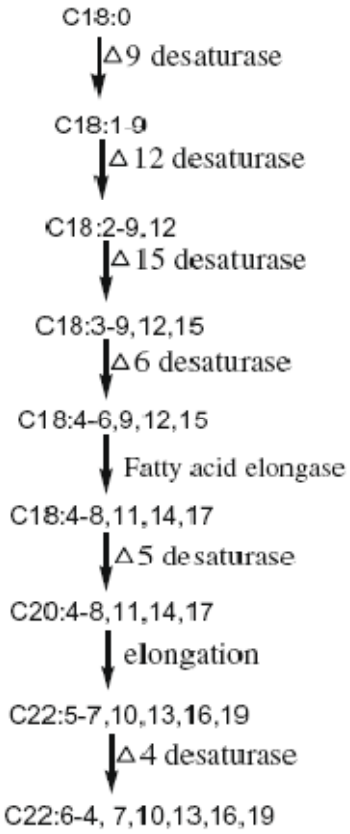


Figure 4: The elongation and desaturation of carbon chain of fatty acids

2.3.4 The biosynthesis of triglycerides in microalgae

Like other higher plant and animal, microalgae are able to biosynthesize triglycerides to store substance and energy. Generally, L-a-phosphoglycerol and acetyl-coA are two major primers in the biosynthesis of triglycerides. The L-a-phosphoglycerol mainly derives from phosphodihydroxyacetone which is the product of the glycolysis process. The reaction steps are shown in **Figure 5**. One of the hydroxyl in L-a phosphoglycerol reacts with acetyl-coA to form Lysophosphatidic acid and later combines with another acetyl-coA to form phosphatidic acid.

These two reactions are catalyzed by glycerol phosphate acyl-transferase. In the following steps, lysophosphatidic acid is hydrolyzed by phosphatidate phosphatase to form diglyceride which is then combined with the third acetylcoA to complete the biosynthesis of triglycerides. The last reaction step is catalyzed by glyceryl diester transacylase.

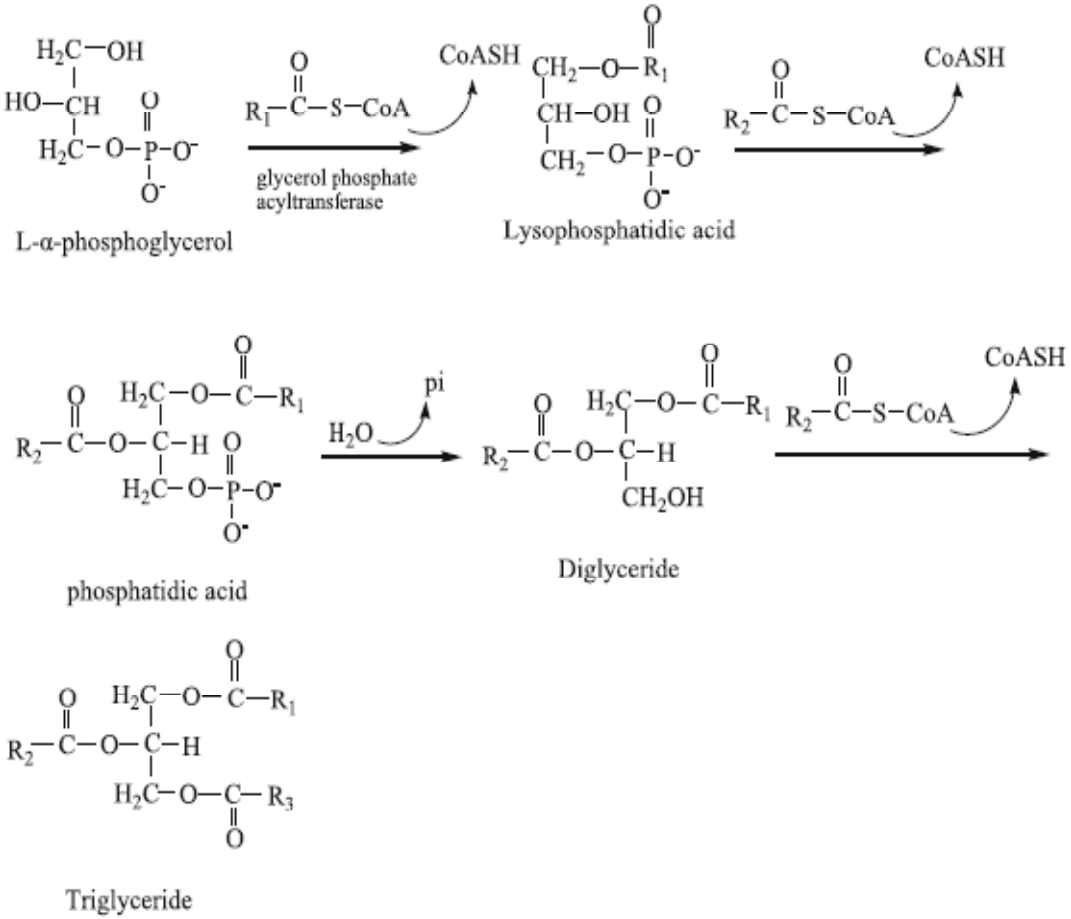


Figure 5: The biosynthesis of triglycerides in microalgae (Huang G et al., 2009)

2.4 Microalgal Cultivation

Microalgae can be cultivated by a large number of systems (Scragg AH et al.,2002). For small scale or laboratory scale, cultivation uses a small fermenter or internally illuminated photobioreactor (Ugwu CU, 2008) or box type water tanks. The fermenter uses four fluorescents lamp as external irradiation or by using other light distributors. One of the major advantages of an internally continuously illuminated photobioreactor or by using a flat plate photobioreactor is that it can be heated sterilized under pressure, and thus, contamination can be minimized.

Figure 6: Fermenter

For large scale cultivation, algae can be grown either in open culture systems or closed systems. The most commonly used systems include shallow large ponds, tanks, circular ponds and raceway ponds (Ugwu CU, 2008). A tubular photobioreactor is one of the most suitable types for outdoor mass cultures. Tubular photobioreactors have advantages over conventional open ponds as they can be erected over any open space, can operate at high biomass concentration and keep out atmosphere contaminants and they seem to be most satisfactory for producing algal biomass on the scale needed for biodiesel production.

Figure 7a: Open pond

Figure 7b: Tubular photobioreator

2.5 Microalgal Harvesting

Algae can be harvested by using microscreens, centrifugation, or flocculation. Alum and ferric chloride are chemical flocculants used to harvest algae. Other flocculant material is chitosin (Morales J. et al., 1985). Harvesting by chemical flocculation is a method that is often too expensive for large operations. Interrupting the carbon dioxide supply to an algal system can cause algae in it to flocculate on its own, which is called autoflocculation. The algal cells grown for a liquefaction process can also be harvested by a centrifugal separator.

Figure 8a: Flocculation

Figure 8b: Centrifugation

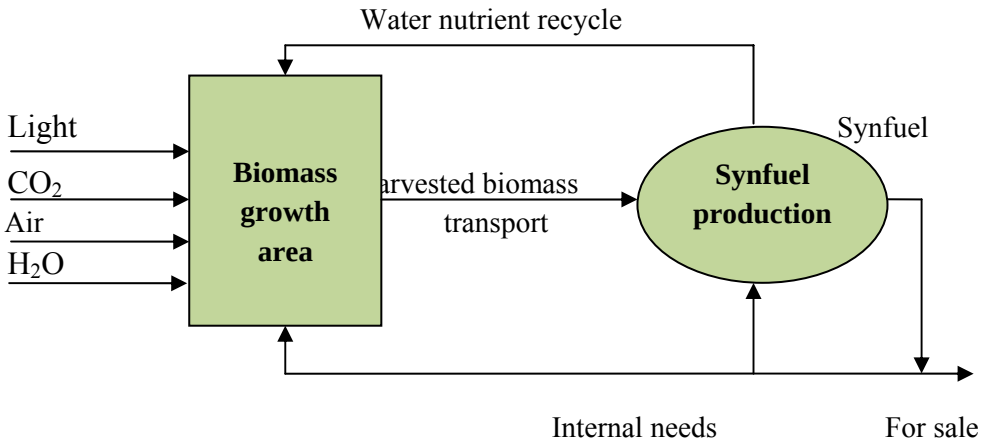


Figure 9: A conceptual model for integrated biomass production

2.6 Cell Disruption of Microalgae

Cell disruption is often necessary for recovering intracellular products from microalgae (Ruane and Mendes). An aliquot (0.5 g) of the dry cell biomass can be blended with 100 mL of distilled water and the mixture can be disrupted using five different methods (Lee, J.-Y et al., 2009) as follows :

i) autoclaving which uses an autoclave, a device to sterilize equipment and supplies by subjecting them to high pressure steam at 121° C or more for 5 minutes.

ii) bead-beating using a bead beater at a high-speed of 2800 rpm for 5 minutes.

iii) microwaves using a microwave oven at a high temperature (about 100° C and 2450 MHz) for 5 minutes .

iv) sonication using a sonicator at a resonance of 10 kHz for 5 minutes (Lee, J.-Y et al., 2009). Sonication is the act of applying sound (usually) ultrasound

energy to agitate particles in a sample, for various purposes sonication may be sufficient to disrupt or deactivate a biological material. For example, sonication is often used to disrupt cell membranes and release cellular contents

v) osmotic shock using a 10% NaCl solution with a vortex for 1 minute and maintained for 48 hour (Lee, J.-Y et al., 2009). Osmotic shock is a sudden reduction in osmotic pressure, this can cause cells in a solution to rupture. Osmotic shock is sometimes used to release cellular components, such as oil.

2.7 Microalgal Oil / Lipid Extraction

When the biomass is processed under high temperature at the absence of oxygen, products derived would be in three phases which are the vapor phase, the liquid phase, and the solid phase. The liquid phase is basically a complex mixture called bio-oil (Yanqun Li et al., 2008). The compositions of bio-oils vary significantly with the types of feedstock, processing conditions and parameters used. Nowadays, there are two methods to extract the oil which mainly consists of lipid from microalgae; by using physical extraction and chemical extraction.

2.7.1 Physical extraction

(a) Expeller/Press Method

Expeller pressing (also called oil pressing) is a mechanical method for extracting oil from microalgae. Firstly, the microalgae are squeezed under high pressure in a single step which are supplied to the press in a continuous feed. Expeller presses can

recover 75% of the oil from algae. An expeller press is a screw type machine, which presses the microalgae through a caged barrel-like cavity.

Microalgae enter one side of the press and waste products exit the other side. The machine uses friction and continuous pressure from the screw drives to move and compress the microalgae. The oil seeps through small openings that do not allow the fiber solids to pass through. Afterward, the pressed microalgae are formed into a hardened cake, which is removed from the machine. Pressure involved in expeller pressing creates heat in the range of 140-210 F (60-99 °C). Some companies claim that they use a cooling apparatus to reduce this temperature to protect certain properties of the oils being extracted.

Figure 10: Oil Expeller (www.ascof.com/oil_expeller.htm)

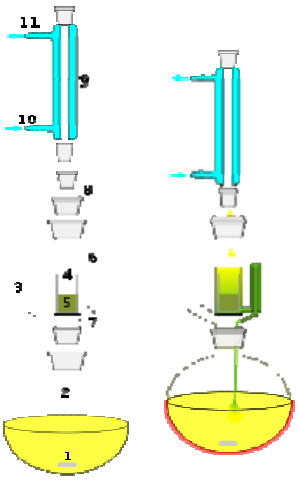
2.7.2 Chemical extraction

(a) Soxhlet Extraction

In this method, oils from the algae are extracted through repeated washing, or percolation, with an organic solvent such as hexane or petroleum ether, under reflux in a special glassware. The value of this technique is that the solvent is reused for each cycle. Normally a solid material containing some of the desired compound is

placed inside a thimble made from thick filter paper. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.

The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the solvent will run back down to the distillation flask by siphone arm. This cycle may be allowed to repeat many times, over hours or days. The advantage of this system is that just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.



No.	Description
1	Stirrer bar
2	Still pot
3	Distillation path
4	Thimble
5	Solid
6	Siphon top
7	Siphon exit
8	Expansion adaptor
9	Condenser
10	Cooling water in
11	Coolong water out

Figure 11: A Soxhlet Apparatus

(Source: http://www.en.wikipedia.org/wiki/Soxhletextraction)

(b) Solvent extraction

Algal oil can be extracted using chemicals. Benzene and ether have been used, but a popular chemical for solvent extraction is hexane, which is relatively inexpensive. The downside to using solvents for oil extraction are the inherent dangers involved in working with the chemicals. Benzene is classified as a carcinogen. Chemical solvents also present the problem of being an explosion hazard.

Hexane solvent extraction can be used in isolation or it can be used along with the oil press/expeller method. After the oil has been extracted using an expeller, the remaining pulp can be mixed with cyclo-hexane to extract the remaining oil content. The oil dissolves in the cyclohexane, and the pulp is filtered out from the solution. The oil and cyclohexane are separated by means of distillation. These two stages (cold press & hexane solvent) together will be able to derived more than 95% of the total oil present in the algae (Oilgae, 2009).

(c) Supercritical CO_2 Extraction

In this method, CO_2 is liquefied under pressure and heated to the point that it becomes supercritical (having properties of both a liquid and a gas), allowing it to act as a solvent. This method offers very attractive extraction characteristics, owing to its favorable diffusivity, viscosity, surface tension and other physical properties (Mukhopadhyay M., 2006). Its diffusivity is one or two orders of magnitude higher than those of other liquids, which facilities rapid mass transfer and faster completion of extraction than conventional liquid solvents (Mukhopadhyay M., 2006). Supercritical CO_2 is an inert, inexpensive, easily available, odorless, tasteless, environmental-friendly, and GRAS (generally regarded as safe) solvent. Further, there is no solvent residue in the extract, since it is a gas in ambient condition. Commercial CO_2 required for supercritical fluid extraction process is already present in the environmental system obtained as a by-product from the fermentation process

or the fertilizer industry (Mukhopadhyay M., 2006). So, its use as an extraction does not cause any further increase in the amount of CO_2 present in the earth's atmosphere. Therefore, there is no additional "green house effect" from using CO_2 as the supercritical fluid solvent.

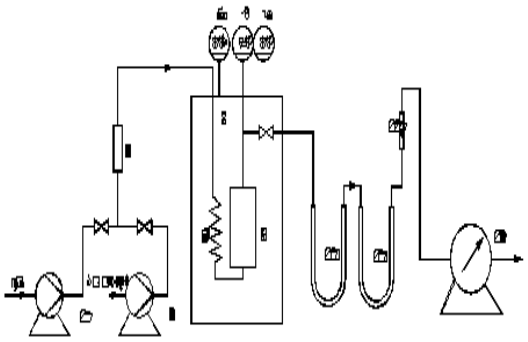


Figure 12: Supercritical CO_2 Extraction Apparatus

2.7.3 Thermochemical Conversion

(a) Pyrolysis

Pyrolyzing microalgae to produce liquid fuel was first put forward in Germany in 1986. It was reported that the method of catalytic pyrolysis could yield gasoline with high content of aromatic hydrocarbon and octane number (Milne TA et al., 1990). Pyrolysis is a phenomenon related to decomposition of biomass under the condition of oxygen deficiency and high temperature. Pyrolysis previously was first used for the production of bio-oils or bio-gases from lignocellulose. However, such a technology may be more suitable for microalgae because of the lower temperature required for pyrolysis and the higher-quality oils obtained. Moreover, the cost of pyrolysis of lignocellulose is relatively higher than that of microalgae. Compared to

lignocellulose, microalgae contain high content of cellular lipids, resolvable polysaccharides and proteins, which are easier to be pyrolyzed to bio-oils and bio-gases. Compared to slow pyrolysis (Minowa T et al., 1995), fast pyrolysis is a new technology, which produces bio-fuel in the absence of air at atmospheric pressure, with a relatively low temperature and high heating rate as well as short gas residence time to crack into short chain molecules and be cooled to liquid rapidly. The main products of slow pyrolysis are char and char-oils with a 15–20% yield, whereas, the products of fast pyrolysis are oils and gases with a yield of approximately 70%. Fast pyrolysis is proven to be a promising way to produce bio-oils compared to slow pyrolysis for the following reasons of less bio-oils were produced from slow pyrolysis, the viscous bio-oils from slow pyrolysis is not suitable for liquid fuels and the fast pyrolysis process is time saving and requires less energy.

(b) Liquefaction

High content of water often exists in microalgae after harvesting which requires a great deal of energy to remove moisture in the algal cells in the period of pretreatment. Liquefaction has been developed to produce bio-fuel directly without the need of drying microalgae (Dote Y et al., 1994). Moreover, wet microalgae can provide hydrogen for hydrogenolysis. It was reported that Dunaliella tortiolecta cells with 78.4% water content converts to oils directly. The yield of oils reached 37% of the total organic matters. Dote et al. reported that B. braunii produced liquid oils at 57–64% of dry weight under the conditions of a N_2 pressure of 10 MPa at 300 °C in warm water and catalyzed by $NaCO_3$. Sawayama et al. investigated the energy balance and CO_2 mitigating effect of a liquid fuel production process from B. braunii using thermochemical liquefaction. The study suggested that microalgae consume low amounts of nutrients and accumulate high caloric materials, and nutrient resources which are produced without energy wasting processes encourage the recovery of oil from microalgae and CO_2 mitigation.

2.8 Transesterification

The viscosities of vegetable oils and microalgal oils are usually higher than that of diesel oils (Fuls J et al., 1984). Hence, they cannot be applied to engines directly. The transesterification of microalgal oils will greatly reduce the original viscosity and increase the fluidity. Nevertheless the technologies of the biodiesel production for vegetable oils can be applied to the biodiesel production of microalgal oils because of the similar physical and chemical properties. In the process of transesterification, alcohols are the key substrates in transesterification. The alcohols that are commonly used are methanol, ethanol, propanol, butanol, and amyl alcohol but methanol is widely applied because of its low-cost and physical advantages.

Alkali, acid, or enzyme catalyzed processes may be applied in transesterification (Canakci M et al., 1999). The use of acid catalyst has found to be useful for the conversion of high free fatty acid feedstocks to esters but the reaction rates for converting triglycerides to methyl esters are too slow (Gerpen JV, 2005). Alkali catalysts have higher reaction rate and conversion than acid catalysts for the for the transesterification of triglyceride. Alkali-catalyzed transesterification is about 4000 times faster than the acid catalyzed reaction (Fukuda H et al., 2001). So, alkali-catalyzed transesterification is most frequently used commercially. The free fatty acid (FFA), however, may react with the alkali catalyst to form soap and water (**Figure 13**) which results in the loss of alkali catalysts in the process of reaction. Therefore, additional catalysts must be added to compensate for the catalyst loss to soap. When the FFA level is above 5%, the soap will inhibit separation of the methyl esters and glycerol and causes emulsion formation during the water washing. Therefore, it is necessary to first convert FFAs to methyl esters (**Figure 14**) in order to reduce the contents of FFAs, and the low FFAs pretreated oil is transesterified with an alkali catalyst to convert triglycerides to methyl esters. In contrast, enzymes exhibit good tolerance to the FFA level of the feedstock, but the enzymes are expensive and may not be able to provide the degree of reaction required to meet the

ASTM fuel specification (ASTM International, 2002). Immobilization of the enzyme and multiple enzymes may provide more choices in the future (Hama S et al., 2006).

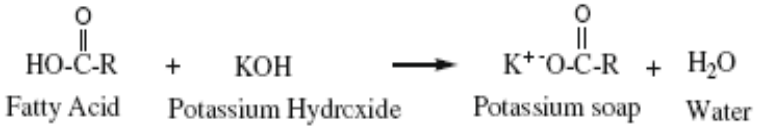


Figure 13: Transesterification by alkali catalyst

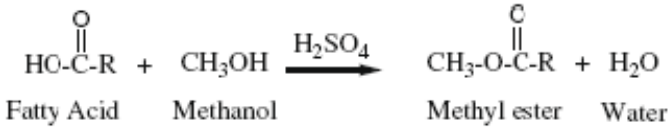


Figure 14: Transesterification by acid catalyst

2.9 Chemical Analysis

2.9.1 Thin Layer Chromatography (TLC)

It is a chromatography technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. It is a simple and cost-effective tehnique (Sarker S.D. et al., 2006).

TLC plates may be sprayed with reagents that react specifically with certain classes of compounds. The use of different spraying reagents can give plenty of information about the chemical classes present in the extract. Moreover, direct combination of TLC with bioassay can provide more information about the active component within the extract mixture.

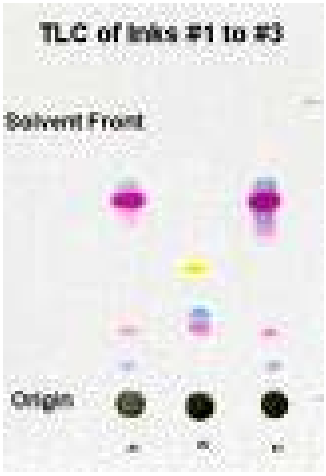


Figure 15: TLC Plate

2.9.1 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules.

C_{18} is the most commonly used stationary phase, with UV (ultraviolet) monitoring or, increasingly PDA detectors to monitor the separations, allowing the comparison of extract chromatograms with known compounds on the basis of their retention times and UV-spectra (Sarker S.D. et al., 2006). Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

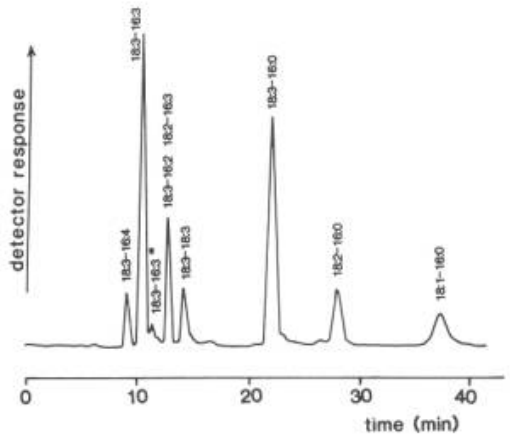


Figure 16: HPLC Diagram

2.9.2 Gas Chromatography

Gas chromatography (GC), is a common type of chromatography used in analytic chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can also be determined). In some situations, GC may help in identifying a compound. In preparative chromatography, GC can be used to prepare pure compounds from a mixture.

In gas chromatography, the moving phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (an homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a gas chromatograph.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. Firstly, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas moving phase, whereas in column chromatography the stationary phase is a solid and the moving phase is a liquid. (Hence the full name of the procedure is "Gas-liquid chromatography", referring to the mobile and stationary phases, respectively.) Secondly, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

2.9.3 Column Chromatography

Column chromatography in chemistry is a method used to purify individual chemical compounds from mixtures of compounds. It is often used for preparative applications on scales from micrograms up to kilograms.

The classical preparative chromatography column is a glass tube with a diameter from 50 mm and a height of 50 cm to 1 m with a tap at the bottom. Two methods are generally used to prepare a column; the dry method, and the wet method. For the dry method, the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely

wet, and from this point is never allowed to run dry. For the wet method, a slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Care must be taken to avoid air bubbles. A solution of the organic material is pipetted on top of the stationary phase. This layer is usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent. Eluent is slowly passed through the column to advance the organic material. Often a spherical eluent reservoir or an eluent-filled and stoppered separating funnel is put on top of the column.

2.9.4 NMR Analysis

NMR spectroscopic analysis plays an indispensable role in the structure elucidation of pure compounds. It can also provide a lot of information on the chemical nature of compounds in a mixture. In effect, it is recommended to obtain ^{1}H- and ^{13}C-NMR spectra for marine extracts. The objectives are to detect the presence / absence of common aritfacts, e.g., plasticizers and to assign the components in the mixture to certain chemical classes. Combination of fractions after any separation step can be decided on the basis of their similar NMR spectra.

CHAPTER 3

METHODOLOGY / PROJECT WORK

3.1 Methodology

This chapter will cover deeply on the methods used by the author upon completing this project. The early stage of this research is done at the Biotoxin Lab of Fisheries Research Institute (FRI), Batu Maung, Penang as the microalgae specieses are easily obtained there. The processes involved at the lab are comprised of cultivation and harvesting. As for extraction, the process is continued at the lab of Chemical Engineering Department, Universiti Teknologi Petronas (UTP).

3.1.1 Microalgae cultivation

The cultivation is performed in the laminar flow cabinet (**Figure 17**). Before starting the process, it is very important to ensure that the laminar flow and lamp are switched on while the Bunsen burner is kept open. Besides, the working area should also be sterilized by ethanol. The basic procedure for microalgae cultivation is as follows:

1. 0.1 ml of Vitamin is added to 1L of sea water. (Vitamin:sea water =1:10000) This solution will act as the media stock for cultivation.

2. After that, 4 test tubes (10 ml) are filled with 10 ml of sea water solution each. By using Finpipette digital, 2.5 ml of *Nannochloropsis* sp. Is added to all the test tubes respectively. (The ratio of culture species to media stock is usually around 1:3 to 1:4)
3. Then, the test tubes are closed with their caps and positioned at the iluminated shelf to allow the algae to increase in density.
4. Step 1 to 3 is repeated by using the *Chlorella* sp.

The production culture starts in test-tube and subsequent cultures can involve respectively in 250-ml flask, 2-litre Erlenmeyer Flasks, 30-liter and up to 300 litre conical bottomed fiberglass tanks. Basically, this system is called batch culture method (Faazaz et al., 1991).

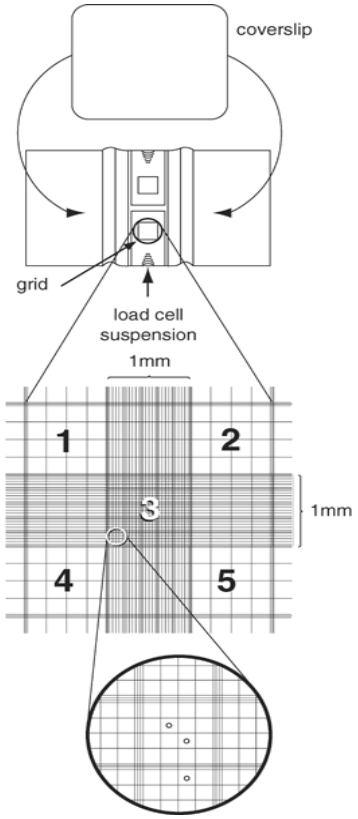
Figure 17: Laminar flow cabinet

Figure 18: The cultivation of microalgae at the culture room

Figure 19 : The culture in Erlenmeyer Flask

3.1.2 Growth evaluation

The growth evaluation of the microalgae is done by performing the cell count. The equipments involved in this process are LEICA microscope with 40 x focusing power and a hemacytometer, that consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The growth of microalgae cells are tracked for about a month from day 0.



Deep = 0.1 mm

Area for 1 square= 0.04 mm^2

Figure 20: Hemocytometer and the laser etched grid

For the cell count, 10 readings are taken for each sample which are 5 readings from the upper part and another 5 from the lower part of the hemacytometer. Calculation of the cell count for each sample is as follows:

Average cell = Sum/10

Number of cells = $\frac{\text{average cell}}{\text{deep x area}}$ (Unit = cell ml^{-1})

(Refer Appendix A for sample of calculation)

Once the growth phase has been plotted based on the cell counting, (time on x-axis and biomass on logarthmic y-axis) careful determination of the exponential (straightline) phase of growth is needed. Two points, x_0 and x, at the extremes of this linear phase are taken and substituted into the equation:

Max specific growth rate, $\mu_{max} = \frac{\ln x - \ln x_0}{t - t_0}$

x= growth at time t

x_0 = growth at time t_0

The calculation of doubling time for the microalgae is as below:

Doubling time, $td = \frac{\mu_{max}}{\ln 2}$

3.1.3 Microalgae harvesting

The *Nannochloropsis* sp. is filled in 45 ml plastic tubes as in **Figure 21**. Then, it is harvested by using centrifugation by maximum rpm in 13 minutes time. As the microalgae has settled down by gravity at the bottom of the tubes, the remaining clean water at the upper layer is taken out. The wet cell mass is then kept in the freezer. Before it is brought back to UTP lab for further extraction, the microalgae is dried using vacuum dryer at 52 x 10^{-3} Bar for 48 hours.

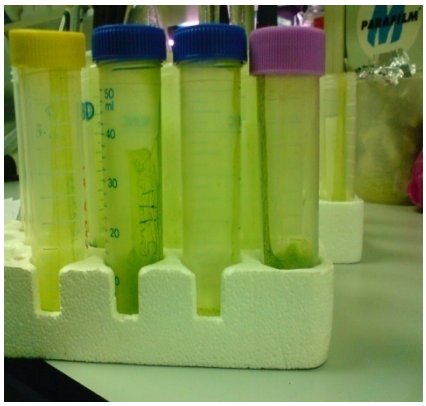


Figure 21: Microalgae filled in 45 ml tubes

Figure 22: Microalgae collected in a 100ml flask before vaccuum drying

3.1.4 Extraction

(a) Sample collection

The dried sample of *Nannochloropsis* are just consist of a small quantity. Hence, more sample is needed for the extraction experiment to be performed. In that case, microalgae, mainly consist of mixed-culture are collected from the pond of UTP, as shown in **Figure 23** below.

Figure 23: Collection of microalgae from the pond

Figure 24: The collected sample of microalgae

After that, the sample is cleaned by removing the sand and any other contaminants that may exists in the microalgae.

(b) Oil extraction

Firstly, the microalgae are placed in the tray and covered with the aluminium foil. Then, the microalgae are dried in the oven for 48 hours at 120 °C for releasing the water. The dried microalgae is then grinded as much as possible by using the grinding machine. The aim of grinding is to improve the subsequent extraction by rendering the sample more homogeneous, increasing the surface area, and facilitating the penetration of solvent into the cells. The grinded sample is shown in **Figure 25**.

Figure 25: The grinded sample of microalgae

Then, three samples of microalgae are weighed with 5 grams each. Three types of solvents are also prepared for the extraction, which are n-hexane, methanol, and chloroform:methanol for 100 ml. Each 5 grams sample are mixed up with the solvents respectively and shaked for about 20 minutes. The objective of shaking is to improve the solvent diffusivity into the cell. Then, the mixtures are kept for about 24 hours for settling as in **Figure 26**.

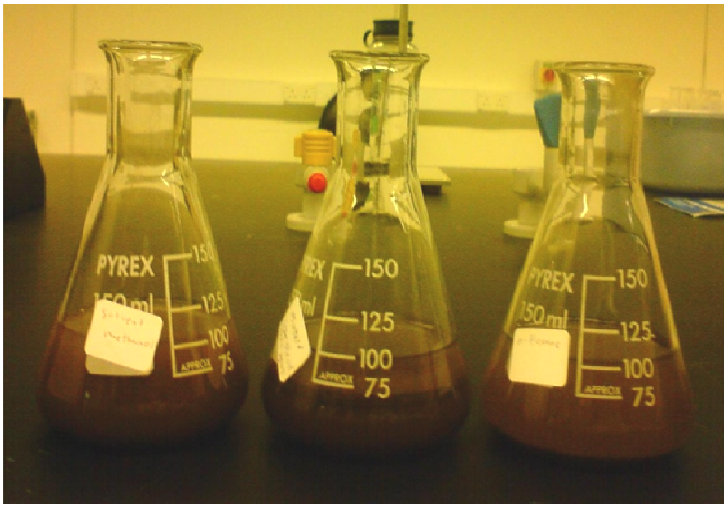


Figure 26: Settling of the mixtures of microalgae and solvents

(c) Biomass collection

The biomass was collected after the filtration by using filter paper .

(d) Evaporation

The extracted oil was then evaporated in vaccuum to release the solvent using rotary evaporator (**Figure 28**) at 60°C with 40 rpm. Rotary evaporators are used to remove solvents from reaction mixtures and can accommodate volumes as large as several liters. A typical rotary evaporator has a water bath that can be heated in either a metal container or crystallization dish. This keeps the solvent from freezing during the evaporation process. The solvent is removed under vacuum, is trapped by a condenser and is collected for easy reuse or disposal.

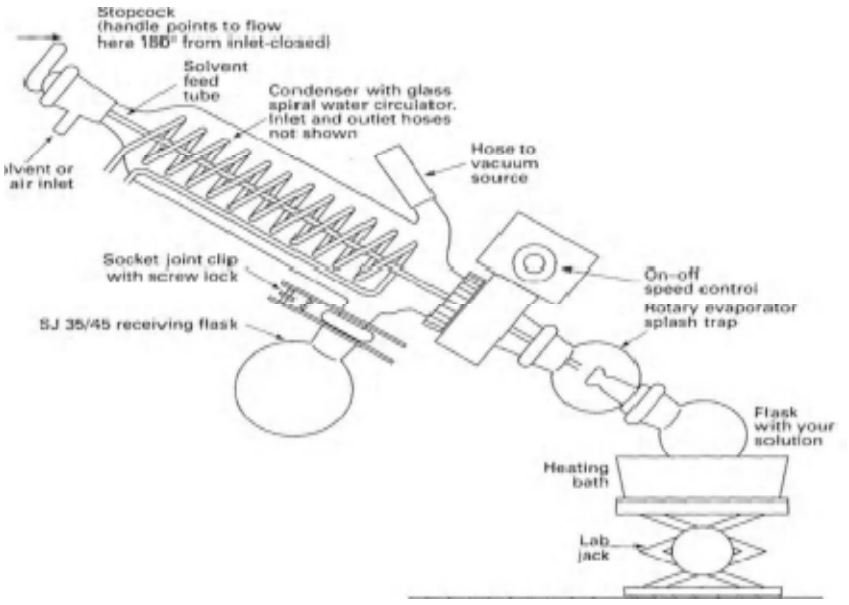


Figure 27 : The diagram of rotary evaporator with main parts

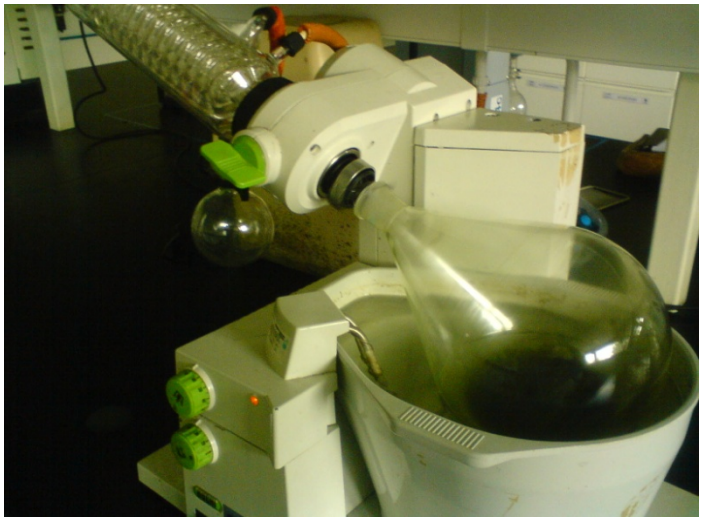


Figure 28: Rotary evaporator

The standard operating procedure of the rotary evaporator is as follows:

1. The solvent collection flask is emptied and replaced on the unit to avoid the incompatible chemicals to be mxied accidentally.
2. The round-bottomed flask is placed with the solution on the rotary evaporator. Most people use a bump trap to prevent their material from accidentally splashing into the condenser (and being contaminated). It is highly advisable to start with a clean bump bulb.
3. A metal or Keck clip is used to secure the round-bottomed flask and the bump trap**.** The green one shown fits 24/40 ground glass joints. Similar blue clips fit 19/22 joints and the yellow ones fit 14/20 joints,
4. The dial on the motor is used for speed control of the flask rotation. A typical rotoevap uses a variable speed sparkless induction motor that spins at 0- 220 rpm and provides high constant torque.
5. The aspirator vacuum is turned on. On most models, the vacuum on/off control is managed by turning a stopcock at the top of the condenser. This stopcock is later also used to vent the setup.
6. The flask is lowered into the water bath or the water bath raised to immerse the flask in the warm water. At this point, the flask should not be more than half filled. On most models, a convenient handle (with height locking mechanism) moves the entire condenser/motor/flask assembly up and down. Often the tilt of the condenser assembly can also be adjusted. The water bath temperature should not exceed the boiling point of the solvent. For small amounts of common solvents you do not need to turn on the bath heater.
7. Once all the solvent has evaporated (no solvent condenses in the trap anymore), the vacuum is released (by allowing air to bleed into the setup), the flask raised out of the water bath and the rotation is turned off. The Keck clip is removed, and then can flask with the remaining material.
8. The bump trap has to be cleaned and the receiving flask is emptied upon completion of the evaporation.

The mocroalgal lipid is then collected after the solvent has been removed by evaporation and weighted.

3.1.5 Transesterification

0.25 g NaOH is mixed with 24 ml methanol and stirred properly for 20 minutes. The mixture of catalyst and methanol is poured into the algal oil in the conical flask. The following reaction and steps were followed (**Figure 29**).

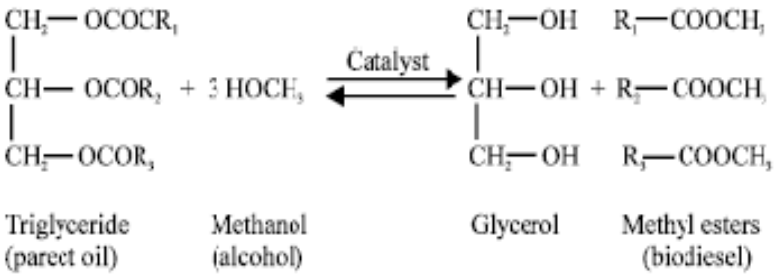


Figure 29: Transesterification of triglyceride

This reaction process is called transesterification. The conical flask containing solution was shaken for 16 hours using electric shaker at 160 rpm. After shaking, the solution was kept for 24 hours to settle the biodiesel and sediment layers clearly. The biodiesel was separated from sedimentation carefully. Biodiesel was washed by 5% water until it was become clean. Biodiesel was dried by using dryer and finally kept under the running fan for 12 hours. Biodiesel production was measured by using measuring cylinder, pH was measured and stored for analysis.

Figure 30: Separation of esterified product from sediments

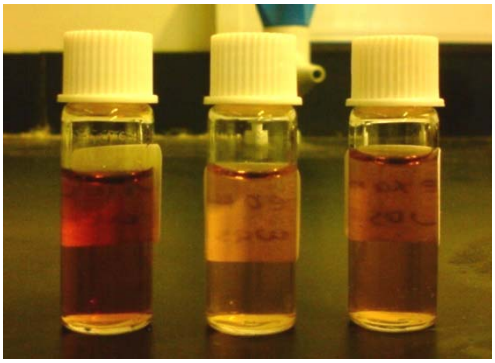


Figure 31: Esterified product of microagal lipid extracted from Chloroform/Methanol, n-Hexane and Methanol

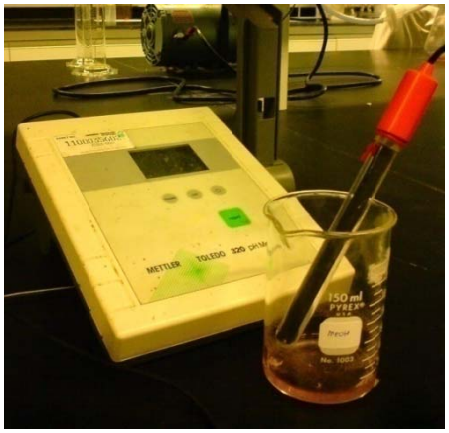


Figure 32: pH analysis of biodiesel

3.1.6 Chemical Analysis

The analysis that is performed for the biodiesel from microalgae is by using Thin Layer Chromatography (TLC). The objective of this method is to examine the main simple lipids from a tissue in one step, for example cholesterol esters, triglycerides, free fatty acids, cholesterol and diacylglycerols, using mobile phases consisting of a mixture of hexane and diethyl ether, with a little acetic acid to ensure that the free acids migrate successfully. Complex lipids such as phospholipids and glycosphingolipids will remain at the origin, and they can then be quantified as if they were a single lipid class. The procedure for the TLC method is as follows:

1. TLC sheet (silica plate) is prepared by cutting it into small strips about 9cm x 2cm for each. The silica plate was marked with the height of 1.5 cm as starting point and 8.5 cm as the final point.
2. Hexane (12 ml), diethyl ether (3ml) and acetic acid (0.5 ml) are added in the TLC chamber.
3. A small pigment spot of the methyl ester (biodiesel) from each sample is applied to the filter paper strip by using capillary tube. The pigment should be as small as possible (less than 4 mm is the best).
4. The papers are suspended in the TLC chromatography chamber as in **Figure 33** below. The spot must be about 1 cm above the solution.

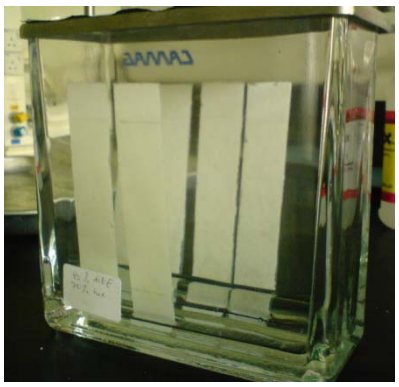


Figure 33: Silica plate are suspended in the TLC chamber

5. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized by putting the silica plate inside an iodine chamber (**Figure 34**).

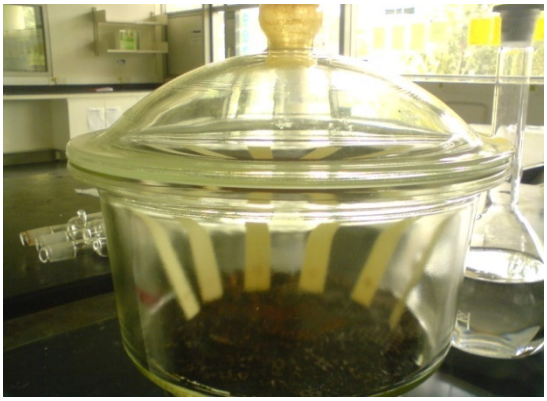


Figure 34: Silica plate in iodine chamber

6. After the plates are placed inside the iodine chamber, identification of the blobs appeared on the plate could be done for further analysis. Each component can be differentiated by their respective retention time. The identification of the components can be done as shown below:

Table 2: Detection Length of Different Components

COMPONENT	DETECTION LENGTH (CM)
Pure biodiesel	5-7
Triglycerides (TG)	2.8-5
Free Fatty Acid (FFA)	2.3-3.3
Diglycerides (DG)	1-2.3
Monoglycerides (MG)	0-0.9

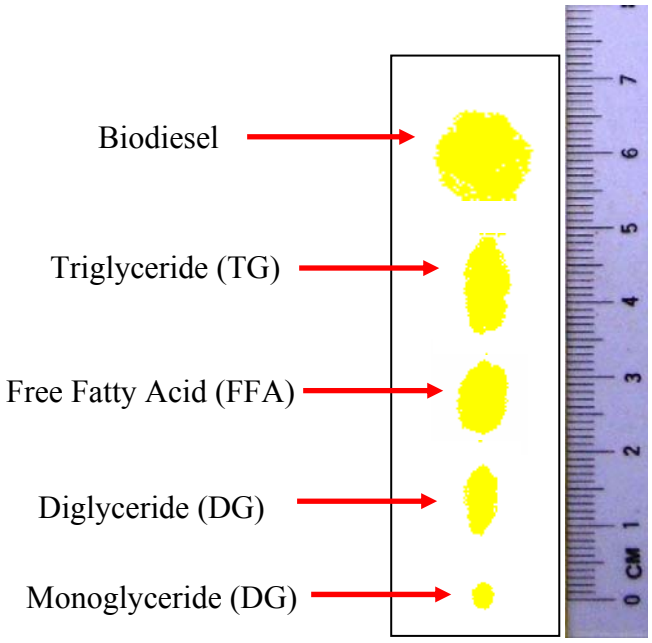


Figure 35: Detection of Product's Components on TLC Plates

3.2 Process Flow of the Project

The following figure shows the process flow of the research method in order to meet the set objectives:

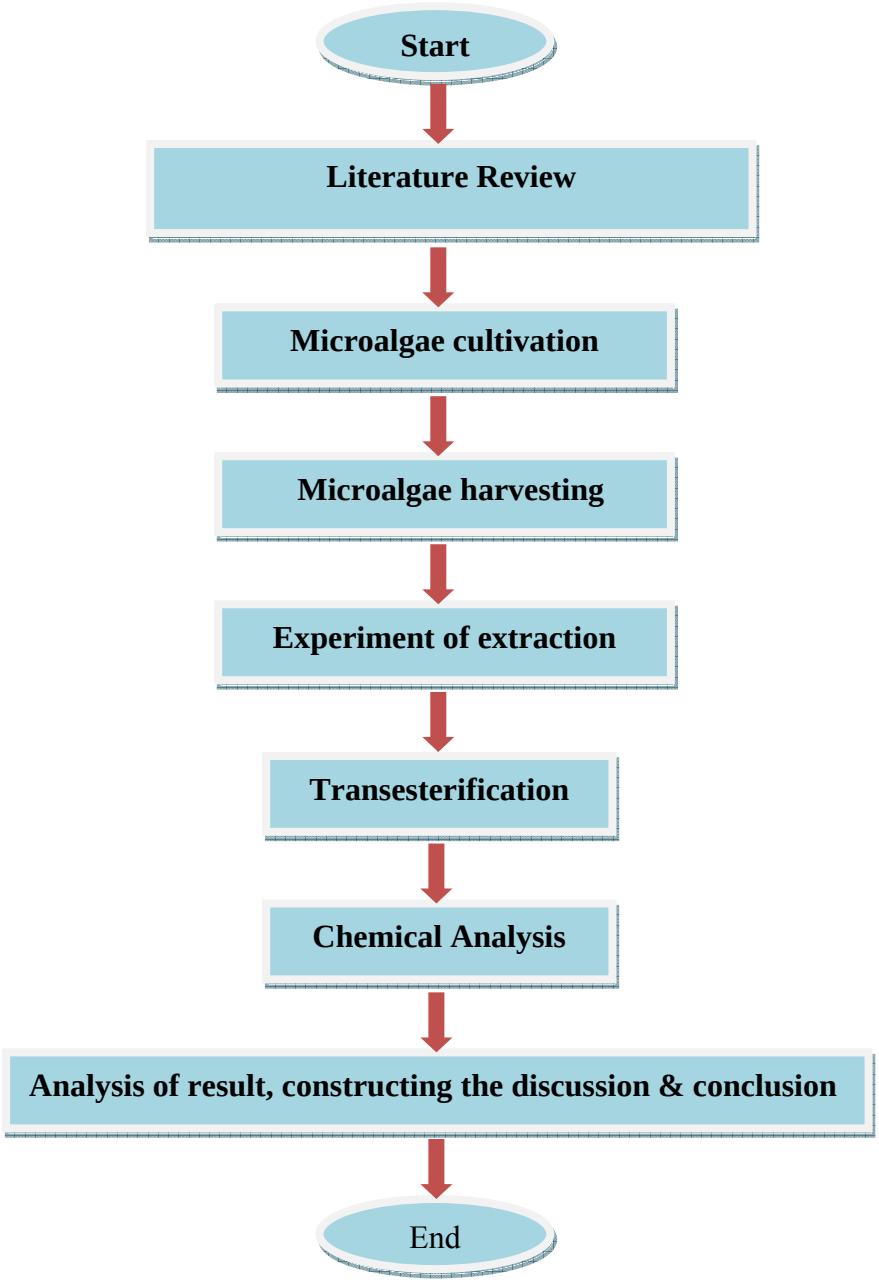


Figure 36: Flow chart of the research methodology

3.3 Gannt Chart

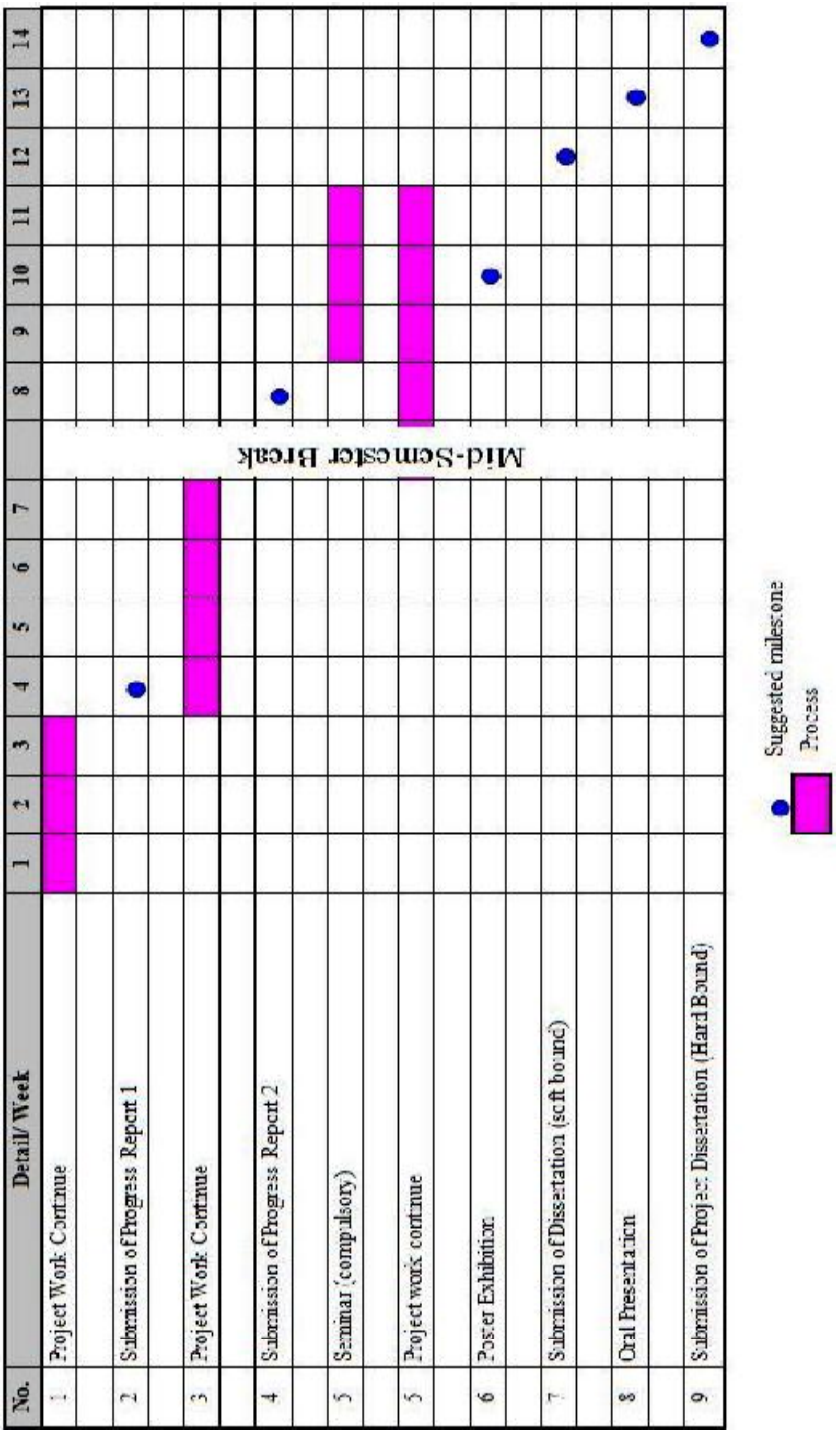


Table 3: Gannt Chart for the Final Year Project 2

CHAPTER 4

RESULT AND DISCUSSION

4.1 Growth Evaluation

Growth rate is one important way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it. From the observation of the cell growth of the *Nannochloropsis* and *Chlorella* species, the result is presented as in the graph below:

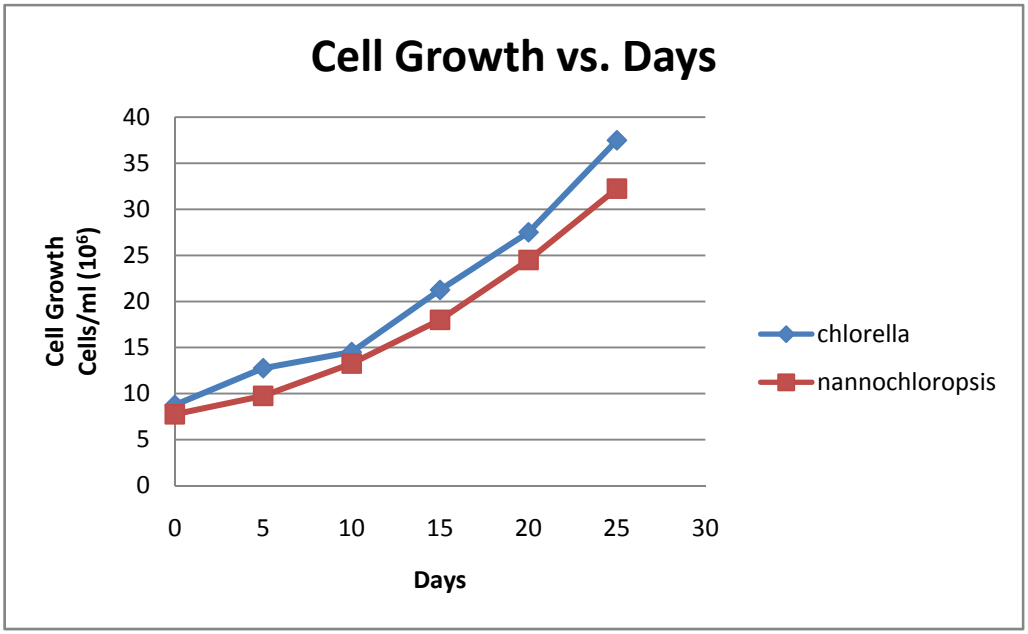


Figure 37: Growth profile of *Nannochloropsis* sp and *Chlorella* sp.

Based on the **Figure 37**, the cell growth of *Chlorella* is significantly higher that *Nannochloropsis* from day 0 until the 25th day but both of the species exhibits the same trend of growth phase. The difference between the growth rate is mainly because of the nature of the species itself. In this case, *Chlorella* has smaller cell size and higher sensitivity to nutrients thus leading to the higher growth rate. From day 0 to day 5, both of the specieses are facing the lag or induction phase and from day 6 to day 25, they have shown the exponential growth profile.

As for *Chlorella*, the cell is growing from the growth rate of 8.75×10^6 cells ml^{-1} to 37.5×10^6 cells ml^{-1} while the growth rate of *Nannochloropsis* is from 7.75×10^6 cells ml^{-1} to 32.25×10^6 cells ml^{-1}. Even though the growth of *Nannochloropsis* is slower than *Chlorella*, it can still be considered as the species with high growing rates if compared to other species such as *Tetraselmis, Isochrysis* and *Dunaliella.* Besides having the high growth rates, *Nannochloropsis* proved to be suitable as raw materials for biofuels production, due to the high oil content (L. Gouveia et al., 2009).

The doubling time is the period of time required for the microalgal cell to double in size or value. The doubling time for both species is calculated to be 2.5 days. The usual doubling time for microalgae is 1 to 2 days and for normal plant, the doubling time is 3 to 4 days. This have shown that microalgae have higher growth rate if compared to normal plant cell. The sample calculation for the doubling time is as below by using the *Chlorella* data:

Max specific growth rate, $\mu_{max} = \dfrac{\ln x - \ln x_0}{t - t_0}$

x= growth at time t

x_0 = growth at time t_0

$$\mu_{max} = \frac{\ln 37.5 \times 10^6 - \ln 14.5 \times 10^6}{(25 - 10)}$$

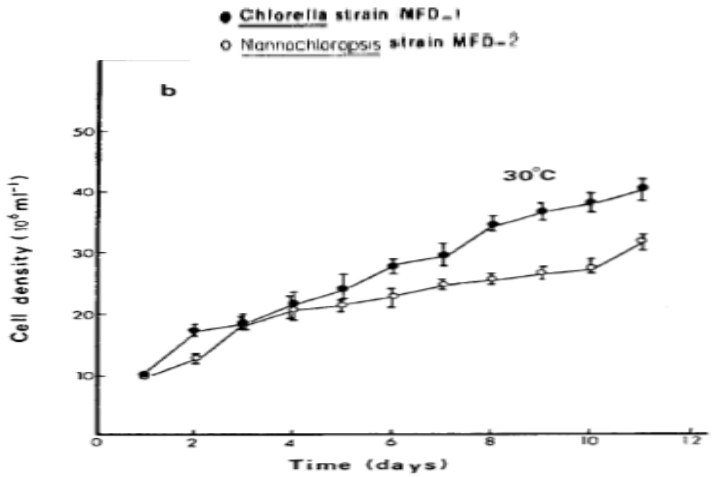
$$= 1.9$$

Doubling time, td = $\frac{\mu_{max}}{\ln 2}$

$= \frac{1.9}{\ln 2}$

≈ 2.5 days

After the lag and exponential phases, the microalgae will undergo the phase of declining relative growth, stationary phase and lastly the death phase. This result is quite similar to the trend of growth profile obtained from previous study as potrayed in **Figure 38**. The slight difference between the 2 graphs is mainly because of the different parameters used in the cultivation procedures.



(Source: James CM et al., 1990)

Figure 38: Growth profile of *Nannochloropsis* and *Chlorella* strain

4.2 Extraction of lipid

The results in the **Table 4** are obtained after 3 runs (by using different solvent systems) of oil extraction using solvent extraction method continued with rotary evaporator.

Table 4: Amount of lipid extracted by using Solvent Extraction method

No.	Mass of algae (g)	Type of solvent	Volume of the solvent (ml)	Yield		
				Mass (g)	Volume (ml)	% of yield
1	5.00	n-Hexane	100	0.13	0.3	2.6
2	5.00	Methanol	100	0.18	0.7	3.6
3	5.00	Chloroform / Methanol	100	0.14	0.4	2.8

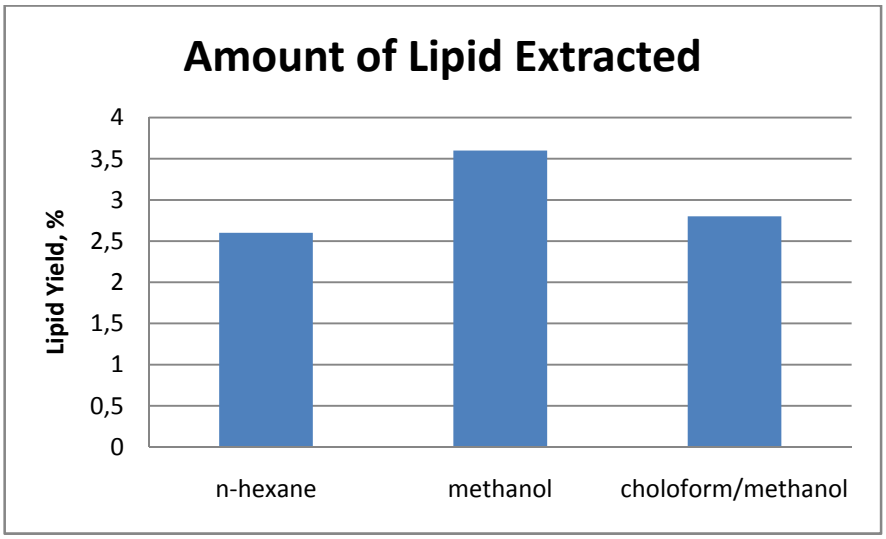


Figure 39: Extraction of lipid by using different solvents

Figure 39 shows the amount of lipid extracted from the microalgae (5 grams for each sample) by using 3 different types of solvents which are n-hexane, methanol and chloroform/methanol. The result shows that 2.6% oil are extracted by uxing n-hexane, 3.6% by methanol and 2.8% by the mixture of chloroform/methanol. It is known that for lipid extraction, alcohol would be good solvents for most lipids. In this case, methanol, as alcoholic solvent has higher ability to increase the cell wall permeability thus facilitating the lipid extraction. Chloroform is a popular solvent, particularly for lipids of intermediate polarity and when mixed with methanol it becomes a general extraction solvent. Among hydrocarbons, hexane is the most popular but is a good solvent only for lipids of low polarity. Its main use is just to extract neutral lipids from mixtures of water with alcohols.

If compared to previous study, the results of extraction methods indicated that the best procedure of lipid extraction is by using *n*-hexane as a solvent (Bligh EG, et al., 1959). This deviation might probably caused by the different extraction procedure and the species used for the extraction. In this research, methanol is proven to be the most suitable solvent for the mixed- culture of microalgae as it possess the highest ability to extract lipid from microalgae.

Referring to **Figure 39**, the amount of the lipid extracted is too small as the sample used (which is 5 grams) is insufficient for the extraction process to perform successfully. So, to increase the efficiency of the extraction process, about 10 grams or 20 grams of dried sample is needed. Besides, the other cause that may contribute to the low quantity of lipid extracted is the species of microalgae used. In this case, the mixed-culture from the pond might not be the best candidate for the bio fuel production as the species is unknown but they still can produce oil in lower quatity if compared to other well-known specieses.

4.3 Transesterification

After transesterification, the biodiesel (methyl ester) produced is weighted and the pH for all samples are determined. **Figure 40** shows the production of esterified product (in grams) from the lipid extracted using n-hexane, methanol and chloroform/methanol.

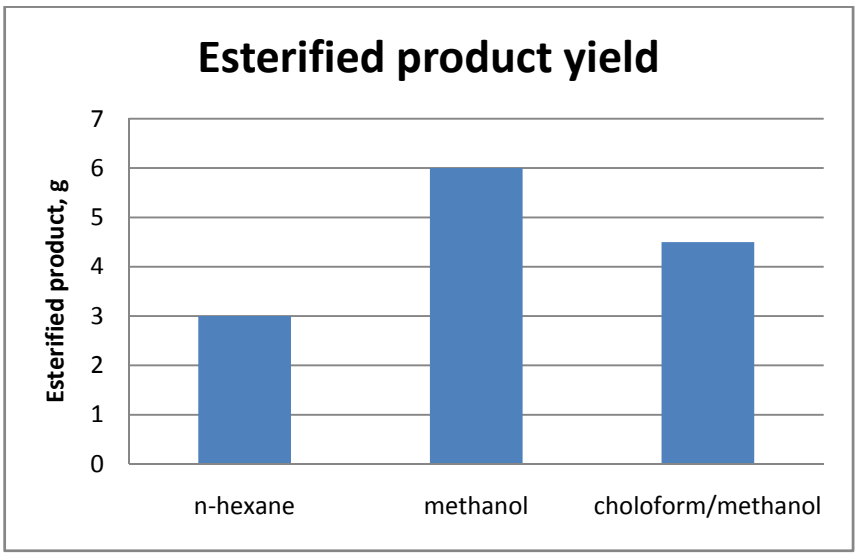


Figure 40: The production of esterified product from the oil extracted using n-hexane, methanol and chloroform/methanol

Based on **Figure 40**, the lipid extracted from n-hexane shows the least amount of esterified product yield, which is only 3 grams. As for the lipid extracted from chloroform/methanol, the esterified product weighted for about 4.5 grams. The highest amount of esterified product yield is by using the lipid extracted from methanol. So, high amount of lipid extracted will lead to high production of esterified product. It can also be concluded that methanol is the best solvent system for extracting lipid from microalgae thus leading to higher production of esterified product.

Table 5: pH measurement of the samples

No.	Type of solvent for extraction	Type of alcohol and catalyst	pH value
1	n-Hexane	Methanol + NaOH	8.89
2	Methanol	Methanol + NaOH	9.24
3	Chloroform/ Methanol	Methanol + NaOH	8.95

Based on the **Table 5**, the pH value of the three samples do not show significant difference. The readings are approximately the same which is in the range of 8.89 – 9.24. When a base is added, it gives up hydroxide (OH) to the solution thus increasing the pH value. The result might not be very accurate as the readings of the pH meter is flunctuating. The pH value that is measured by the author from the esterified product of microalgae is around 9. This is due to the unproper washing procedure of the biodiesel. If the step of washing is performed efficiently, the readings should be closer to 7 which indicates neutral.

4.4 Chemical Analysis

From the analysis obtained by using Thin Layer Chromatography (TLC), blobs are observed on the plates (**Figure 41**). The result is summarized as shown in **Table 6**.

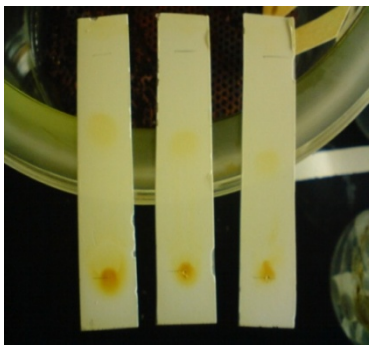


Figure 41: Blobs appear on TLC plates

Table 6: Identification of components by TLC

No.	Type of solvent for extraction	Length of blob detected (cm)	Lipid identification
1	Chloroform/Methanol	4.8	Triglyceride
2	n-Hexane	4	Triglyceride
3	Methanol	3.75	Triglyceride

Based on the result observed from the analysis (**Table 6**), it is clearly identified that the main lipid contained in the esterified product is the triglyceride. Triglycerides as neutral lipids are the main materials in the production of biodiesel. Triglycerides are also split into their components via transesterification during the manufacture of biodiesel. The presence of triglyceride in the final product proves that the esterified product has not reach the standard of pure biodiesel yet. So, improvement in the transterification process need to be done by looking at the reaction parameters and the catalyst used.

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

At the early stage of the research, the author has batch-cultivated the *Nannochloropsis* sp and *Chlorella* sp to perform the growth evaluation of microalgae. Then, centrifugation has been selected as the harvesting technique which later predeeded with vacuum drying. As the amount of dried species are too small, the author has collected new sample which are consist of the mixed-culture from the pond to proceed with the extraction procedure.

For this project, the author has selected the solvent extraction method continued with the rotary evaporator to extract the lipid from microalgae. The solvents that are used for the extraction are n-hexane, methanol and chloroform/methanol. The result from the extraction has shown that methanol is the best solvent to extract the lipid from the mixed-culture of microalgae. The selection of this technique is made based on the ease of application.

Transesterification is also performed in order to convert the oil to methyl ester. Based on the result, the lipid extracted by methanol exhibits the highest yield of esterified product. The pH value of the esterified product is approximately equal for all samples which are in the range of 9.

The chromatographic technique used for identifying simple main lipids is by using thin-layer chromatography (TLC). From the analysis, triglycerides are identified as the main lipid contained in the final esterified product. This has shown that the esterified product derived from transesterification has not reach the standard of pure biodiesel yet. So, improvemnet on reaction parameters need to be done to further purify the esterified product. TLC is chosen for the chromatographic procedure as it is fast, simple, and reproducible if it is to be performed repeatedly.

5.2 Recommendation

Upon completing this research, there are several recommendations to be made in order to produce better methodology and technique of bio oil extraction in the future:

1. Different types of microalgae samples or specieses from different locations should be used to optimize the methodology of bio oil production.
2. The amount of dried algae to be extracted should be increased for at least 10 grams to increase the efficiency of extraction process.
3. The samples should be analyzed by using Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) method instead of using Thin Layer Chromatography (TLC) to obtain precise composition in the product quantitatively.
4. The physical properties for bio oil / biodiesel such as density, kinematic viscosity and specific gravity need to be tested and determined for comparison with the standard of biodisel.
5. The chlorophyll from the microalgae have to be removed first before using them for extraction as they will affect the purity of the oil extracted.
6. The stoichiometric of equation for the reactions should be analysed in detail in order to develop the most efficient method for extraction or transesterification.
7. The composition of the lipid extracted from microalgae need to be investigated further.

REFERENCES

1. Barkley WCW, Lewin RA, Cheng L. In: Sadler T, editor. Development of Microalgal Systems for the Production of Liquid Fuels. Villeneuve d'Ascq, France, Elsevier Applied Science, 1987.
2. De la Noue J, De Pauw N. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol Adv. 1988;6:725–770.
3. Demirbas A. Biomass resource facilities and biomass conversion processing for fuels and chemicals. Energy Convers Manage. 2001;42:1357–1378.
4. Demirbas_ A. Biomass resources for energy and chemical industry.Energy Educ Sci Technol. 2000;5:21–45.
5. Milne TA, Evans RJ, Nagle N. *Catalytic conversion of microalgae and vegetable oils to premium gasoline, with shape-selective zeolites*. Biomass 1990;21: 219–32.
6. Yanqun Li et al. Biofuels from microalgae. Biotechnol. Prog. (2008), doi 10.1021/bp.070371k
7. Amin, S. (2009). *Review on biofuel oil and gas production processes from microalgae,* Agency for Assessment and Application of Technology (BPPT). Retrieved on April 19, 2009 from www.elsevier.com/locate/enconman.
8. Tsukahara K, Sawayama S. Liquid fuel production using microalgae. J Jpn Petrol Inst 2005;48(5):251–9.
9. McKendry P. Energy production from biomass (part 2): conversion technologies. Biores Technol 2003;83:47–54.
10. Morton Satin, Chief Agro-Industries and Post-Harvest Management Service. (2008). *Microalgae.* Retrieved from http://www.fao.org/ag/ags/Agsi/MICROALG.htm

11. FAO Corporate Document Repository. (2004). *Renewable biological systems for alternative sustainable energy production.* Agriculture and Consumer Protection: Author. Retrieved from http://www.fao.org/docrep/w7241e/w7241e0h.htm
12. Benemann, J., "Overview: Algae Oil to Biofuels", NREL-AFOSR Workshop, Algae Oil for Jet Fuel Production, Arlington, VA, February 19, 2008, *www.nrel.gov/biomass/pdfs/benemann.pdf*
13. Yang C, Hua Q, Shimizu K. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic lightautotrophic/ dark-heterotrophic conditions. Biochem Eng J 2000;6:87–102.
14. Akazawa K, Okamoto K. Biosynthesis of sucrose. In: Preiss J, editor. The biochemistry of plants, vol. 3. New York: Academic press; 1980. p. 199–218.
15. Stumpf PK. Fatty acid biosynthesis in higher plants. In: Fatty acid metabolism and its regulations. New York: Elsevier; 1984.
16. Meireles LA, Guedes AC, Malcata FX. Lipid class composition of the microalga Pavlova lutheri: eicosapentaenoic and docosahexaenoic acids. J Agri Food Chem 2003;51:2237–41.
17. Shen T, Wang JY, editors. Lipids [A]. In Biochemistry [M]. 2nd ed. Beijing: Advanced Education Press; 1989. p. 44–51.
18. Huang G et al. Biodiesel microalgal biotechnology. Appl Energy (2009), doi:10.1016/j.apenergy.2009.06.016
19. Sawayama S, Minowa T, Yokoyama SY. Possibility of renewable energy production and CO2 mitigating by thermochemical liquefaction of microalgae. Biomass Bioenergy 1999;17:33–9.
20. Fuls J, Hawkins CS, Hugo FJC. Tractor engine performance on sunflower oil fuel. J Agri Eng Res 1984;30:29–35.
21. Canakci M, Vangerpen JH. Biodiesel production via acid catalysis. Trans ASAE 1999;42:1203–10.
22. Gerpen JV. Biodiesel processing and production. Fuel Process Technol 2005;86:1097–107.

23. Fukuda H, Kondo A, Noda H. Biodiesel fuel production by transesterification of oils. J Biosci Bioeng 2001;92:405–16.
24. American Society for Testing and Materials. Standard specification for biodiesel fuel (b100) blend stock for distillate fuels. Designation D6751-02. West Conshohocken (PA): ASTM International; 2002.
25. Hama S, Tamalampudi S, Fukumizu T, Miura K, Yamaji H, Kondo A, et al. Lipase location in Rhizopus oryzae cell immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. J Biosci Bioeng 2006;101:328–33.
26. Scragg AH, Ilman AM, Carden A, Shales SW. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy 2002;23:67–73.
27. Ugwu CU, Aoyagi H, Uchiyama H. Photobioreactors for mass cultivation of algae. Biores Tech 2008;99:4021–8.
28. Dote Y, Sawayama S, Inoue S, Minowa T, Yokoyama, Shin-ya. Recovery of liquid fuel from hydrocarbon-rich microalgae by thermochemical liquefaction. Fuel 1994;73:1855–7.
29. Wikipedia. Algaculture. http://www.en.wikipedia.org/wiki/Algaculture
30. Morales J, Noue J, Picard G. Harvesting marine microalgae species by chitosan flocculation. Aquacull Eng 1985;4:257–70.
31. Lee, J.-Y., et al. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. (2009), doi:10.1016/j.biortech.2009.03.058
32. Oilgae.com. Extraction of Algal Oil by Chemical Methods. http://www.oilgae.com/algae/oil/extract/che/che.html
33. Mukhopadhyay M., 2006, "*Natural Extracts Using Supercritical Carbon Dioxide"*, Department of Chemical Engineering, Indian Institute of Technology, Bombay, pg3-5.
34. Aresta, M., Dibenedetto, A., & Barberio, G. (2004). *Utilization Of Macro-Algae For Enhanced CO_2 Fixation And Energy Production.* University of Bari, Department of Chemistry.

35. Wikipedia.Expeller Pressing. http://www.en.wikipedia.org/wiki/Expellerpressing
36. Wikipedia.SoxhletExtraction. http://www.en.wikipedia.org/wiki/Soxhletextraction
37. Sarker SD, Zahid Latif, Gray AI., 2006, "*Natural Products Isolation*", Humana Press Totowa, New Jersey, pg 307.
38. Ruan R, *Mass Culture of AlgaeMass Culture of Algae from Waste Water for Biofuels Production*, Department of Bioproducts and Biosystems Engineering, 2007
39. Mahesar, S.A., Sherazi, S.T.H., Abro, K., Kandhro, A., Bhanger, M.I., Van de Voort, F.R., Sedman, J., 2008. Application of microwave heating for the fast extraction of fat content from the poultry feeds. Talanta 75, 1240–1244.
40. Bligh EG, Dyer WJ (1959) A rapid method of lipid extraction and purification. Can J Biochem Physiol 37:911–917
41. European Standard EN 14111 (2003) Fat and oil derivatives— fatty acid methyl esters (FAME)—determination of iodine value
42. Ehrman T, *Standard Method for the Determination of Extractives in Biomass*, Laboratory Analytical Procedure #010, 1994
43. V Palanisamy, Faazaz, Rashidah, *A Guide on the Production of Algal Culture for Use in Shrimp Hatcheries*, K. Lumpur: Department of Fisheries, Minister of Agriculture Malaysia, 1991.
44. James CM, S. Al-Hinty and AE Salman, *Aquaculture*, 77 (1989) 337-351, Elsvier Science Publishers B.V., Amsterdam

APPENDIX A

Sample of calculation for cell count:

Nannochloropsis (day 5)

Above	Below
45	30
42	35
43	43
38	39
41	34

$$\text{Average cell} = \frac{45 + 42 + 43 + 38 + 41 + 30 + 35 + 43 + 39 + 34}{10}$$

$$= 39$$

$$\text{Number of cells} = \frac{\text{average cell}}{\text{deep x area}} \quad (\text{Unit} = \text{cell ml}^{-1})$$

$$= \frac{39 \text{ cell}}{0.1 \text{ mm} \times 0.04 \text{mm}} \times \frac{1000 \text{ mm}^3}{1 \text{ ml}}$$

$$= 9.75 \times 10^6 \text{ cell ml}^{-1}$$