Effect Of Nutrient Deficient Media & Environmental Stressors On Microalgal Growth

by

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Dissertation submitted in partial fulfillment of

the requirement for the

Bachelor of Engineering (Hons)

(Chemical Engineering)

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CERTIFICATION OF APPROVAL

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Approved by,

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(AP DR M Azmuddin Abdullah)

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH PERAK

MAY 2011

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 persons.

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(Ahmad Syazwan b Zainal Ariff)

ABSTRACT

Research on microalgae has been greatly developed as microalgae are one of the new fast solution's to many of world's problems such biofuel, environmental remediation, aquaculture and hunger. Microalgae are great factory because of their components that can be extracted and converted into many kinds of product. Every single cell of microalgae is a tiny factory which produces certain useful products. A few of the important extracted product are lipid, carbohydrate and protein, and different species of algae produce different composition of this amount.

In order to produce valuable products, it is very important to view algae culture under different environmental stressors to maximize algae production. The studies in this project are based on four different species, which are *Isochrysis galbana spp*., *Tetraselmis batan spp*., *Nannochloropsis spp*. and *Pavlouva lutheri spp*. The optimum number of cells and kinetic growth of 25^0C, pH 7, and 24 hours normal light intensity are determined. The extractions of lipid from both fresh and dried algae are done. Different environmental stressors are given to *Pavlouva lutheri spp* (salinity and photoperiod) and the optical density and amount of lipid content are verified.

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INTRODUCTION

1.1 Background

Moving into the new era, the annual world primary consumption has estimated oil to be at 11, 000 million tones equivalent. Fossil fuels has been accounted for 88% of the primary energy consumption, with oil (35% share), coal (29%) and natural gas (24%) as the major fuels, while nuclear energy and hydroelectricity account for 5% and 6% of the total primary energy consumption, respectively. Given the current technological progress, potential reserves, and increased exploitation of newer unconventional reserves, it is highly probable that fossil fuels will continue to be available for a considerable period of time.(Brennan et al, 2010)

Unfortunately, the potential threat of global climate change has increased, and for a major part, this has been attributed to greenhouse gas emissions from fossil fuel usage. The associated climatic change projections could have major consequences for nature as well as human systems, which creates uncertainty regarding the sustainability of current fossil fuel use, not only in relation to the finiteness of the resource, but also on the negative effects of CO2 emissions.(Brennan et al, 2010) Hence, because of many of these reasons, the stress to find alternative to fossil energy has increased.

As the world tries to wean off fossil fuels, it is turning to renewable energy such as wind, solar, hydroelectric and biomass. Algae has emerged as a promising feedstock for future bioenergy due to its high energy content, energy yield per acre, fast growth and ability to grow in water of varying quality. Algae's potential, at least in theory, is remarkable. According to the U.S. Department of Energy (DOE), algae may be able to produce 100 times more oil per acre than soybeans, currently the leading source of U.S. biodiesel or any other terrestrial oil. Among the important factors is because of its high energy content and oil from algae can be refined into biodiesel, green gasoline, jet fuel or ethanol. Lastly, algae need only water, sunlight and CO2 to grow and it grows rapidly.[1] Today, algae have been solution to many of world's problem such as hunger in Africa, and aquaculture.

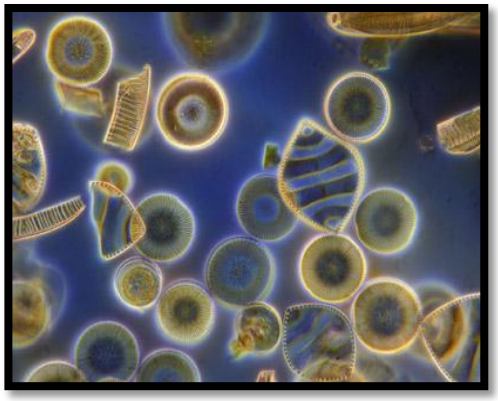


Figure 1 : Diatom microalgae Figure 2 : *Cladophora*

1.2 Problem Statement

Microalgae are an ideal choice as it is not interfering with world food chain, animal and plant eco population. Nowadays, microalgae have been developed for many purposes. To achieve this objective, large scale of strong and healthy known species microalgae need to be ready. The basic principle of microalgae cultivation for some species is unknown to man. Hence, culture condition of microalgae need to be studied to increase growth rate of microalgae. Likewise, algae are a kind of plant and basic source for life are light, water, salt, nutrients (food). This conditions need to be studied to obtain best growth of algae. As microalgae produces lipid for biofuel, protein and carbohydrate for supplement, whether environmental stressors can produce significant amount of these component or not is still a debatable point.

1.3 Objetive/ Scope of Study

The objectives of this research are:

a) To establish kinetics of microalgal cell growth
b) To study the effect of different environmental stressors such as temperature, light period, pH and salinity on cell growth and lipid content of algae
c) To study lipid content on algae with nutrient deficient media and environmental stressors

CHAPTER 2

LITERATURE REVIEW

2.1 Algae

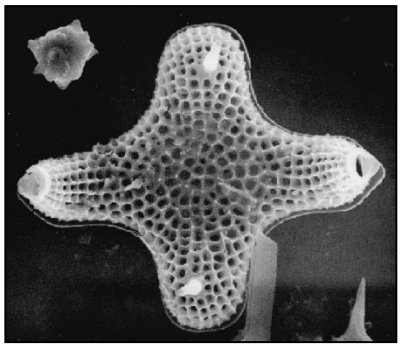
Algae are heterogeneous assemblage of photosynthetic organisms, one of the most vast and diverse groups of ancient eukaryotic organisms, about 30 000 known species. Algae are classified into two, macroalgae and microalgae. Mainly, algae are microscopic and macroscopic, unicellular, colonial, or multicellular, mobile and immobile, attached and free-living. Algae are widespread in water, like seas, lakes, rivers and soil habitats, at different geographic latitudes, and on all continents. They occur in waters with different degrees of salinity, organic matter, hydrogen ions, and at various temperatures. They include planktonic, periphytonic and benthic organisms. Algae are unique model organisms in evolutionary biology and also are used in various genetic, physiological, biochemical, cytological, and other investigations. Algae have practical significance as edible or poisonous plants, as indicator organisms in the monitoring of ecological systems, as agents of self-purification of polluted waters and in the purification of sewage, as the primary producers in the trophic chains of hydrobionts in marine and freshwater, and also as organisms for biotechnology.[2]

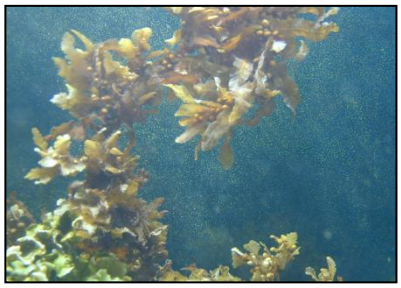
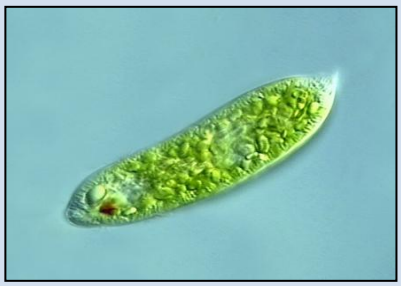
Algae can reproduce sexually and asexually. Most algae reproduce asexually, but however, the proper environmental stimulus may initiate sexual reproduction, heterogamy and isogamy. The algae have evolved many variations in sexual reproduction such as different types of gametes, different means of gamete transfer, and different locations of fertilization. Asexual reproductions of algae are through daughter colony formation and sporulation.[3] The number of algal species estimates to be one to ten million, and most of them are microalgae (Laura et al, 2005).

Algae can be classified into seven phylum. These divisions are separated on the basis of various features including their morphology and the biochemistry of their pigments, cell walls and energy storage compounds. The colors of these various algae types differ according to their particular mixtures of photosynthetic pigments, which typically include a combination of one or more chlorophylls and various accessory pigments.

Table 1 : Seven Phylums of Algae[4]

Algae	Classification
Chlorophyta (Green Algae) 	• May be unicellular, multicellular, or colonial • Include *Spirogyra*, *Ulva*, & *Chlamydomonas* • Contain chlorophyll a & chlorophyll b and carotenoids (orange & yellow pigments) as accessory pigments • Store food as starch • Cell walls mainly cellulose, but some marine forms add $CaCO_3$ • Habitat may be freshwater, moist surfaces, or marine environments • Some have whip-like flagella for movement • May live symbiotically as lichens • Thought to have given rise to terrestrial plants
Phaeophyta (Brown Algae) 	• Contain chlorophyll a & chlorophyll c and fucoxanthin (brown pigment) as accessory pigments • Most are multicellular growing in cooler marine habitats • Include kelps & seaweeds • Largest protists • Specialized rootlike holdfasts anchor thallus to rocks • Specialized air bladders keep leaflike blades afloat near surface to get light for photosynthesis • Stemlike structures are called the stipe and support the blades • Store food as a carbohydrate called laminarin
Rhodophyta (Red Algae) 	• Multicellular algae that mainly grow deep in warm marine waters • Some freshwater species exist • Highly branched thallus • Contain chlorophyll a & phycobilins (red pigments) to trap sunlight for photosynthesis • Store food as starch • Cell walls contain cellulose and agar (used as a base in culture dishes to grow microbes)

	• Some species contain carageenan in their cell walls used for gelatin capsules & in some cheeses
Bacilliarophyta (Diatoms) 	• Abundant in marine & freshwater habitats • Called phytoplankton & start many aquatic food chains • Contain chlorophyll a & c, carotenoids (orange pigments), & xanthophyll (yellow pigments) • Store food as starch & contain mainly cellulose in their cell walls • Lack cilia & flagella • Have glass like shells or valves containing SiO_2 that fit together in 2 parts • Centric diatoms are marine & have circular or triangular shells • Pennate diatoms are found in freshwater & have rectangular shells • When diatoms die, they form a layer called diatomaceous earth that is abrasive and used in detergents, toothpaste, fertilizers
Dinoflagellata 	• Major producers in marine habitats • Small, unicellular organisms making up plankton • Many are photosynthetic, but some are colorless heterotrophs • Photosynthetic dinoflagellates are yellow to brown in color due to chlorophyll a & c and carotenoids • Have 2 flagella that spin and move the dinoflagellate through water • Store food as starch • Some dinoflagellates are covered with armor like plates & spines made of cellulose • Often undergo algal blooms where their numbers greatly increase • Produce a toxic substance and cause poisonous red tides

Chrysophyta (Golden Algae) 	• Most are live in freshwater habitats, but some are marine • Unicellular algae containing chlorophyll a & c and the brown pigment fucoxanthin and carotenoids • Many have flagella for movement • May be naked or have cellulose cell walls or silica scales or shells • May form highly resistant cysts to survive beneath frozen lake surfaces in winter
Euglenophyta 	• Unicellular algae that lack cell walls • Have a flexible protein covering called the pellicle • Called euglenoids • Possess chlorophyll a & b and carotenoids • Store food as paramylon (polysaccharide) • Most live in freshwater, but some live in moist soil & the digestive tracts of certain animals

2.1.1 Microalgae

Microalgae comprise a vast group of photosynthetic, heterotrophic organisms which have an extraordinary potential for cultivation as energy crops. Microalgae are called microfytes. Its size range from few micro meters to hundred micro meters. Up till now, there are around 200 000 to 800 000 species of miro algae has been found.(Hyuen Wu, 2010). They can be cultivated under difficult agro-climatic conditions and are able to produce a wide range of commercially interesting byproducts such as fats, oils, sugars and functional bioactive compounds.

As a group, they are of particular interest in the development of future renewable energy scenarios. Now, the harvesting and transportation costs of algae species are lower that with conventional crops and their small size allows for a range of cost-effective processing options. They are easily studied under laboratory conditions and can effectively incorporate stable isotopes into their biomass, thus allowing effective genetic and metabolic research to be carried out in a much shorter period than conventional plants. Microalgae are indispensable in the commercial rearing of various

species of marine animals as a food source for all growth stages of bivalve molluscs, larval stages of some crustacean species, and very early growth stages of some fish species.

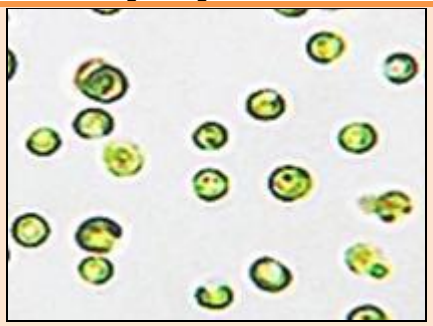
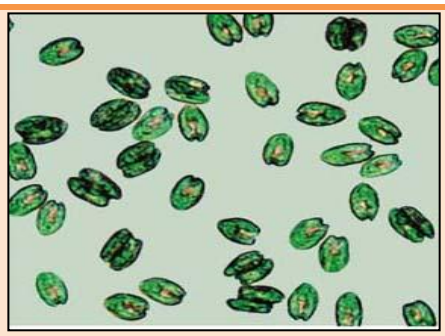
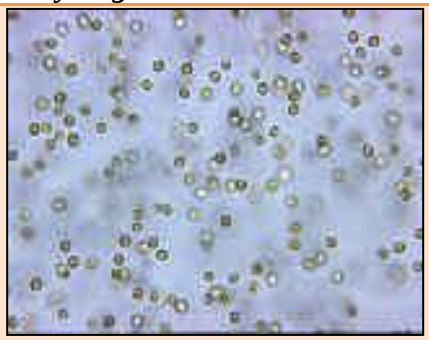
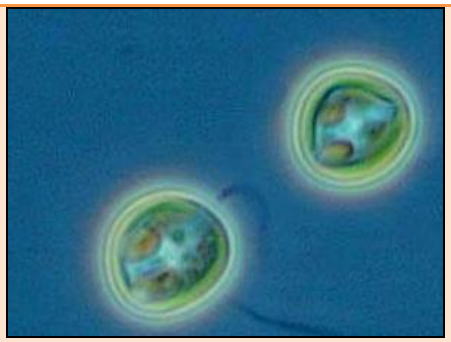
Nanochloropsis sp	***Tetraselmis batan***
	
Isochrysis galbana	***Pavlouva lutheri***
	

Figure 3 : Microalgae

2.1.2 Macroalgae

Macroalgae are primitive photosynthetic plants that include the single celled 'phytoplankton' of the multi-celled macroalgae, or seaweeds, that can range in size from microscopic to the massive bull kelps (*Durvillaea*) and giant kelps (*Macrocystis*) . The latter are closely related to land plants as they have roots, vascular tissue produce flowers and pollen.[5]

Macroalgae derive all their nutrients directly from the surrounding water through their tissue, a bit like a sponge soaks up moisture, and their holdfasts are purely for

physically anchoring the thallus to the seabed. Macroalgae reproductive structures are mostly microscopic and require fine dissection to be revealed.

Macroalgae are strictly benthic plants; that is they are always attached to the seabed or a solid substratum such as natural reef, rocks, shells, mangrove roots, boat hulls, jetty piling mooring lines. When dislodged, most macroalgae have a limited lifespan as free floating seaweed drift and they may only live for hours to several months. Macroalgae grows both intertidally and subtidally. Because they derive their nutrients by diffusion through their tissue, the water movement across fronds has to be continually refreshed and by being anchored to the seabed, they increase their chances of this. When floating with the curents and tides, the water surrounding them immediately is not replenished as rapidly.[5] .Macroalgae also have potential usage as functional food and helps into human health. (Fitzgerald et al, 2011)

Since macroalgae are true photosynthetic organisms, they can only grow in the photic zone of the coastal regions, where the light penetrates sufficiently for photosynthesis to occur. In clear waters, macroalgae can survive and grow at depths of over 200 metres, but in murky water this is reduced to only a few metres. [5]

Hormosira Banksii	*Oodium Isthmoclaclum*
	
"Nuisance" Macroalgae	*Caulerpa Nummularia*
	

Figure 4 : Macroalgae

2.2 Usage of Algae

2.2.1 Microalgae for Biofuel

Microalgae can potentially be employed for the production of biofuels in an economically effective and environmentally sustainable manner. Microalgae have been investigated for the production of a number of different biofuels including biodiesel, bio-oil, bio-syngas, and bio-hydrogen. The production of these biofuels can be coupled with flue gas CO2 mitigation, wastewater treatment, and the production of high-value chemicals. Microalgal farming can also be carried out with seawater using marine microalgal species as the producers. Developments in microalgal cultivation and downstream processing (e.g., harvesting, drying, and thermochemical processing) are expected to further enhance the costeffectiveness of the biofuel from microalgae strategy. (Li et al,2008). The advantages of using microalgae-derived biofuels are:

a) microalgae are capable of all year round production, therefore, oil productivity of microalgae cultures exceeds the yield of the best oilseed crops(Brennan et al 2009)
b) they grow in aqueous media, but need less water than terrestrial crops therefore reducing the load on freshwater sources (Brennan et al, 2009)
c) microalgae can be cultivated in brackish water on non-arable land, and therefore may not incur land-use change, minimizing associated environmental impacts while not compromising the production of food(Brennan et al, 2009)
d) National Energy Security is an achievable goal with Algae Oil
e) Environmental Enhancement through zero sulfur and the sequestration of CO2
f) After oil is extracted, the remaining press cake is highly valuable product

Biodiesel is produced by a mono-alcoholic transesterification process, in which triglycerides reacts with a mono-alcohol (most commonly methanol or ethanol) with the

catalysis of alkali, acids, or enzymes. It has combustion properties similar to those of diesel and has been produced commercially or in backyard facilities to fuel vehicles. Significant technical advances have been achieved to optimize the transesterification process. Some microalgae species could accumulate lipids to a significant portion of their biomass (30–50% on dry weight basis), serving as a promising alternative source of lipids for biodiesel production. One major challenge of biodiesel production is the high costs of feedstock. Currently, biodiesel production relies on animal fats and plant oils. This agricultural approach will eventually compete for land resource against food industry.(Li et al 2008)

The free fatty acids were converted into esters using a proprietary method developed by Renewable Energy Group, Inc. The determination of the fatty acid profile was based on AOCS Method Ce 1c-89 using a PerkinElmer Inc. Clarus 600 GC-FID equipped with a Supelco SP 2340 fused silica column The helium flow was 2.0 mL min_1 at 1.6 psi and the FID temperature was 210 C. Biodiesel was diluted to a 1% solution in heptane before injection. The core properties of biodiesel such as free glycerin and total bound glycerin were measured in a GC . (Chinnasamy S et al, 2010).

The microalgal biomass has relatively high water content (80-90%) and this is major bottleneck for usage in energy supply. As most other virgin biomass, the high water content and inferior heat content makes the microalgal biomass difficult to be used for heat and power generation. Thus necessitating pre-treatments to reduce water content and increase the energy density. As consequence the energy cost increases and makes the alternative less economically attractive. Direct hydrothermal liquefaction in sub-critical water conditions is a technology that can be employed to convert wet biomass material to liquid fuel. .(Vishwanath P et al, 2008).

This technology is believed to mimic the natural geological processes thought to be involved in the formation of fossil fuel, but in the time scaleof hours or even minutes. A number of technical terminologies have been used in the literature to refer to this technology, but it essentially utilize the high activity of water in sub-critical conditions in order to decompose biomass materials down to shorter and smaller molecular

materials with a higher energy density or more valuable chemicals.(Vishwanath P et al, 2008).

An example of production of microalgae for biofuel at large scale is shown below.

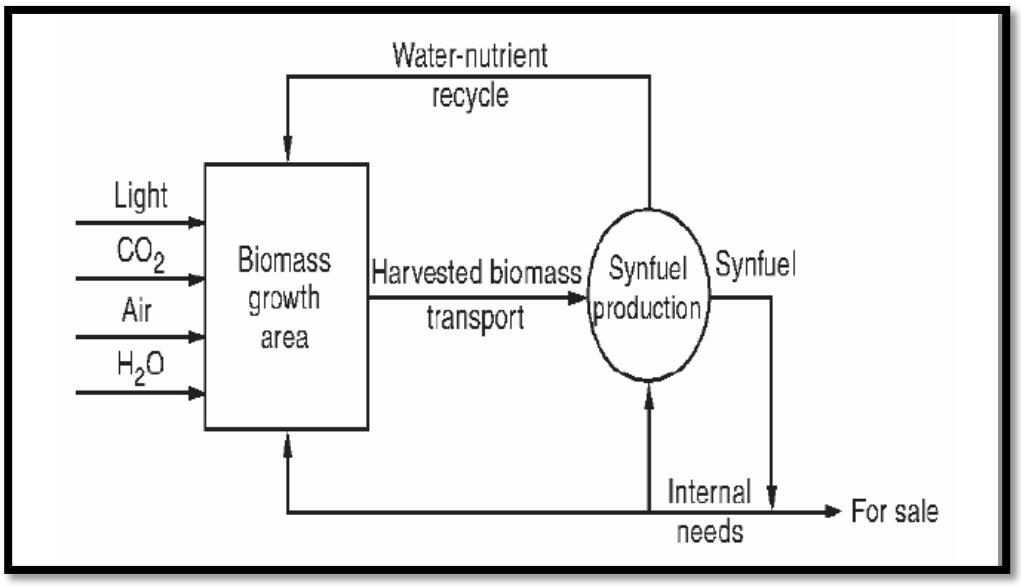


Figure 5 : A conceptual model for biomass production of algae

2.2.2 Microalgae in Aquaculture

Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs likes oysters, scallop and clams for the larval or early juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food chains. Over the last four decades, several hundred microalgae species have been tested as food, but probably less than twenty have gained widespread use in aquaculture. Microalgae must possess a number of key attributes to be useful aquaculture species. They must be of an appropriate size for ingestion, for example from 1 to 15 μm for filter feeders; 10 to 100 μm for grazers and readily digested. They must have rapid growth rates, be amenable to mass culture, and also be stable in culture to any fluctuations in temperature, light and nutrients as may occur in hatchery systems. Finally, they must have a good nutrient composition, including an absence of toxins that might be transferred up the food chain.(Brown, 2002)

Isochrysis Galvana., Pavlova lutheri and *Chaetoceros calcitrans* are the most common species used to feed the larval, early juvenile and broodstock (during hatchery conditioning) stages of bivalve mollusks, these are usually fed together as a mixed diet. Many of the strains successfully used for bivalves are also used as direct feed for crustaceans (especially shrimp) during the early larval stages, especially diatoms such as *Skeletonema spp*. and *Chaetoceros spp.*

2.2.3 Algae in Environmental Remediation

Another important usage of algae is that it can act as an environmental remediation in waste water treatment and also as carbon avoidance (Brune et al, 2010). It can reduce the need for amounts of toxic chemicals products than are already in used. It can also capture some fertilizer in runoff of farms and thus, reduce the pollution with surplus nitrogen or phosphorus. In addition, with algae respiration and photosynthesis process, it recycles carbon in the atmosphere.

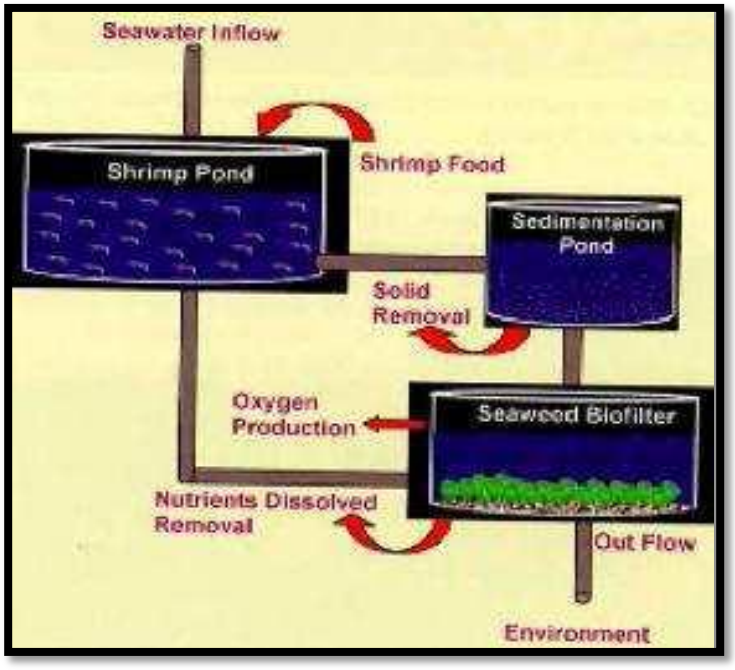


Figure 6: Sewage treatment using algae

In this system, the waste water goes to different tanks, where the nutrient salts are absorbed by the algae and other microorganisms. The water then flows until the environment, free from the majority of pollutants.

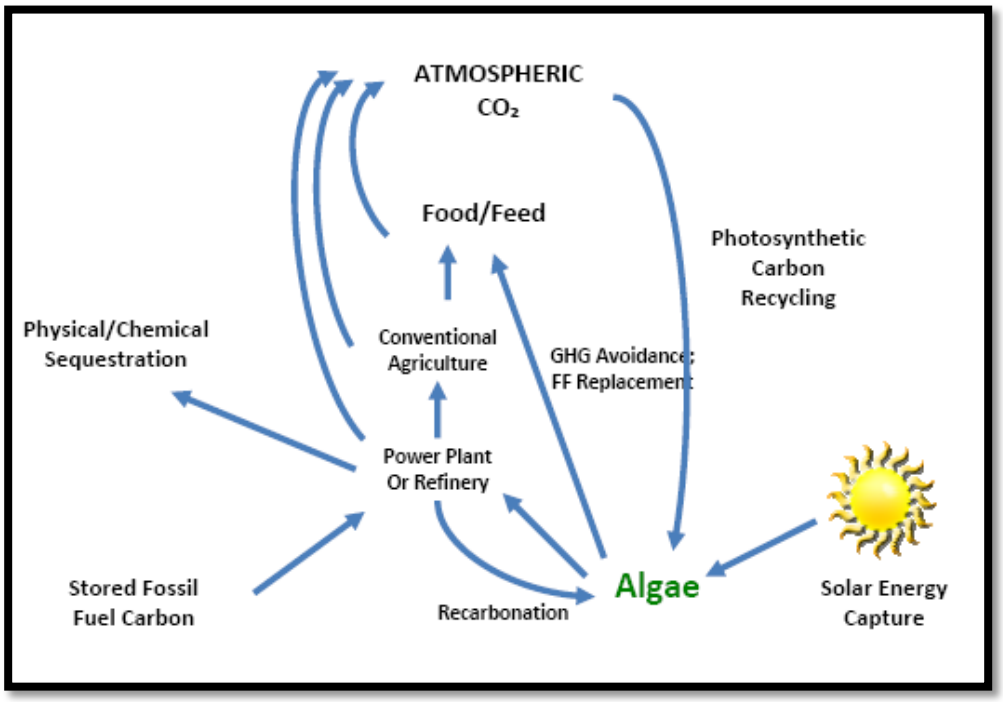


Figure 7: Algae for Carbon Avoidance and Energy Efficient

2.3 Microalgae Cultivation

2.3.1 Photobioreactor

A photobioreactor is a bioreactor which incorporates some type of light source to provide photonic energy input into the reactor. The term photobioreactor mostly only refers to closed systems, systems closed to the environment having no direct exchange of gases and contaminants with the environment. Many other microalgae are promising for the production of an enormous variety of compounds. Also, to cultivate these algae and their products, monocultures have to be maintained and for that, enclosed photobioreactors have to be used.(Tredici. M.R.et al 1999) A photobioreactor can be described as an enclosed, illuminated culture vessel designed for controlled biomass production of phototrophic liquid cell suspension cultures. Photobioreactors, despite their costs, have several major advantages :

- prevent or minimize contamination, permitting axenic algal cultivation of cultivating monocultures (culture consisting of only one species of microalgae),
- offer better control over biocultural conditions (p_H, light, carbon dioxide, temperature).
- lower carbon dioxide losses due to out gassing,
- permit higher cell concentrations.

On the other hand, certain requirements of photobioreactors: cooling, mixing, control of oxygen accumulation and biofouling, make these systems more expensive to build and operate than ponds. New cheaper innovative systems are being designed and waste streams are used to make the production of microalgae commercially attractive. (Tredici. M.R. et al ,1999)

Figure 8 : Tubular Photobioreactor

Figure 9 : Algae bioreactor

Figure 10 : Photobioreactors in Negava Desert

Figure 11 : Photobioreactor In "Sculpture"

2.3.2 Open pond

Open ponds are highly vulnerable to contamination by other microorganisms, such as other algal species or bacteria. Thus cultivators usually choose closed systems for monocultures. Open systems also do not offer control over temperature and lighting. The growing season is largely dependent on location and, aside from tropical areas, is limited to the warmer months.

Open pond systems are cheaper to construct, at the minimum requiring only a trench or pond. Large ponds have the largest production capacities relative to other systems of comparable cost. [14]Also, open pond cultivation can exploit unusual conditions that suit only specific algae. For instance, *Spirulina sp.* thrives in water with a high concentration of sodium bicarbonate and *Dunaliella salina* grow in extremely salty water. Open culture can also work if there is a system of culling the desired algae and inoculating new ponds with a high starting concentration of the desired algae.(AlgaeTech Laboratory 2011)

Some chain diatoms fall into this category since they can be filtered from a stream of water flowing through an outflow pipe. A "pillow case" of a fine mesh cloth is tied over the outflow pipe allowing other algae to escape. The chain diatoms are held in the bag and feed shrimp larvae (in Eastern hatcheries) and inoculate new tanks or ponds.

Enclosing a pond with a transparent or translucent barrier effectively turns it into a greenhouse. This solves many of the problems associated with an open system. It allows more species to be grown; it allows the species that are being grown to stay dominant; and it extends the growing season – and if heated the pond can produce year round.[14]

Figure 12 : Open pond for Spirulina

Figure 13 : Large Scale Cultivation

Table 2 : Comparison of open pond and bioreactor

Parameters	Relative	Notes
Contamination risk	Ponds > PBRs	Much reduced for PBRs
Space required	Ponds > PBRs	A matter of productivity
Productivity	Ponds < PBRs	PBRs 3-5 times more productive
Water losses	Ponds > PBRs	Some PBRs is designed with temperature control
CO2 losses	Ponds ~ PBRs	Depends on pH, alkalinity
02 Inhibation	Ponds < PBRs	O2 greater problem in PBRs
Process Control	Ponds < PBRs	Very important in PBRs
Biomass concentration	Ponds < PBRs	3-5 times in PBRs
Capital/Operating Cost ponds	Ponds << PBRs	Ponds are cheaper to construct and operate

(AlgaeTech Laboratory, 2011)

2.4 Growth Curve

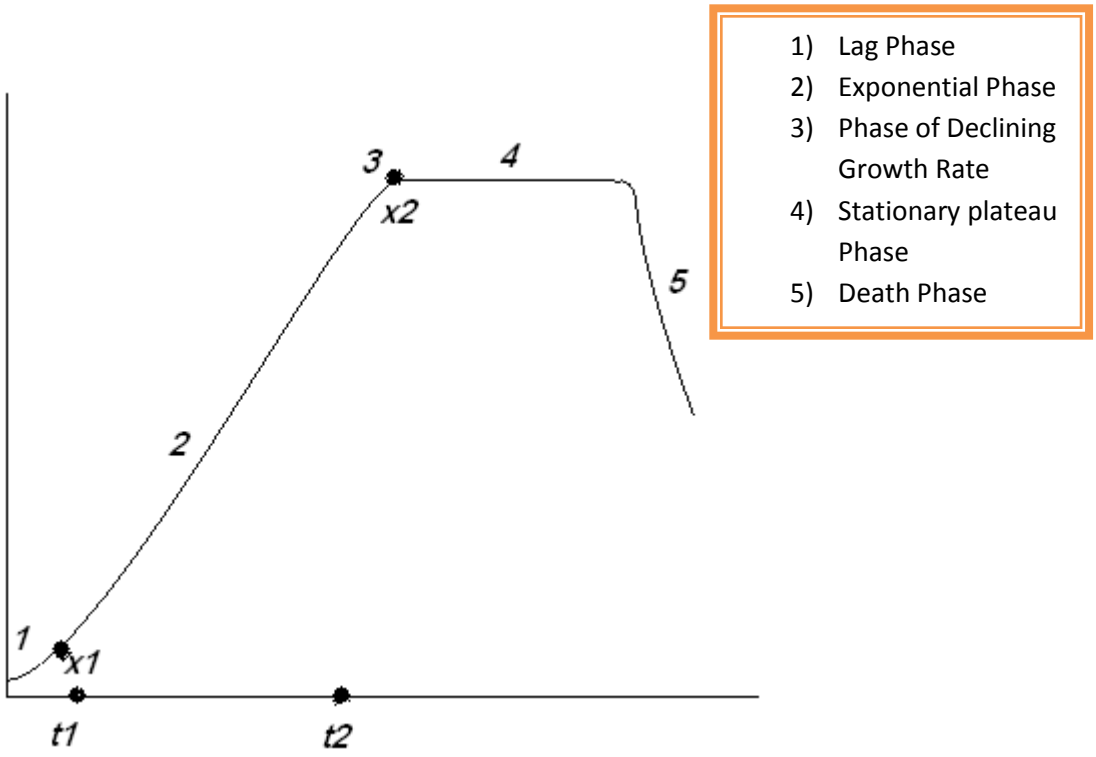


Figure 14 : Graph of Algae's Growth

This is the basic growth curve of microalgae. Lag phase occurs during which little increase in cell density, relatively long when an algal culture is transferred from an agar or broth culture to another liquid. The lag in growth is attributed to the physiological adaption of the cell metabolim to growth, such as the increase of the levels of enzymes and metabolites involve in cell division and carbon fixation. (AlgaeTech Laboratory, 2011)

The growth rate of a microalgal population is a measure of the increase in biomass over time and it is determined from the exponential phase. The duration of exponential phase in cultures depends upon the size of the innoculum, the growth rate and the capacity of the medium and culturing conditions to support algae growth. (AlgaeTech Laboratory, 2011)

$dx / dt = u$(Specific Growth Rate)

$(1/x)\, dx = u\, dt$

$\ln x2/x1 = (t2-t1)\, u$

$x2 = 2x$

$2x/x = 2$

$u = \ln 2/ (t2-t1)$

Doubling Time = $u / \ln 2$ (Richard et al, 2001) x1=density at t1(initial)

x2 =density at t2(final)

At phase of declining growth rate, cell division slows down when nutrients, lights, pH, carbon dioxide or other physical and chemical factors begin to limit growth. In the fourth stage, the limiting factor and the growth rate are balanced, which results in a relatively constant cell density. During the final stage, water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth. Cell density decreases rapidly and the culture eventually collapse. (AlgaeTech Laboratory, 2011)

2.5 Effect of Nutrient Deficient Media and Environmental Stressors on Microalgae

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as nutrients or nutritional requirements(Todar K et al, 2008).Many bacteria can be grown in the laboratory in culture media which are designed to provide all the essential nutrients in solution for bacterial growth.. Microorganisms can be identified in the environment by inspection or

using genetic techniques, but attempts to isolate and grow them in artificial culture has been unsuccessful.

2.5.1 Importance Nutrients for Algae's Growth

All of the solutions(components/elements) in conway media provide own important minerals for algae's growth. Here, it is describes their important at all aspects. This discussion is based on the need of microorganism. (Todar K, 2008)

Table 3 : Basic Needs for Microorganisms

Main Nutrients	
Nitrogen	from nitrate solution is a constituent of amino acids, nucleic acids nucleotides, and coenzymes.
Hydrogen	from H20 and organic compound it functions as main constituent of organic compounds and cell water
Phosporus	build up nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	a constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	main cellular inorganic cation and cofactor for certain enzymes
Calcium	for inorganic cellular cation, cofactor for certain enzymes and a component of endospores
Magnesium	inorganic cellular cation, cofactor for certain enzymatic reactions
Iron	component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

Enough nutrient and good stressors will lead to algae bloom. An algal bloom is a rapid increase or accumulation in the population of algae (typically microscopic) in an aquatic system. Algal blooms may occur in freshwater as well as marine environments. Places where blooms are frequent often support a thriving marine population. Since the plants need nutrients like iron to grow, fertile waters are often near a continental shelf in areas

where cool water from the ocean's depths pushes to the surface. This upwelling water carries with it nutrients that had settled to the ocean floor; the nutrients allow the water to sustain large algae blooms. Algae influence global climate by regulating gases in the atmosphere. Like all plants, algae absorb carbon dioxide and release oxygen as they grow. When the plants die, they sink to the ocean floor, carrying the absorbed carbon with them. Over the course of the Earth's history, the oceans have become the primary sink for atmospheric carbon dioxide. Since carbon dioxide is a greenhouse gas (it traps heat at the Earth's surface), the Earth would be a much warmer place without these algae.[13]

Figure 15 : Algae Bloom In China Quingdao

Figure 16 : Algae Bloom in Tai Lake, China, 2007

2.5.2 Effect of Silica in Media Culture

Some of the nutrients needed for other microorganisms are not mentioned in this media culture.. For example, silicon is a major limiting nutrient for diatom growth and hence is a controlling factor in primary productivity. Numerous studies have characterized parameters of silicic acid uptake by diatoms, and molecular characterization of transport has begun with the isolation of genes encoding the transporter proteins. Multiple types of silicic acid transporter gene have been identified in a single diatom species, and multiple types appear to be present in all diatom species. The controlled expression and perhaps localization of the transporters in the cell may be factors in the overall regulation of silicic acid uptake. (Martin V et al, 2003).

Transport can also be regulated by the rate of silica incorporation into the cell wall, suggesting that an intracellular sensing and control mechanism couples transport with incorporation. Cell wall silicification and silicic acid transport are tightly coupled

to the cell cycle, which results in a dependency in the extent of silicification on growth rate. (Martin V et al, 2003).

Silica dissolution is an important part of diatom cellular silicon metabolism, because dissolution must be prevented in the living cell, and because much of the raw material for mineralization in natural assemblages is supplied by dissolution of dead cells. Perhaps part of the reason for the ecological success of diatoms is due to their use of a silicified cell wall, which has been calculated to impart a substantial energy savings to organisms that have them.(Martin V et al, 2003)

2.5.3 Temperature Effect

Temperature has a major effect on the types of fatty acids produced by micro algae. Many microalgal species respond to decreased growth temperature by increasing the ratio of unsaturated to saturated fatty acids. However, the response to growth temperature varies from species to species, with no overall consistent relationship between temperature and fatty acid unsaturation (James et al., 1989; Thompson et al.,1992; Renaud et al., 1995). The chemical composition of micro algae is influenced by environmental conditions, including temperature and light (Richmond, 1986; Tomaselli et al., 1988; James et al.,1989; Thompson et al., 1992; Renaud et al., 1995; Oliveira et al., 1999; Thompson, 1999). High growth temperature has been related to significant decrease in protein content, together with increases in lipids and carbohydrates. However, other studies have found that the response of micro algae chemical composition to high and low growth temperatures varies from species to species. High growth temperature has been associated with increases in protein content and decreases in carbohydrate and lipid in some species.(M. Renaud et al, 2001).

2.5.4 Light Effect

Light is another factor that effect both primary and secondary metabolites such as enzymes, amino acid, terpens, volatile oils.(Seibert & Kadkake, 1980). Effect of light depend on wavelength, intensity, duration of illumination and characteristic of the cultures. Light may affect phytohormones concentrations which in turn influence the

accumulation of precursors from primary metabolism. Light has major impact on micro organism photosynthesis process. This is a process that converts carbon dioxide into organic compounds, especially sugars, using the energy from sunlight. Through this process, oxygen is given out to the environment. For algae's growth only visible light can be use. This is known as Phtosynthetically Available Radiation (PAR) and makes up only 43% of the solar radiation that hits the earth(red and blue portion of the visible spectra). For both green and brown algae, the wavelength is 435 nm and 675 nm. On the other hand, one the most important issues besides colour is light intensity. The required light intensity for algae's growth is around 2500 to 10 000 lux. An intensity which is above 15 000 lux inhibits growth. Normal bright sunlight has an intensity of 80 000 lux.(AlgaeTech Laboratory, 2011)

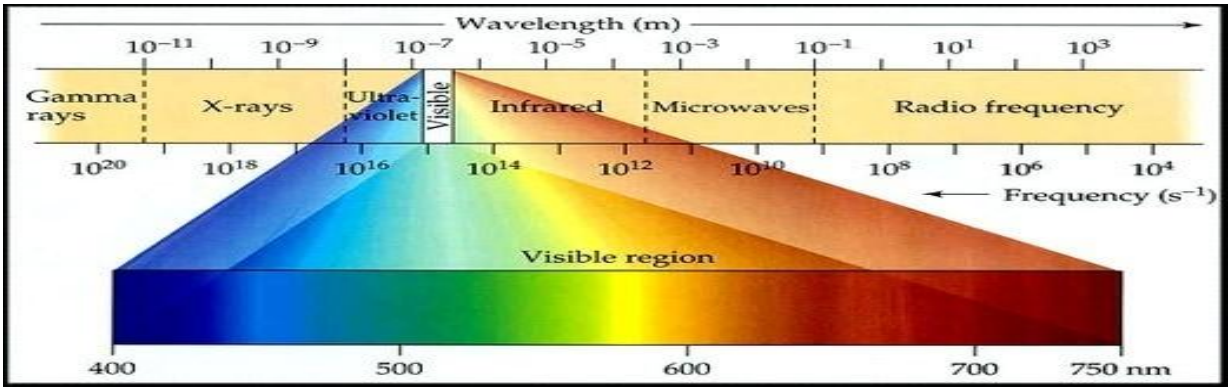


Figure 17 : Visible Light Wavelength for Algae's Growth

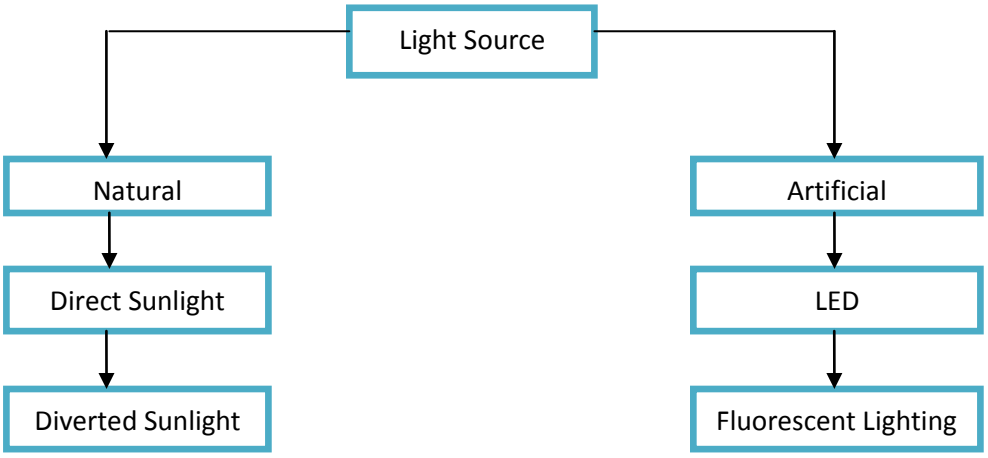


Figure 18 : Light Source for Algae's Growth

2.5.5 pH Effect

One major factor to be considered is the effect on pH. pH is a measure of the acidity or basicity of a solution. It approximates but is not equal to p[H], the negative logarithm (base 10) of the molar concentration of dissolved hydronium ions (H_3O^+); a low pH indicates a high concentration of hydronium ions, while a high pH indicates a low concentration. This parameter can easily be manipulatedas it changes with ionic balance or by efflux and influx of anions and cations.(Jardin et al, 1991) Initial pH of a plant tissue culture media prior to autoclaving is generally adjusted between pH 5 to 6.5(Thorpe 2000). After autoclaving it usually drops by 0.6 to 1.3 units. This parameter affects cell growth and product formation by influencing the breakdown of substrates and transport of both substrates and products through cell membrane or cell wall. The acidic pH may directly destabilize the cross linking of wall polymers. In culture media, cell expension will be sensitive towards buffers, therefore, changes in extracellular pH will affect cell growth and secondary metabolism.(Rozita Omar, 2003).

2.5.6 Salinity Effect

Salt concentration in an environment is the major contributor to the osmotic effects of ions on algae growth. Algae requires ions that are provided by salt and typically tolerate moderate salt concentration. High salt or high sugar in the environment normally leads to loss of water from cells and ultimately to death. This is the basis for preserving foods using high concentrations of salt or sugar. (Theresa Thiel, 1999). Salt affects plant growth when toxicity from excessive uptake of salt substances such as sodium and reduced water uptake, known as water stress. Then, this reduce the uptake of essential nutrients particularly potassium. Early signs of salinity damage are brighter color of algae than the normal color of green. [9]Scientist has just recently found that algae cells are in association with salt tolerant bacteria, and this has prompted them to hypothesize that the algae provided a source of energy for the salt trapped bacteria. [8]

CHAPTER 3

METHODOLOGY

3.1 Materials and Equipments

This research requires materials and chemicals which are less hazardous. Based on the material safety data sheet and risk assessment, all materials and chemicals involved (Appendix I) are being specified according to the respective methodology (V. Palanisamy et al., 1991, Satyajit D. Sarket et al., 2005).

3.2 Research Methodology

To achieve the objective of this project, the following steps are being conducted for microalgae:

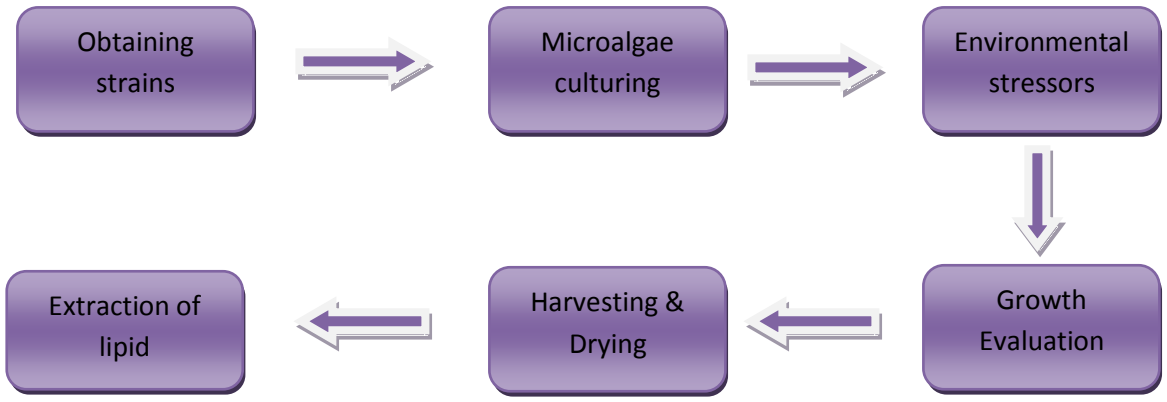


Figure 19 : Experimental works methodology

3.2.1 Obtaining Strains

For this project four species were collected. The species were taken from Fisheries Institute of Penang.. Those are :

i. *Isochrysis galbana spp*.
ii. *Tetraselmis batan spp*.
iii. *Nannochloropsis spp*.
iv. *Pavlouva lutheri spp*.

Nannochloropsis spp. and *Pavlouva lutheri* is more reliable on its adaption for growth and analysis.

3.2.2 Culturing with Environmental Stressors

A culture can be defined as an artificial environment in which the algae grow. In theory, culture conditions should resemble the alga's natural environment as far as possible; in reality many significant differences exist, most of which are deliberately imposed. Here, continuous cultures, resources are potentially infinite: cultures are maintained at a chosen point on the growth curve by the regulated addition of fresh culture medium. In practise, a volume of fresh culture medium is added automatically at a rate proportional to the growth rate of the alga, while an equal volume of culture is removed.[10] Culturing of *Nannochloropsis spp*., *Isochrysis galbana spp*., *Tetraselmis batan spp*. and *Pavlouva lutheri spp*. are important to get mass production of collected microalgae. At first stage, the most important parameters regulating microalgae growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature (Barsanti, Paolo Gualtieri, 2005).In second stage, environmental stressors such salinity and photoperiod are given. This procedure shall be conducted in an aseptic condition under microalgae free environment and sterilization method. All equipments e.g. glasswares, flasks, measurement cylinder etc. and culture media are first sterilized using auto clave.

Figure 20: Media for algae growth is being sterilize in an auto clave machine at 121^0C for 15 minutes

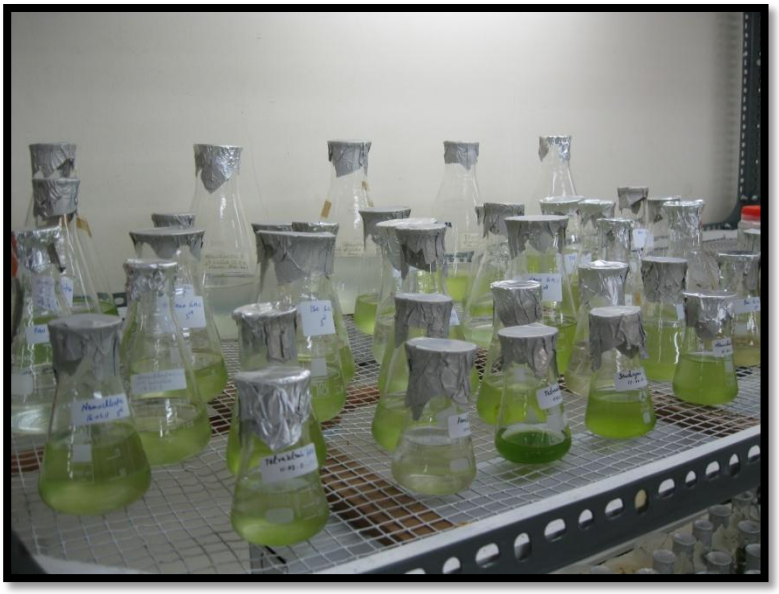


Figure 21 : Culturing of microalgae in 250 ml flasks

Figure 22 : Microalgae with agitation(bubling agitation)

3.2.3 Growth Evaluation

3.2.3.1 Evaluation with Hamocytometer

The growth evaluation of the microalgae is done by performing 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.

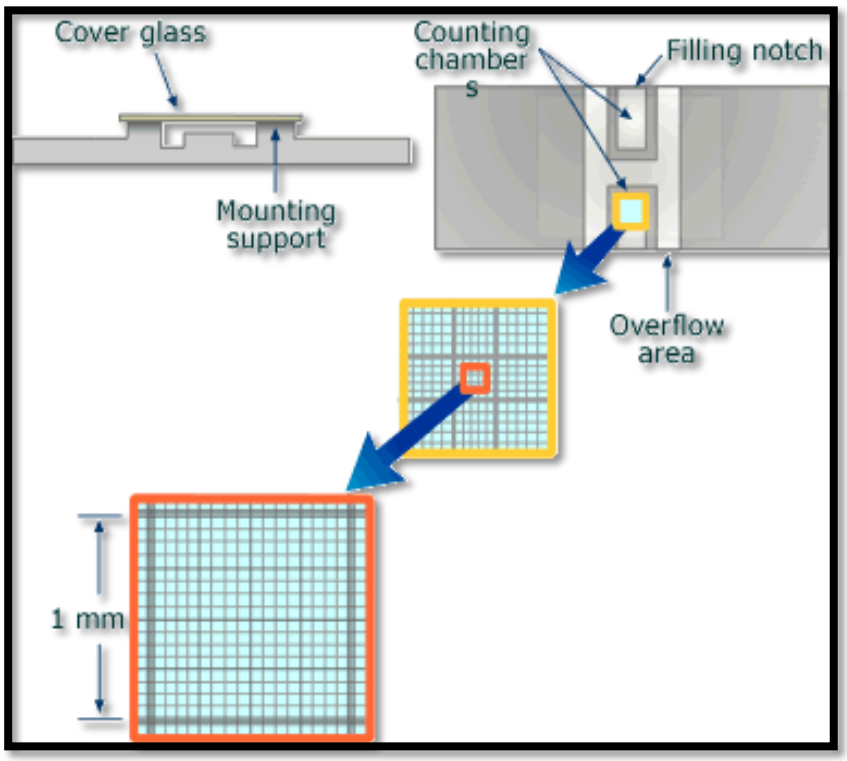


Figure 23 : Hamocytometer

For cell count, 8 readings are taken for each sample which are the readings from the upper part and another 4 from lower part of the hamocytometer. Calculation of the cell count of each sample is as follows :

Average cell = {a(4 boxes from upper) + b(4 boxes from lower)} / 8 * 10000 * 1000(dilution)

(AlgaeTech Laboratory, 2011)

3.2.3.2 Evaluation with Spectrophotometer

A spectrophotometer consists of two instruments, namely a *spectrometer* for producing light of any selected color (wavelength), and a *photometer* for measuring the intensity of light. The instruments are arranged so that liquid in a cuvette can be placed between the spectrometer beam and the photometer. The amount of light passing through the tube is measured by the photometer. The photometer delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.If development of color is linked to the concentration of a substance in solution then that concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. For this growth evaluation on the species, wavelength of 620nm is selected as recommended.

Figure 24 : UV Spectrophotometer

3.2.4 Harvesting

The term algae harvesting refers to concentration of diluted algae suspension until a thick algae paste is obtained. Harvesting of microalgae from algae cultivation employ several techniques. Normally harvesting of microalgae can be a single step process or two spep process which involves harvesting and dewatering. Harvesting microalgae is

difficult because of the small size of the algae.[11]Choosing the effecting harvesting process for a particular strain depends on size and properties of algae strain. Centrifugation process has been chosen for concentrating the diluted microalgae because the centrifuge equipment is easy to be cleaned and sterilized. In the centrifugal separation process the feed is subjected to centrifugal forces which make the solids move through the liquid. Equipment available for centrifugation is divided into fixed wall devices (hydrocyclone) and rotating wall devices (sedimenting centrifuges). A sedimenting centrifuge is an imperforate bowl into which a suspension is fed and rotating at high speed. Liquid is removed through a skimming tube or over a weir, while solids remain in the bowl (batch processing) or are continuously or intermittently removed from it (Shelef et. al, 1984). The variant on zonal centrifugation known as continuous sample-flow with isopycnic banding offers a number of theoretical advantages in the concentration(and simultaneous purification)of particles:large capacity, more efficient recovery at substantially lower speeds than are required for conventional continuous-flow centrifugation, and avoidance of pelleting. Plankton including algae, have been collected in sucrose gradients in the zonal rotors, but we do not know either the efficiency of recovery or the integrity of the recovered algae.

3.2.5 Drying

The next step is usually drying the dewatered slurry to a moisture content of 12-15%. By drying or dehydration, the microalgae biomasses are converted to a stable storable product. At this stage, the utilization of oven (tray dryer) is used. Oven is generated at 100^0C – 200^0C and microalgae aseptic conditions are always kept as a prior procedure (G. Shelef et. al, 1984).

Figure 25 : Example of industry belts harvest and drying process of algae(Brune et al, 2010)

3.2.6 Lipid Extraction & Transesterification Process

3.2.6.1 Lipid Extraction from Fresh Algae

Nannochloropsis spp. sample of 200 ml is taken into centrifuge for 10 minutes and 3500 rpm to extract the fresh weight of sample in pellet form. Fresh weight (pellet) is weighed. Distilled water, methanol and chloroform are put into a 250 ml Erlenmeyer flask with ratio of 4: 10: 5. Pellet of *Nannochloropsis spp.* is transferred into the flask and put on the shaker for overnight stay at 120 rpm. Distilled water and chloroform are put together in the flask after overnight stay with ratio 5:5. Sample in the flask is transferred in centrifuge tube to be centrifuged for 10 minutes and 3500 rpm. This would be two layers; upper and lower chloroform layer where lower chloroform layer contains lipid. These two layers are separated using dropper. The lower chloroform layer is taken out and put into a preweight vial. It then is evaporated at 55^0C until 1/3 is left and put in oven at 105^0C for 1 hour to extract lipid from chloroform. The percentage of lipid content is calculated (Bligh, E.G. et al., 1959).

Figure 26 : Preheated chloroform

3.2.6.2 Lipid Extraction from Dried Algae

Nannochloropsis spp. sample of 2.36 g is taken for lipid extraction. Extraction solvent chloroform/methanol are added separately at 100ml , with 2:1 ratio. Then, the solvent is shake for 20 minutes are mixture of chlorofm/water are added 50ml at 1:1 ration v/v. Algae residue is then filtrated. The filtrated solution is then put up in a rotary evaporator to make sure all chloform are evaporated. This is followed by 3 hours drying process in oven at 100^0C. Then, the dried percentage of lipid is calculated.

Figure 27 : Rotary Evaporator evaporates chloroform

3.2.6.3 Transesterification Process

The process used to convert oil to biodiesel is called transesterification. The Transesterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and glycerol. A triglyceride has a glycerine molecule as its base with three long chain fatty acids attached. The characteristics of the fat are determined by the nature of the fatty acids attached to the glycerine. The nature of the fatty acids can in turn affect the characteristics of the biodiesel. During the esterification process, the triglyceride is reacted with alcohol in the presence of a catalyst, usually a strong alkaline like sodium hydroxide. The alcohol reacts with the fatty acids to form the mono-alkyl ester, or biodiesel and crude glycerol. In most production methanol or ethanol is the alcohol used (methanol produces methyl esters, ethanol produces ethyl esters) and is base catalysed by either potassium or sodium hydroxide.[11] In this experiment, lipid from dried algae undergoes transesterification process together with 24 ml of methanol and 0.25 g of sodium hydroxide. The solvent are mixed and shake for 16 hours. Using a flask separator, the solution is transferred and leaves for 24 hours. Glycerin and biodiesel are obtained from this experiment.(M. R Afify et al, 2010)

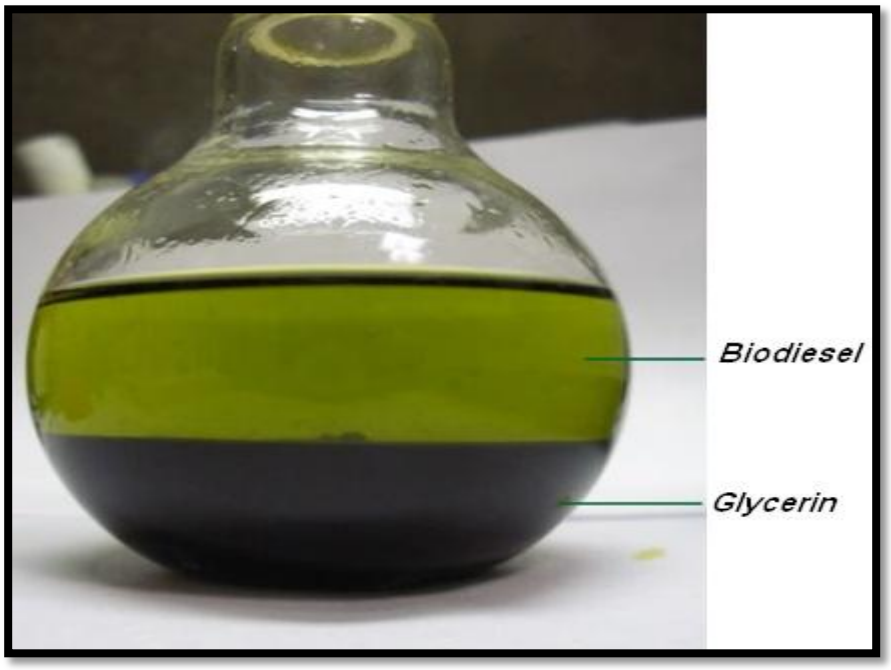


Figure 28 : Results of transesterification process

CHAPTER 4

RESULT & DISCUSSION

4.1 Growth Profile on Different Species

Generating growth profile for *Nanochloropsis sp, Tetraselmis Batan and Pavlouva Lutheri* and *Isochrysis galbana sp.*

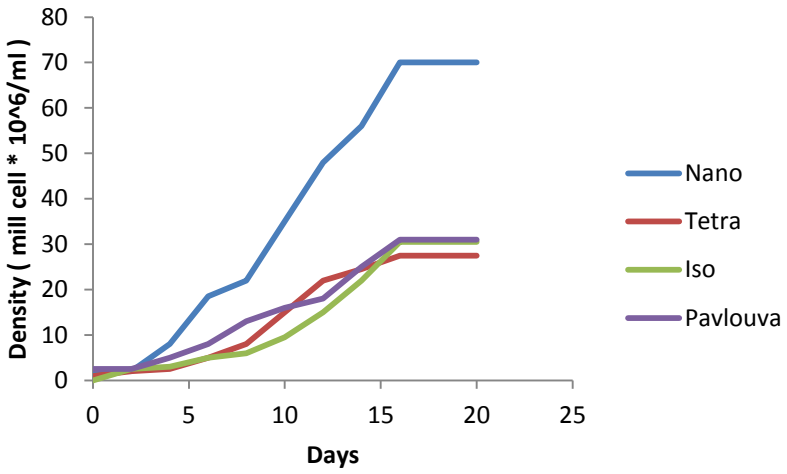


Figure 29 : Growth Rate for Different Species

Species	Nanochloropsis sp	Tetraselmis Batan	Iso chrysis	Pavlouva Lutheri
Doubling Time (Days)	2.33	3.46	3.87	4.55
Specific Growth Rate	0.296	0.199	0.178	0.152

Table 4 : SGR & Doubling Time

From this experiment, *Nanochloropsis sp* shows good growth rate. It gives the highest number of cells after two weeks which is *70*10^6/ml.* Thus, better experiment towards the efficiency of biofuel can be easily achieved by using *Nanochloropsis sp* as strains. *Tetraselmis Batan and Pavlouva Lutheri* also shows good growth rate but the average number of cells after two weeks are much lesser than *Nanochloropsis sp.* Their average

number of cells are around *30 *10 ^6/ml.* Doubling time for *Nanochloropsis sp* is better than others. The growth rate is around 2.33 days which is the minimum if compared to others.

4.2 Generating Growth Profile of *Pavlouva Lutheri* on Different Salinity

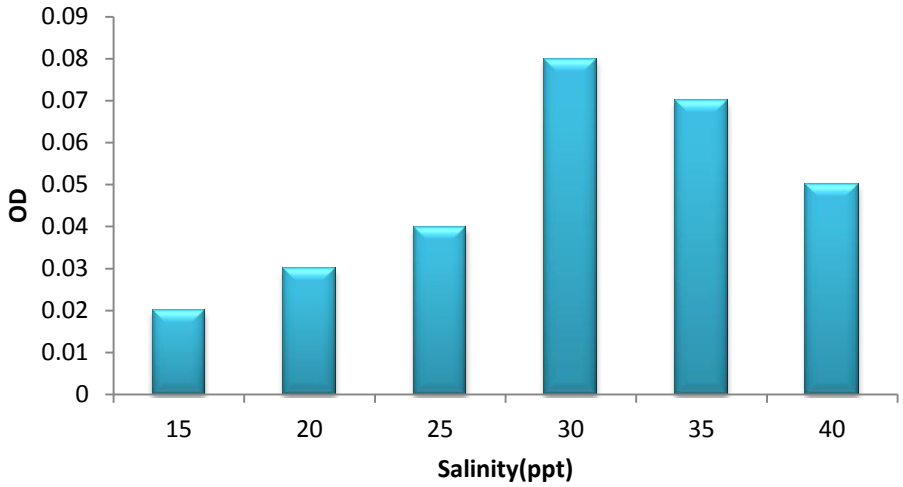


Figure 30 : Growth Rate for Different Salinity

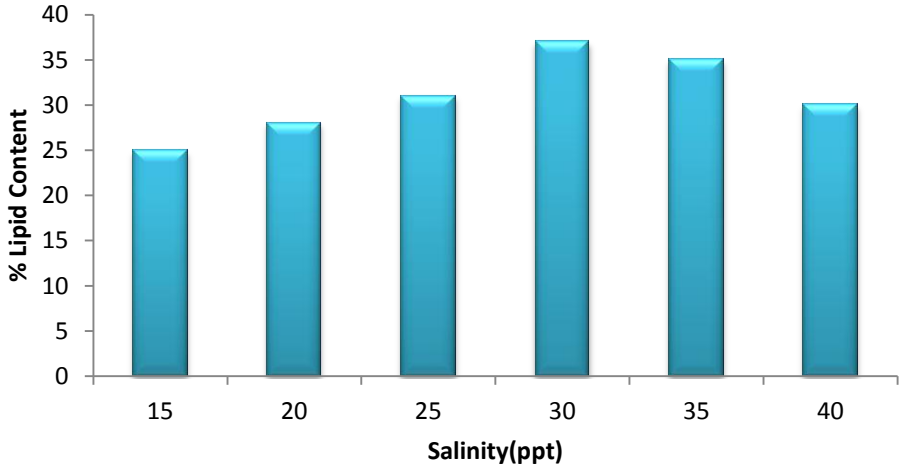


Figure 31 : % Lipid Content Based on Different Salinity

From the second experiment, different salinity is applied to the media to view the growth rate. In this experiment, instead of using hemocytometer, a spectrophotometer is used. Thus, the optical density(OD) on each day is obtained. The graph shows optical density on 16th day. From graph tabulated, *Pavloua Lutheri* on salinity of 30ppt(g/L) and

35 ppt(g/L) shows the best growth rate among all. The amount of cell slowly decreased when more than 35 ppt of salinity is applied. This shows that higher salinity might kill algae as well. Lipid content for all species is slightly the same but the highest among them are to 30 ppt and 35 ppt. Total lipid content produce will effect amount of biodiesel production. At 30 ppt and 35 ppt, the growth rates are good because the algae receive enough ions for its growth provided by the salts. Higher ions provided leads to decrease in growth rate. This is because, when more ions get into algae, it leads to loss of water from algae itself. Thus this reduces algae potential to live. Moreover, it can be concluded that at 30 ppt and 35 ppt, the osmotic effect of ions are optimum on algae growth.

4.3 Generating Growth Profile of *Pavlouva Lutheri* on Different Photoperiod

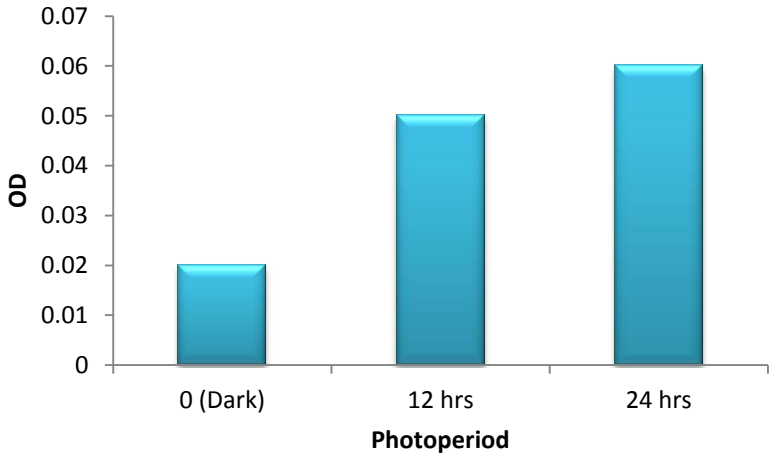


Figure 32 : Growth Rate on Photoperiod Effect

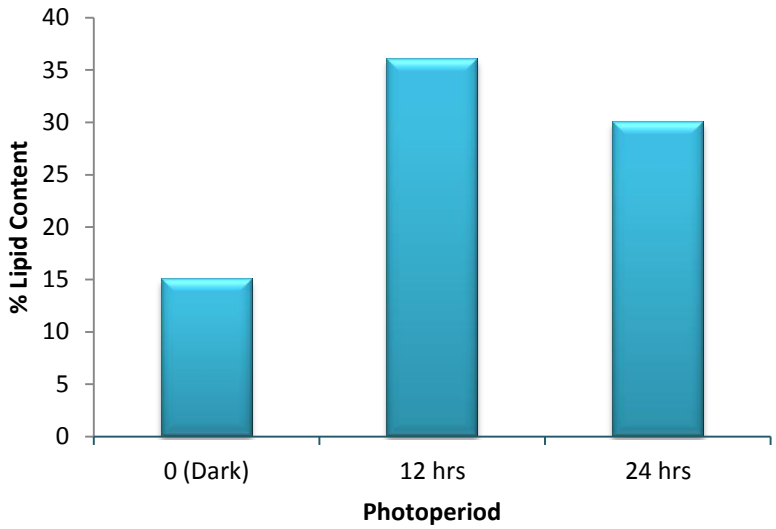


Figure 33 : % Lipid Content for Different Photoperiod

For the third experiment, spectrophotometer is used for cell calculation. Normal intensity of visible light in use. From the graph, 24 hours light gives best growth among all. However, it is only a slight different with 12 hours light. Light affect phytohormones concentrations which in turn influence the accumulation of precursors from primary metabolism. Light has major impact on micro organism photosynthesis process. This is a process that converts carbon dioxide into organic compounds, especially sugars, using the energy from sunlight. But, 24 hours light produce less lipid content than 12 hours. 24 hours like light meaning that microorganism can perform photosynthesis continuously and 12 hours light means the microorganisms can perform respiration with photosynthesis. It is highly recommended for algae to have 12 hours light because the growth rate is good and it can maximize its lipid content.

4.4 Results for Transesterification Process

Table 5 : Amount of Glycerin and Biodiesel Obtained

Dried Algae (g)	Amount of Lipid (g)	% Glycerin	% Biodiesel
2.36	0.42	3.8	8

Based on table above, from 2.36 dried *Nanochloropsis sp*, amount of lipid obtained is around 0.42 g. This amount of lipid undergoes transesterification process and produce 8% of biodiesel and 3.8 % of glycerin. Biodiesel colour in this experiment is light brown. A small amount of biodiesel is obtained because of small amount of dried algae. It is forecasted that if bigger amount of dried algae is obtained, large amount of biodiesel can be produced.

CHAPTER 5

CONCLUSION & RECOMMENDATION

From the first experiment, it shows that *Nanochloropsis sp* gives the best result on growth rate in this environment. Thus it is better if all experiment on biodiesel is focused on *Nanochloropsis sp* only. Moving on to the second experiment, the salinity effect on 30 ppt gives good growth rate with lowest of 12.5% and lipid content lowest of 2% if compared to other salinity. The stressors prove that it yields high amount of lipid content too. On the other hand, the third experiment proves that stressors on 12 hours light produce higest growth rate with 16.66% from 24 hours photoperiod and produce higher lipid content of 7% . Last but not least, for dried algae extraction, it may produce a significant amount of biodiesel. It is predicted that when huge amount of dried algae is obtained, huge amount of biodiesel can be produce through a process known as transesterification. Microalgae needs good growth rate with the best environmental stressors for its to yield its maximum lipid content towards enhancement of biodiesel.

It is also highly recommended for students to be able to observe the microorganisms condition all day in a microbiological lab condition so that the light period and other stressors can be maintain. In order to enhance microalgal growth, more agitation with air should be given. With complete equipments and utilities etc pump for air supply, doubling time of microalgae may decrease.

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APPENDIX I

Chapter 3

Methodology

3.1 Materials and Equipments

3.1.1 Materials:

3.1.1.1 Chemicals in Conway solution:

- Main mineral solution:
    1) Sodium nitrate and/ or potassium nitrate
    2) Disodium ethylenediaminetetraacetic acid (EDTA)
    3) Boric acid
    4) Sodium phosphate monobasic tetrahydrate
    5) Ferric chloride hexahydrate
    6) Manganese chloride tetrahydrate
- Trace metal solution:
    1) Zinc chloride
    2) Cobalt chloride hexahydrate
    3) Ammonium molybdate tetrahydrate
    4) Cupric sulfate pentahydrate

3.1.1.2 Vitamin solution:

1) Thiamine chlorhydrate, B_1
2) Cyanocobalamin, B_{12}

3.1.1.3 Silicate solution:

1) Sodium silicate

3.1.1.4 Nitrate solution:

1) Potassium nitrate

3.1.1.5 Artificial seawater solution

1) Sodium chloride

3.1.1.6 Chemicals used for extraction and further screening in Gas Chromatography- Mass Spectrometry (GCMS), High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC):

1) Sodium hydroxide
2) N- Hexane
3) Ethanol
4) Methanol

3.1.1.7 Other materials:

1) Microalgae species:
    i. Isochrysis galbana spp.
    ii. Tetraselmis batan spp.
    iii. Nannochloropsis spp.
    iv. Pavlouva lutheri spp.
2) Distilled water

3.1.2 Equipments and Tools

1) 20 ml test tubes and stand
2) 250 ml, 1 L and 2 L Erlenmeyer flasks
3) 500 ml and 1000 ml beakers
4) Magnetic stirrer
5) Spatulas
6) Filter funnel
7) Aluminum foil
8) Micro pipette
9) Centrifuge machine
10) Oven
11) Rotary evaporator
12) HPLC,GCMS and TLC instrument