CULTIVATION OF MICROALGAE *SPIRULINA* SP. IN PALM OIL MILL EFFLUENT FOR ESSENTIAL FATTY ACIDS PRODUCTION

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ABSTRAK

Asid lemak penting dalam memberikan manfaat kesihatan dan telah meningkatkan penggunaannya sebagai makanan tambahan. Asid lemak omega-3, kebiasaannya berasal dari ikan berlemak, juga terdapat dalam haiwan, tanaman transgenik, kulat dan banyak mikroorganisma sehingga memberi tekanan berlebihan kepada stok ikan global. Microalga merupakan sumber primer yang kaya dengan asid lemak penting and mengandungi asid lemak omega-3 yang menjanjikan. Kos media nutrien menjadi halangan untuk mengkultur microalga dalam indsutri yang lebih besar. Dalam kajian ini, sejumlah 16 mikroalga asli telah dikenal pasti dan enam monokultur dari pelbagai spesies seperti *Amphora* sp., *Anabaena* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., dan *Tetraselmis* sp., telah diasingkan dalam keadaan makmal dengan menggunakan teknik pencairan bersiri dan pelat bergaris dari sampel laut yang dikumpulkan dari kawasan pantai Timur Kuantan, Pahang. Spesies ini diuji untuk pembesaran kuantiti dan daya tahan dalam campuran 5% (v/ v) POME + medium konvensional. Antara spesies ini, *Spirulina* sp. menunjukkan pembesaran yang lebih tinggi dan daya tahan yang lebih tinggi semasa menilai dan dipilih untuk kajian selanjutnya. Pertumbuhan *Spirulina* sp. diuji dengan menggunakan POME sebagai satu-satunya medium dengan kepekatan yang berbeza (10-50% v/v) untuk memaksimumkan pengeluaran biomas. Diikuti dengan kesan intensiti cahaya yang berbeza (1000-4000 lux) dan suhu (21-36 ± 2 ℃). Kadar pertumbuhan *Spirulina* sp. diukur dengan kepekatan biojisim (mg/L) dan kandungan klorofil (µg/mL). *Spirulina* sp. menghasilkan biojism yang tertinggi dalam keadaan optimum iaitu 30% v/v POME, intensiti cahaya 3000 lux dan suhu 31 ± 2 dengan hasil biojisim tertinggi iaitu 1.54 ± 0.11 g/L setelah 18 hari dikultur. Biojisim *Spirulina* sp. dipisahkan dari medium kultur dengan sentrifugasi pada 6000 g dan dikeringkan pada suhu 70 ± 3 ℃. Biojisim yang telah kering dilarutkan dengan heksana dan kandungan lipid tertinggi diperolehi daripada pengekstrakan secara Soxhlet (44.9±6.1%) berbanding dengan kaedah ultrasound (32.3±6.5%) dan kaedah Bligh dan Dyer (12.3±4.0%). Lipid yang diekstrak melalui transesterifikasi dengan menggunakan KOH telah dilarutkan dalam metanol (1:1 v/v) selama 120 minit pada suhu 60±2 ℃ bagi tujuan mendapatkan metil ester asid lemak. Sampel metil ester asid lemak dicampurkan dengan asid heptadekanoik sebagai standard dalaman dicairkan dalam oktana pada kepekatan 15 mg/ml. 1 µL sampel telah dianalisis dengan menggunakan gas kromatografi. Di antara 13 asid lemak yang dikenalpasti, didapati asid oleik (70.16%), asid palmitoleik (13.07%), asid linolik (8.62%), asid stearik (2.11%) dan asid γ-linolenik (0.43%) sebagai asid lemak dominan. *Spirulina* sp. lipid berpotensi tinggi untuk digunakan dalam produk nutraseutikal dan farmaseutikal kerana komposisi asid lemak pentingnya.

ABSTRACT

Essential fatty acids providing health benefits and has increased the consumption as dietary supplements. Omega-3 fatty acids, usually derived from fatty fish, are present in animals, transgenic plants, fungi and many microorganisms, bringing excessive pressure on global fish stocks. Fortunately, microalgae are the primary which are rich in essential fatty acid and present a promising source of omega-3 fatty acid. The cost of nutrient media becomes an obstacle for cultivation of microalgae in larger industry. In this study, total 16 indigenous microalgae been identified and six monoculture of different species such as *Amphora* sp., *Anabaena* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Tetraselmis* sp., were isolated in laboratory environment by using serial dilution and streaking plate techniques from the marine sample collected East costal region of Kuantan, Pahang. These species were tested the growth and survivability in mixture of 5% (v/v) POME + conventional medium. Among these, *Spirulina* sp. shown higher growth and higher survivability while evaluating and chosen for further experiment. The growth of *Spirulina* sp. was tested by feeding POME as an only medium with different concentration (10-50% v/v) to maximize the biomass production. Followed with different effect of light intensities (1000-4000 lux) and temperature (21-36±2 ℃). The growth rate of *Spirulina* sp. was measured by biomass concentration (mg/L) and Chlorophyl content (µg/mL). The highest biomass production of *Spirulina* sp. was found in the optimum conditions of 30% v/v of POME, 3000 lux light intensity and 31±2 ℃ temperature with highest biomass yield 1.54±0.11 g/L after 18 days of cultivation. The biomass of *Spirulina* sp. was separated from the culture medium by centrifugation at 6000 g and dried at 70±3 ℃. Dried biomass was treated with hexane and extracted the highest lipid from Soxhlet extractor (44.9±6.1%) as compared to ultrasound assisted (32.3±6.5%) and Bligh and Dyer method (12.3±4.0%). The extracted lipids were trans esterified using methanolic KOH (1:1 v/v) for 120 min at 60±2℃ with aim of obtaining fatty acid methyl ester. Fatty acid methyl ester sample added with internal standard heptadecanoic acid which diluted with octane at 15 mg/ml. 1 µL of samples were analyzed using gas chromatography. Among 13 fatty acids found that, oleic acid (70.16%), palmitoleic acid (13.07%), linoleic acid (8.62%), and stearic acid (2.11%) and γ-linolenic acid (0.43%) as dominant fatty acids. *Spirulina* sp. lipid have great potential to be use in nutraceutical and pharmaceutical product due to its composition of essential fatty acids.

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℃	Temperature
%	Percentages
M	Molarity
µl	Microliter
mL	Milli litre
L	Litre
cm	Centimetre
mm	Millimetre
m	Meter
v/v	Volume per volume
g	Gram
mg	Milligram
g	Gravity
γ	Alpha
Δ	Delta
∑	Total
µg	Microgram
mL	Millilitre
sp.	Species
t	Time

LIST OF ABBREVIATIONS

FA	Fatty acid
AA	Arachidonic acid
ALA	Alpha-linolenic acid
APHA	American Public health Association
ATP	Adenosine triphosphate
BBM	Bold Basal Medium
BOD	biochemical oxygen demand
BP	Biomass production
CO_2	carbon dioxide
COD	Chemical oxygen demand
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC	Gas chromatography
GC-FID	Gas chromatography flame ionization detector
GC-MS	Gas Chromatography Mass Spectrometry
LA	Linoleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
O_2	Oxygen
OD	Optical density
PBDE	polybrominated diphenyl ethers
PCB	polychlorinated biphenyls
POME	Palm oil mill effluent
PUFA	Polyunsaturated fatty acids
TAG	Triacylglycerols
UNECOSOC	Economic and Social Council of the United Nations
LC-PUFA	Long chain polyunsaturated fatty acid

CHAPTER 1

INTRODUCTION

1.1 Background of study

Lipids are important macromolecules for all species, including human beings. Indeed, they are key components of the cell-delimiting plasma membrane and are essential for the integrity and functionality of all cell membranes (Lupette and Benning et al., 2020). Because humans are unable to synthesize all the required lipids themselves and the lipids supplied externally are metabolized into various bioactive molecules, the consistency of the diet from which lipids are supplied is crucial for the maintenance of human lipid homeostasis (Laye et al., 2018; Long et al., 2020). Due to the absence of delta-12 desaturase (Δ12DES) in mammals, the human metabolism is not able to produce two FAs which are, therefore, essential FAs: linoleic acid ($18:2^{\Delta 9,12}$, LA) derived from the *n*-6 pathway and alpha-linolenic acid ($18:3^{\Delta 9,12,15}$, ALA) from the *n*-3 pathway of fatty acid modification (Lupette and Benning et al., 2020; Lupette and Marechal et al., 2018).

In the absence of Δ12DES, these VLC-PUFAs are readily supplied through the diet. A major dietary source of *n*-3 and *n*-6 are oils from fish (Ex. anchovy, herring, sardine, salmon, tuna, mackerel, halibut) which are rich in triacylglycerols (TAGs, corresponding to the esterification of three FAs on a glycerol-3-phosphate backbone) (Levasseur et al., 2020). However, fish oil can be subject to contamination with heavy metals (mercury, lead, cadmium), dioxins, polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs). Moreover, ocean overfishing is also a threat to processing fish oils on a sustainable scale. Finally, oils from fish are rich in VLC-PUFAs because phytoplankton is the source of their food (Randhir et al., 2020; Lupette and Benning et al., 2020).

Therefore, microalgae have long been regarded as sustainable tools for the production of essential fatty acids with presents of oil contents of between 10–50%, (w/w) and produce a high percentage of total lipids (up to 30–70% of dry weight). he

accumulation of fatty acids is closely linked to microalgal growth stages, functioning as an energy stockpile during unfavourable conditions or cell division. Omega-3 is accumulated due to its high energy content, as well as the good flow properties crucial for cellular functions (Koyande et al., 2019). The ω-3 fatty acid content of different strains of microalgae has been studied. Strains of the *Phaeodactylum*, *Nannochloropsis*, *Thraustochytrium* and *Schizochytrium* genera have shown high lipid accumulation (Stokes et al., 2020). Among microalgae species. *Spirulina* cyanobacterium become trendy health food due to their abundant source of nutritious composition, including protein, vitamins, lipids, minerals and carbohydrates and natural pigments (Seghiri et al., 2019; Ran et al., 2020).

Spirulina require a wide range of inorganic nutrients such as nitrogen, phosphorus, and vitamins and trace elements such as iron, manganese, selenium, zinc, cobalt, and nickel. The growth and the biomass productivity of any species depend on the type of nutrient sources, its quantity, and the relative proportions of C, N, and P of the culture medium. (Kim et al., 2015). Wastewater may serve as the medium for assimilating microalgae with organic carbons and nutrients. Palm oil mill effluent (POME) containing high organic, nitrogen and phosphorus supplies demonstrated a strong potential for large-scale production of microalgae and remediation of contaminants (Acien et al., 2017; Udaiyappan et al., 2020). These components are supporting microalgae such as *Chlorella sp.*, *Nannochloropsis sp.* and *spirulina sp.* growth (Ahmad et al., 2011). Microalgae have utilised the POME and produce biomass as end product and reduce the pollutants in the wastewater (Gupta et al., 2016). Use of POME for nutrients and the availability of organic carbon, the application of supplements is needed to further maximise biomass and lipid production. Nevertheless, the cost of the medium may be impacted by supplementation, as it reported to be between 35% to 80% of the medium cost, depending on the nutrient source (Cheah et al., 2018).

Therefore, in this study the native microalgae *Spirulina* sp. was isolated from Kuantan east coastal area and cultured in POME with different concentration (v/v) and enhanced the growth and biomass production under different light intensities and temperature. The accumulation of lipid in the biomass of *Spirulina* sp. been studied. And followed by transesterification to breakdown the macromolecules of lipid into micro molecules. The fatty acid compositions of *Spirulina* sp. were quantified by using gas chromatography mass spectroscopy.

1.2 Problem statement

Environmental conditions affect the growth of microalgae population. In order to obtain desirable potential microalgae especially indigenous species require skill full techniques of isolation and purification. All of the isolates need to categorized based on the morphological appearance of the culture and the microscopic cellular appearance of the isolated colonies. These steps help to isolate, purification and characterised the indigenous species.

Cultivation of microalgae required large investment to buy fertiliser (expensive nutrient medium) for mass production. In order to minimised the cost, utilization of wastewater for the microalgal growth is considered beneficial idea. Malaysia is the world's second largest producer and exporter of palm oil and thus produces vast amount of POME annually. It is estimated that approximately 2.5 to 3.5 tons of POME is generated for each ton of crude palm oil produced (Osman et al., 2020). POME cause water pollution due to high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) that reduces biodiversity and the capacity of aquatic ecosystems (Kumar et al., 2011; Mishra et al., 2017).

Utilizing POME as supplements source to culture microalgae. The unicellular species use as food nutrition for live feed culture. Meanwhile nitrogen sources play key role in promoting microalgae growth. To grow microalgae basic nitrate concentration required in the range of 200-400 mg/L. other minerals such as Fe, Zn, P, Mg, Ca and K required for microalgae growth are available in POME. This condition helps to limiting the utilization of freshwater, expelling nitrogen and phosphorus from the wastewater. In addition, it generating microalgal biomass as bioresources for value added by product.

Algae biomass contain nutritious compounds especially essential fatty acid which important for healthy diet. The composition of biomass contains various substances. To obtain desirable compound require various technique to perform and followed by quantification composition.

1.3 Objectives of study

There are three objectives which been achieved in this study.

i. To isolate and characterize the marine microalgae (*Spirulina* sp.) from Kuantan coastal area, Pahang.

ii. To enhance the growth and productivity of microalgae *Spirulina* sp. by different palm oil mill effluent concentrations (% v/v), light intensity (lux) and temperatures (°C).

iii. To identify and quantify the essential fatty acids from *Spirulina* sp. lipid using GC-MS.

1.4 Scope of study

The scope of study is to isolate, characterize and maximize the *Spirulina* sp. biomass production for essential fatty acid. In order to achieve the objectives, the following research scopes had been identified as supporting tools.

i. Collection of marine algal sample and isolate the indigenous microalgae species from Kuantan costal region of Pahang, Malaysia by using serial dilution and streaking plate techniques.

ii. Utilization of waste water as medium to cultivate microalgae to produce biomass.

iii. Maximize the growth of *Spirulina* sp. for higher biomass and lipid productivity by enhancing with different POME concentration (v/v), light intensity (lux) and temperature (°C).

iv. Extraction of lipid from the biomass by using Bligh and Dyer, ultrasound-assisted and Soxhlet extraction method.

v. Transesterification of *Spirulina* sp. extracted lipid with optimum reaction condition of methanol to lipid volume ratio, catalyst amount and reaction temperature.

vi. Analyse the composition of fatty acid methyl ester through gas chromatography-mass spectrometry (GC-MS).

1.5 Significant of the study

The finding of this study will be beneficial for the human community, in term of health, economy and green environmental development. Novel and potential microalgae *Spirulina* sp. have been isolated and cultivated. Palm oil mill effluent are rich in nutrient medium which used to cultivate the *Spirulina* sp. and obtained comparable amount of dry biomass as compared to conventional BG-11 medium. *Spirulina* sp. is relatively high in lipids and consists of essential fatty acid as linoleic and linolenic. It has great potential to be use in nutraceutical and pharmaceutical industrial product.

CHAPTER 2

LITERATURE REVIEW

2.1 Marine microalgae

Microalgae are primarily a variety of microorganisms that include eukaryotic photoautotrophic protists and prokaryotic cyanobacteria (Occasionally called blue-green algae). Such microbes contribute to half of global photosynthetic production and are located in euphotic niches digitally (Singh and Gu, 2010). Microalgae are a diverse group of eukaryotic photosynthesizing microorganisms. Another large group of photo-synthesizers of prokaryotes, cyanobacteria, are often discussed together with microalgae. This convention is going to be followed here, although the two groups of microorganisms are quite distinct. The exact number of algal species is not known (Ajayan et al., 2012; Guiry, 2012). Estimates vary from 45,000 to more than 100,000 species. There are many species of freshwater cyanobacteria and microalgae were dispersed and disconnected bodies of water in world widely (Venkatesan et al., 2015).

Microalgae (phytoplankton) are a diverse assemblage of plant-like, predominantly aquatic, unicellular, multicellular, or colonial forms; photosynthetic; chlorophyll 'a' containing organisms found in the atmosphere, on the tops of the mountains, and 200-300m below the sea-air interface (Malcata et al., 2018). Microalgae are evolutionarily diverse in two domains, Eukaryota and bacteria, including, but not limited to, Cyanobacteria (blue-green algae), Charophyte (chara), Chlorophyte (green), Rhodophyte (red), Ochrophyte (brown), Glaucophyte, Euglenophyte, Cryptophyte, Chrysophyte, Bacillariophyceae (diatoms) and dinophyceae (dinoflagellates) (Heimann and Huerlimann, 2015).

Microalgae or microphytes are microscopic eukaryotic species, typically found in freshwater and marine environments, use solar energy to generate ATP, which is transformed by its metabolic process to lipid, carbohydrate and protein. Microalgae give great promise to contribute to sustainable bioenergy, as they have a high lipid content, a rapid growth rate and an atmosphere of aquatic growth using solar energy (Yousuf, 2019). Many of the researchers reported that microalgae feedstock is an excellent alternative

source of energy, and the fuel produced has a similar property profile as vegetable oil, which has been proven to be an efficient alternative source (Schenk et al., 2020). The products developed during the preparation of biofuels can be used for different applications such as food supplements, lipids, enzymes, biomass, polymers, pesticides, pigments, tertiary wastewater treatment, and "green energy," it has been regarded as a highly potential source of biofuels in the last few decades (Singh and Gu., 2010).

Algae have traits both plant-like and animal-like. Plant-like algae usually occur in aquatic ecosystems that contain chloroplasts and are photo synthesizable. Unlike higher plants, algae are lacking in vascular tissue and have no roots, branches, leaves or flowers. Animal-like algae have flagella and centrioles and can feed on organic material in their metabolism (Chaisutyakorn et al., 2018). Algae sizes range from single cell to giant multicellular organisms, and they may live or expand in multivariate conditions such as fresh water, salt water, or wet soil, or on damp rocks. They can be transmitted by sexually or asexually, or by the alternation of generations through a mixture of both mechanisms (Goldstein, 2015).

Algae are divided loosely into two categories: macroalgae and microalgae. Between 30,000 and more than 1 million species were estimated by June 2012, of which 44, 000 names were likely written, and 33 248 names were processed (Greenwell et al., 2009: Yousuf, 2020). Microalgae are simple heterotrophic microscopic or autotrophic photosynthetic species often called phytoplankton by biologists ranging in diameters from 1 to 50 mm (Kumar et al., 2016). These usually occur in pond, humid areas or in marine environments. Most species contain chlorophyll, which synthesizes carbohydrates using photonic energy (light), carbon dioxide (CO_2), and water (energy storage) and make biomass (algae growth) (Huang et al., 2010).

Algal growth experiences five distinct phases within a batch culture system. Through this system inoculum is charged into the system with a fixed volume of the nutrient media. The nutrients depleted as the microalgae repeated, and the final product was collected. The following stages are as follows. (1) Lag phase: Initial slow growth cycle in which microalgae take time to adapt to the new climate. The duration of the lag process depends on both inoculum size and environmental shock. (2) Exponential: Rapid growth and sometimes cell division happens as the cells and microalgae expand very rapidly. (3) Relative growth decline: This step occurs when cell division growth requirements are reduced. (4) Stationary: The cell division is slowing because of the lack

of resources required for development. (5) Death / lysis: Cells continue to die because of a lack of nutrients (Yousuf, 2020).

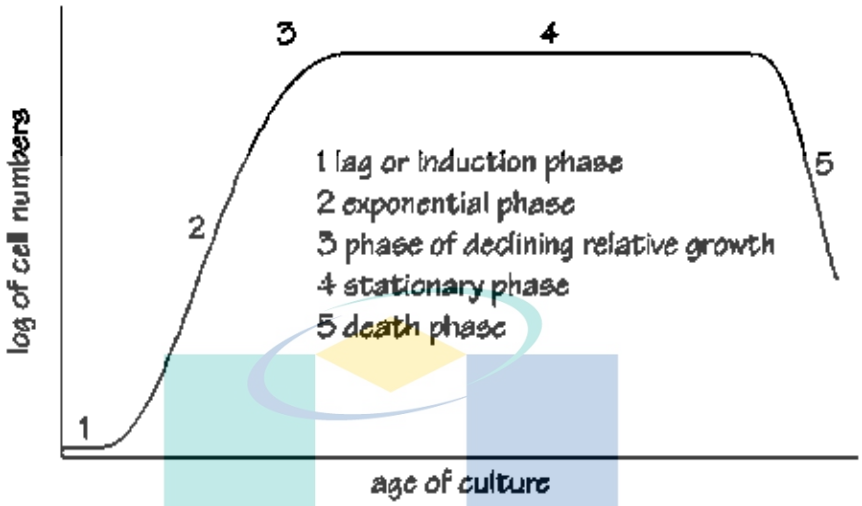


Figure 2.1 Schematic growth curve of a microalgae batch
Source: Yousuf, (2020)

Microalgae are easy to grow, and can tolerate a wide range of pH, salinity, and temperature. Commonly they double up every 24 hours. Some microalgae can double every 3.5 hours during the peak growth process. Microalgae oil content typically varies from 20% to 50% (dry weight), although some species can reach as high as 80%. This has the potential to withstand much more harsh situations, which causes higher lipid content (Halim et al., 2012; Yousuf, 2020).

There are various commercial applications for microalgae such as production of biofuels, use biomass to enhance the nutrition value of food, extract high value compounds, capture CO_2 from environment, and using in wastewater treatment. Alternatively, fast duplicating microalgae have been identified as a promising third generation source of biofuels (Christi, 2007). They are also used as biofilters to remove pollutants from wastewater and as part of fertilizers and feed for livestock. Microalgae are also an energy source capable of producing different types of biofuels.

2.2 Major advantages of microalgae

Microalgae been selected for this study due to advantages of microalgae. It does not need arable land and can be cultivated in desert regions and on poor soils, because the demand for land is used only as support for the method of cultivation (Kumar et al., 2011). Even growing in aqueous media, they require less water than terrestrial plants, and the residual water may be reused in the process depending on the process used in biomass concentration, minimizing global freshwater consumption (Han et al., 2019; Osman et al., 2020).

Algae do not compete with farming and It can produce throughout the year and are not reliant on seasons and crops (Cruz et al., 2018). They can contain more than half the natural oxygen. Microalgae have high biomass productivity and easily accumulate lipids in many organisms, between 15 and 50 % in dry mass (Cruz et al., 2018). A growing does not require the use of herbicides or pesticides (Khatun et al., 2017). The nutrients can be collected from residual waters and agro-industrial waste for their cultivation (Cheah et al., 2018; Lim & Biswas, 2019).

It can contain a number of other useful items besides lipids, such as proteins, carotenoids and carbohydrates that can be used as foods or fertilizers, fermented to produce ethanol or other goods with high added value (Yue et al., 2019). By photosynthesis, they effectively fix atmospheric carbon, or even contaminants from industrial processes (each ton of biomass produced absorbs 1.7 tons of CO_2, 10–20 times more than is absorbed by oilseed crops) (Gao et al., 2019).

2.3 Bioactive compounds from microalgae

Microalgae are able to turn carbon dioxide (CO_2) into oxygen (O_2) and biomass in the presence of sunlight (Bhatia et al., 2017). Microalgae work has now increased exponentially, as there is an interest in exploiting its potential for CO_2 mitigation and wastewater treatment (Daneshvar et al., 2019). In addition, biomass from microalgae is a rich source of natural compounds such as carotenoids, astaxanthin, lutein, and fatty acids (Patil et al., 2019). Such bioactive compounds have both beneficial and harmful effects for all living beings.

In addition, they are used as natural drugs, food additives and UV-light protective agents in the pharmaceutical, nutraceutical, cosmetic, and food industries (Ercolano et al., 2019; Molino et al., 2020). Microalgae have been recognized as a source of bioactive compounds, such as *Spirulina, Botryococcus, Chlorella, Dunaliella, Haematococcus* and *Nostoc*. Natural pigments are essential for the metabolism of photosynthetic algae and feature many beneficial biological activities, such as antioxidants, anti-carcinogenic, anti-inflammatory, anti-obesity, anti-angiogenic and neuroprotective (vaz, 2016).

However, Natural microalgae products remain largely unexplored in contrast to those derived from land plants, though algae have many advantages over terrestrial plants. Mathimani et al. (2019) stated that, microalgae have been found to be a competent source of biofuel development due to certain advantages, such as improved lipid and biomass efficiency, preferred fatty acid profiles, strong photosynthetic levels, low crop requirements and tolerance to different environmental conditions (De Lorgeril et al., 2012)

Several factors lead to chemical composition of microalgae such as size, shape, digestibility, formation of toxic compounds, biochemical compositions and conditions of the animal feeding on microalgae (Bhuvana et al., 2019). Raposo et al. (2013) explains the variability, together with differences between species and production method. The protein, lipid and carbohydrate amounts are 12-35%, 7.2-23%, and 4.6-23% respectively. This fluctuation degree can be affected by the conditions of culture (Shahidi et al., 2018). Fast growth, for example, and high production of lipids can be achieved in a way of stressing the culture (Brown et al., 1992).

The biochemical composition of microalgae varies greatly shown in Table 2.1, even when grown under normal conditions. Gross composition varies between species but this is not the main deciding factor for food value for many species (Long et al., 2018). Of all microalgae, the protein content is good. The composition of sugar is complex and can affect the nutritional value in some cases (Kumar et al., 2019).

Table 2.1 Main bioactive compounds extracted from microalgae

Microalgae	Bioactive compounds
Spirulina sp., *S. platensis, S. fusiformis, S. maxima*	Phycocyanin, Polysaccharides, C-phycocyanin, allophycocyanin, phenolic acids, tocopherols (vitamin E), polyunsaturated fatty acids, oleic acid, linolenic acid, palmitoleic acid, diacylglycerols, alkaloids, flavonoids
Chlorella sp., *C. vulgaris, C. minutissima, C. ellipsoidea, C. protothecoides*	Sulphated polysaccharides, alkaloids, phytol, phenol, carotenoids, astaxanthin, sterols, PUFA(n-3) fatty acids, peptides, eicosapentaenoic acid (EPA), zeaxanthin, violaxanthin, lutein, canthaxanthin, terpenoids and oleic acids.
Haematococcus pluvialis	Oleic acids, lutein, astaxanthin, zeaxanthin, canthaxanthin, β-carotene and canthaxanthin
Dunaliella salina	All trans-β-carotene, cis-betacarotene, all trans- luteins, oleic acid, palmitic acid, linolenic and sterols
Botryococcus braunii	Linear alkadienes and triene
Nostoc sp., *N. muscorum. N. spongiaeforme, N. linckia, N. humifusum,*	Phenolic, phycocyanin, cryptophycin, borophycin, alkaloids, terpenoids, phycobilins.

Source: de Morais (2015); da Silva vaz (2016)

2.4 Lipid

The lipids are important macromolecules for all species, including humans. They are indeed main constituents of the cell-limiting plasma membrane and are necessary for the integrity and functionality of all cell membranes (Alhusseiny et al., 2020). Furthermore, lipids construct membrane vesicles or lipid droplets role in the transport of proteins, hormones or fat-soluble vitamins (A, D, E and K) in cells and extracellularly,

e.g. in the bloodstream (Lupette et al., 2020). Microalgal lipids can be classified into two groups: nonpolar neutral polar (acylglycerols, sterols, free fatty acids, wax and sterile esters) and polar lipids (phosphoglycerides, glycosylglycerides and sphingolipids). Such lipids play various but essential roles in cycles of growth and microalgal metabolism. (Mata et al., 2010; Zhu et al., 2016). Human beings cannot synthesize all the required lipids themselves and the externally supplied lipids are metabolized into different bioactive molecules (Rahmawaty et al., 2020), and the consistency of the diet by which lipids are supplied is crucial for maintaining lipid homeostasis in humans (Ji and Ledesma-Amaro, 2020). Preferentially, mammalian metabolism includes n-3 and n-6 sequence fatty acids. FA are carboxylic acids that have an aliphatic variable-length carbon chain (Lupette and Marechal, 2018).

Fatty acids are the most essential components of marine microalgae sources because they are structurally diverse and because of their taxonomic specificity have gained significance (Mathimani et al., 2018). They have become a rich source of primary productivity in a wide range of habitats, including fresh, sea and brackish water. In this period of energy scarcity there is a rising need for renewable fuels to replace fossil fuels to meet commercial viability (Kumar et al., 2019). Over the past decade, focus has been shifted to the development of third-generation biofuels from microalgae. It has been found to be a competent source of biofuels production due to certain advantages, such as increased lipid and biomass productivity, and preferable fatty acid profiles, high photosynthetic levels, low crop requirements, and tolerance to different environmental conditions (Bhuyar et al., 2020).

Microalgae lipid production is around 15–300 times higher than oil-bearing crops such as sunflower, corn, soybean, and palm oil. Although many challenges remain in the production chain of microalgae biodiesel, especially during microalgae biomass harvesting and dewatering processes (Han et al., 2019). Microalgae can be considered a very good alternative source of lipids since they have a dry weight content of between 15 and 75% depending on the form and conditions in which they are grown (Huerlimann et al., 2010). Table 2.2 shown the lipid content in various microalgae. Microalgae have potential to be a possible source of phenolic compounds and other antioxidant compounds, and bioactive compounds derived from microalgae have applications in the pharmaceutical, healthcare and food industries (Bhuyar et al., 2019). In aquaculture feeds microalgal lipids are considered valued constituents (Venkatesan, et al., 2015). Among

the added value products derived from microalgae, the production of polyunsaturated fatty acids (PUFA) or long-chain fatty acids is commonly known for their beneficial effects on human health and the nutritional value of microalgae is primarily related to their essential fatty acid content (Vidyashankar et al., 2015; Harwood, 2019).

Table 2.2 List of lipid content of selected microalgae

Microalgae	**Lipid content (%)**
Schizochytrium sp.	50-77
Botryococcus braunii	25-75
Nannochloropsis	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	16-37
Cylindrotheca sp.	20-35
Nannochloris sp	20-35
Isochrysis sp.	25-33
Chlorella sp.	28-32
Phaeodactylum tricornutum	20-30
Tetraselmis sueica	15-23
Dunaliella primolecta	23
Monallanthus salina	>20
Crypthecodinium cohnii	20
Spirulina sp.	**20-50**

Source: Cruz (2018)

2.4.1 Polyunsaturated fatty acids

All essential lipids can be synthesized by humans except those belonging to long-chain fatty acids, which can be classified into three omega-families (including ω-3, ω-6 and ω-9), depending on the location of the first double bond. The erection-3 and erection-6 are polyunsaturated fatty acids (PUFAs), while most of the ω-9 FAs are

monounsaturated (De Meester et al., 2012). In biological environments, fatty acids usually have an equal number of carbon atoms.

$$O = C\ (OH) - CH_2 - CH_2 - (CH_2)n - CH3$$

Figure 2.2 Molecular structure of polyunsaturated fatty acids

Fatty acids typically have an equal number of carbon atoms in their biological system. Membrane phospholipids usually have between 14 and 24 carbon fatty acids (Rosenfeldt, 1999). The carboxylic end of the fatty acid is where the carbon numbering starts. The last carbon in the omega (ω) chain and this carbon forms the methyl group. Therefore, 18:3:3 refers to 18-carbon Omega-3 fatty acid of 3 double bonds, which known as α-linolenic acid. Whereas 18:2:6 refer as 18-carbon omega-6 fatty acids with 2 double bonds, which known as linoleic acid (Palanisamy et al., 2021). Saturated fatty acid absents with double bonds, example is 18:0 stearic acid. A monounsaturated fatty acid has one double bond, which known as 18:1 oleic acid. (Brown and Miller, 1992 and Rosenfeldt, 1999).

Normally Unsaturated fatty acids typically have lower melting points than saturated fatty acids because a greater number of double bonds (degree of unsaturation) confers the number lower melting point, and hence more fluidity (Elagizi et al., 2018). Significant omega-6 PUFAs (predominant in animal tissue along with cholesterol) are linoleic acid (18:2:6), the precursor of linolenic acid (via unique desaturase and elongases) (18:3:6) and arachidonic acid than, (AA, 20:4:6). AA is the main omega-6 PUFA in membrane phospholipids, and (after interaction with phospholipase, Cyclooxygenase, lipoxygenase). The important omega-3 PUFA are α-linolenic acid (18:3:3), the precursor of eicosapentaenoic acid (EPA, 20:5:3) and docosahexaenoic acid (DHA, 22:6:3) (Rosenfeldt, 1999).

2.4.2 Benefit of polyunsaturated fatty acids

Long chain polyunsaturated fatty acids (LC-PUFAs) such as EPA (Eicosapentaenoic acid, ω-3 C20:5) and DHA (docosahexaenoic acid, ω-3 C22:6) is believed to have a wide range of beneficial health effects and high potential for niche markets. PUFA may have applications such as biosurfactants, bio-lubricants, fatty agents or biodegradable plastics (De Morais et l., 2015). There many PUFAs have antioxidant,

anti-inflammatory and antibacterial activity. The cellular effects of some-three fatty acids on human cardiovascular diseases have been extensively reported and include altering the biophysical properties of cell membranes, modulating membrane protein function and transporting ions, affecting gene expression (Hamilton., 2015; Huang et al., 2016).

The cellular effects of individual-3 fatty acids on human cardiovascular diseases have been extensively documented, including altering the biophysical properties of cell membranes, modulating membrane protein activity and transporting ions, affecting gene expression or serving as substrates for the development of lipid mediators (De Oliveira et al., 2017).

2.5 *Spirulina* sp.

Spirulina sp. is a blue-green alga, a filamentous cyanobacterium that is sometimes used as a single cell protein. This microalga contains essential amino acids, proteins, fatty acids, pigments with antioxidants, carotenoids, beta-carotene and phycocyanin (Seyidoglu et al., 2017). *Spirulina* sp. is a member of the family phormidiaceae. It is a cyanobacterium, filamentous and multicellular, which is found as a cylindrical filament. This is also a photosynthetic bacterium and is known to be in eukaryotic organisms according to Bergey 's Manual of Determinative Bacteriology (Smibert et al., 1974).

In fact, there was only one more alga in this family, named *Arthrospira*, which was confirmed by Gomont in 1989 (Boone and Castenholz, 2001). He explained that *Spirulina* and *Arthrospira* differ because of their characteristics such as helix type, cell wall, microscopic visibility, diameters and filaments (Gershwin and Belay, 2007). Botanics claim the name is S. Platensis was initially named *Arthrospira platensis* because of its oxygenic photosynthetic properties, but today the name "Spirulina" is used by researchers worldwide for microalgae (Boone and Castenholz, 2001).

That was reported by the World Health Organization S. Platensis does not pose a risk and is a good health food supplement (Seyidoglu et al., 2017). The Intergovernmental Institution, included in this issue, studied this malnutrition microalgae (IIMSAM) in 2003 and developed a charter with the Economic and Social Council of the United Nations (UNECOSOC). They agreed that *Spirulina* should be used against human malnutrition, particularly.

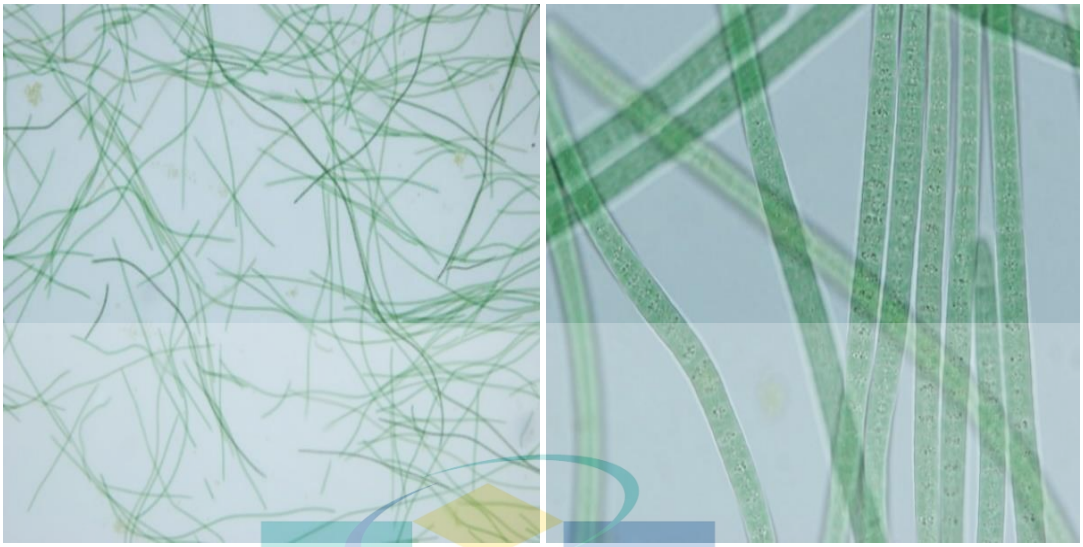


Figure 2.3 Microscopic view of microalgae *Spirulina* sp. at 100X magnification

2.5.1 *Spirulina* sp. as source of fatty acids

The bioactive compounds such as protein, amino acids, minerals, vitamins, pigments, nucleic acids, carbohydrates and lipids. Since our study focus on lipid and fatty acids, here explained mainly about composition of *Spirulina* sp. as shown in Table 2.3 (Otles and Pire, 2001; Sevidoglu et al., 2017). The polyunsaturated content present in *Spirulina* sp. about 4-7% but it contains the important essential fatty acids for human such as gamma-linolenic acid and linolenic acid (Ho et al., 2018; Mühling et al., 2005). These components are act as immune mediators and cardiovascular system due to their precursor effects of prostaglandins and leukotrienes (Banakar et al., 2020).

Table 2.3 Fatty acid composition of *Spirulina* sp.

Fatty acids	Composition (%)
Myristic acid	0.23
Palmitic acid	46.07
Palmitoleic acid	1.26
Oleic acid	5.26
Linoleic acid	17.43
Gamma-Linolenic acid	8.87
Others	20.88

2.6 Cultivation of microalgae in wastewater

The concept of increasing microalgae in wastewaters derives from the idea of resource conservation and nutrient recovery. Algae cultivation in wastewater streams allows for a double beneficial effect as urban and agro-industrial wastewater is treated and valuable biomass is generated at the same time. Since wastewater is used as a source of nutrients, the application of the grown biomass (Hupfauf et al., 2016). Microalgae can play an important role in wastewater treatment, especially at the level of removing nutrients and reducing the operating costs of wastewater treatment (Chinnasamy et al., 2010). Agricultural wastewaters are highly rich in carbon, nitrogen, phosphorus and other minerals, which have to be removed before the effluent released int water bodies. Excessive organic carbon and nutrients released into rivers and lakes can lead to reduced dissolved oxygen, aquatic toxicity and to eutrophics. Table 2.3 shown the pollutants presents in wastewater and their effect and treatment methods.

During their growth microalgae assimilate significant amounts of nutrients and metals in natural aquatic environments. Microalgae can digest inorganic nitrogen sources, such as ammonium, nitrite and nitrate. Oswald and Gotass (1957) were proposed the use of microalgae in the treatment of waste water. Microalgae can be a good alternative to urban wastewater tertiary treatment because they need large amounts of nitrogen and phosphorus to develop, including protein synthesis (40–60%, dry weight), nucleic acids and phospholipids (Swanson et al., 2012).

Microalgae-based wastewater treatment is an environmentally friendly process, without secondary emissions, and allowing for effective nutrient recycling. Commercial product such as fertilizer, animal feed, fine chemical, biofuel can be produced from microalgae biomass. And thereby it reducing the total cost treatment plant (Butler et al., 2017). The microalgae-based treatment system is cheaper and environmentally safer technology, compared to physical and chemical processes, with additional technology benefits of recovery and recycling of resources (Jaiswal et al., 2020; Palanisamy et al., 2021). The aerobic photosynthetic pathway is particularly interesting, as it makes it possible to do so. Reduction of operating costs associated with the aeration of biological treatment (Christenson and Sims, 2011).

Table 2.4 Main pollutants in wastewater, their effects and treatment methods

Wastewater contaminants	**Environmental impacts**	**Treatment methods**	**Disadvantages**
Nutrients (Phosphorus & Nitrogen)	Contamination of groundwater with nitrate Direct reduction of oxygen due to ammonia Ammonia toxicity Eutrophication	Phosphorus-Biological phosphorus removal, Chemical precipitation, adsorption.	Processes for the removal of biological nutrients are effective but are complicated and expensive to operate. Chemical processes cause increased sludge amounts.
Organic matter	Reduces oxygen levels caused by bacterial respiration.	Biodegradation mostly by aerobic bacteria.	Conventional treatment to extract dissolved organic matter is very effective. There are still certain organic traces that are hard to biodegrade. As for detergents and pesticides.
Pathogens (as tracker species, coliforms)	Waterborne illnesses	Getting a physical or chemical disinfection primarily after secondary treatment These processes include UV and chlorination	Product disinfection, particularly of disinfectants derived from chlorine and chlorine, is of concern.
Strong suspended, taste, colour	Aesthetic problems & turbidity Sludge deposits	Tertiary treatment	-
Metals	Toxicity of aquatic organisms. Reduces the effectiveness of biological treatments. Concern where irrigation wastewater is used.	Biological adsorption treatment, chemical precipitation.	They are difficult to remove from waste water; chemical precipitation is widely used but land use of final sludge may be limited.

2.6.1 Palm oil mill effluent

Palm oil mill effluent is the wastewater generated through the processing of oil palm and consists of various materials that are suspended. The waste products produced during the processing of palm oil are oil palm trunks, oil palm fronds, empty fruit bunches, palm pressed fibres and palm kernel shells, less fibrous material such as palm kernel cake and liquid discharge (Abrams et al., 2016). The waste is in the form of a high concentration of organic matter such as cellulosic waste with a combination of carbohydrates and oils (Palanisamy et al., 2021). The discharge of untreated POME has a negative environmental impact (Tan & Lim, 2019). POME is a thick, brownish liquid released at 80-90 °C of temperature and 3.4-5.2 acid pH (Madaki and Seng, 2013). It is 100 times more toxic than urban wastewater with a high demand for biochemical oxygen (BOD) and a high demand for chemical oxygen (COD) (Andiappan et al., 2018). The effluent also contains higher levels of organic nitrogen, phosphorus and various supplements (Kamyab et al., 2018). It will pose environmental problems due to the vast oxygen absorbing potential to the oceanic system (Hasanudin et al., 2015). However, it known to be a good nutrient source (Salama et al., 2017; Altunoz et al., 2020). Table 2.4 shown the characteristics of POME.

POME needs decent treatment before disposal because of its hazardous and eutrophic characteristic such as high viscosity, thick brownish appearance, acidic, high discharge temperature (80-90 °C), high biological oxygen demand (BOD) of about 25,000 mg/L and, high chemical oxygen demand (COD) of up to 54,000 mg/L, 8,000 mg/L of oil and grease content, gross suspended solids reading of 44,000 mg/L (Chin et al., 2020). POME produces a bad odour due to the production of organic matter such as phenolic, pectin and carotene (Ho et al., 1984). POME properties are sensitive to variations in batch, facilities and processing time and may change depending on fruit maturity and form as well as treatment technology (DOE, 1999; Tan and Lim, 2019).

The potential to pollute the environment raises the difficulty of treatment with POME to consider economical, ecological and environmental issues in the treatment decision. POME handling is a source of grievance in the palm oil industry, as the procedure is not advantageous to the industry, but is mandated by regulatory standards (Govindan et al., 2019). POME includes residual oil that affects the atmosphere and the value of edible oil and fats derived from palm fruit. One of the major environmental issues is the handling and storage of oily wastewater such as POME. It has existed for

years but their effects on environment are at present more noticeable. To avoid problems that are known to be dangerous contaminants, particularly in aquatic ecosystems, oily waste must be removed because they are highly toxic to aquatic organisms. Discharging the effluents or by-products on the land or releasing them to the river will lead to contamination and the surrounding environment that deteriorate (Akpor et al., 2014; Kamyab et al., 2015).

Table 2.5 Characteristics of palm oil mill effluent

Parameter	Value	Unit
Temperature	80-90	℃
pH	6-9	-
Chemical oxygen demand (COD, mg L^{-1})	50,000+2,000	mg/L
Biological oxygen demand (BOD)	25,000±1,000	mg/L
Total suspended solids (TSS), mg L^{-1})	77 ± 6	mg/L
Total solid (TS)	40,000±1,000	mg/L
Total volume solid (TVS)	34,000±800	mg/L
Ammonium (mg L^{-1})	9.9 ± 0.4	mg/L
Phosphate (mg L^{-1})	5.9 ± 0.3	mg/L
Nitrite (mg L^{-1})	1.5 ± 0.2	mg/L
Nitrate (mg L^{-1})	3.5 ± 0.6	mg/L

Source: Akhbari and Govindan (2019)

2.7 Challenges in microalgae using wastewater

Microalgae or microalgae-bacteria symbiosis have great potential to reduce the pollutant substances by removing organic compounds and nutrients elements such as nitrogen, phosphorus, heavy metals, and hazardous contaminant particles (Gertsakis et al., 2003). The different environmental microalgae cultivation condition and biomass harvesting are the main challenges in application of water treatment by microalgae (Chye & Wahidul, 2019). The concentration and composition of wastewater which released from industry affect the growth rate and survivability of microalgae. Selection of the desired species, and finding and fixing an optimal ratio of algae/bacteria, and micropollutants removal, and a need for external CO_2 present additional obstacles (Zerrouki & Henni, 2019).

The productivity of microalgae increases with increasing temperature until it reaches an optimum temperature because respiration and photorespiration of microalgae affect productivity. The suitable temperature evaluated under highest growth conditions (optimal conditions of nutrients and light) different species require different optimum temperature. However, temperature between 28 and 35 °C were accepted by most species (Arumugam et al., 2020).

Microalgae productivity affect by quality, intensity and light period. The solar radiations and its availability depend on the geographical location, seasonality and climate. The period of light and dark cycles has different impacts on the treatment efficiency (Cruz et al., 2018). Apart from this, coexistence of microorganisms in waste water affects the productivity of microalgae such as bacteria, fungus. The interaction between bacteria and microalgae affects the CO_2 consumption, organic compounds consumption, vitamin production, and nitrogen compounds (Yin et al., 2020).

The separation of microalgae biomass from the effluent remains a limitation while harvesting. Since the microalgae size is very small, it is very difficult to separate from effluent medium. Due to these various techniques were applying such as coagulation, flocculation, gravitational sedimentation, bio flocculation, flotation, filtration and centrifugation. Researchers are still looking for harvesting techniques with in low cost and high efficiency for commercial production of microalgae.

2.8 Microalgae growth factor

A growth medium with proper nutrients, a light source for photosynthesis, and CO_2 or air flow are the key components for algal growth. For successful cultivation of microalgae, all these growth factors must be specified for a specific purpose.

2.8.1 Nutrients

Different species of microalgae may involve specific nutritional needs at different ratios from each other. Sodium bicarbonate and CO_2 are the only source of carbon used for autotrophic culture, especially in large-scale cultivation where CO_2 from the atmosphere is used. This allows for lower manufacturing costs and helps to minimize CO_2 emissions (Wang et al., 2014). As for heterotrophic culture, it is possible to use fructose, glucose, acetate, glycerol, and sucrose as a source of fuel. Instead, a mixture of

those two carbon sources is used for mixotrophic culture. The pH system would usually increase to pH 11 due to accumulation of OH^- during photosynthesis, which is not optimal for algae development (Kaplan et al., 2017).

Nitrogen is the most essential nutrient which contributes to the production of biomass. Although it may vary depending on the species of microalgae and the amount of nitrogen supplied, the nitrogen content in biomass may usually range from 1% to more than 10% (Khoo et al., 2020). Lack of nitrogen in the culture can reduce the growth of microalgae and the yield of biomass. The widely used source of nitrogen is nitrate (NO_3^-), ammonia (NH_4^+), and urea (Kaplan et al., 2017). To improve microalgae growth and cellular processes an optimum phosphorus level is necessary. Phosphorus concentration affects the biomass composition, in particular the lipid and carbohydrate content, as they are influenced by its internal and external supply (Khan et al., 2018).

In nature, nitrogen is abundant because it fixes continuously by bacteria, while phosphorus is minimal, which is effectively bound in sediment as orthophosphate. Nutrient levels, especially in combination with nitrogen and phosphorus, are very important in lipid production. Nitrogen deficiency enhance the lipid accumulation in the microalgae cells. Nutrient shortages and excess nutrients can cause changes in microalgae both physiologically and morphologically (Jayakumar et al., 2017).

2.8.2 pH

Photosynthesis is affected by alteration of the medium 's pH system which will stress the growth rate of microalgae. Microalgae have been deemed to be able to produce more biomass when cultivating under alkaline conditions as it allows for easier CO_2 capture from the atmosphere (Sakarika and Kornaros, 2016). As pH increases, current carbon formation in poor alkaline, HCO_3^- is formed from CO_2, and this HCO_3^- is consumed by microalgae (Ren, 2014). It was reported that, most microalgae grow well within pH ranging from 6 to 8.76. however, chlorophyll content decrease if the pH goes from 8.5-9.5 (Khoo et al., 2020).

Cultivation under acidic conditions at pH 3 to 4 is also recommended, since this range of pH may prevent contamination of lethal fungus in the cultivation system (Hwang et al., 2019). However, different species of microalgae have their own optimal pH and salinity which causes damage to the cells when they are too low or too high.

The optimum pH of microalgae growth is generally 6-8 and the photosynthesis process take in the neutral to alkaline pH range. Growth of microalgae can be affected by various ways. Assimilation of CO_2 during photosynthesis process increased the pH during daytime. pH dropped at night time due to the respiratory process in microalgae. There is a link between the microalgal culture medium's CO_2 concentration and pH. It is related to the chemical equilibrium of organisms such as CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}.

The balance of these species depends on pH if CO_2 predominates at pH below 7.0 and CO_3predominant pH above 10. Increasing CO_2 can lead to higher accumulation of biomass, but it lowers the pH value, which adversely affects the physiology of microalgae. But, if pH rises too high, photosynthesis may be restricted due to CO_2 scarcity (Sakarika and Kornaros, 2016).

2.8.3 Temperature

Temperature plays a crucial role in algae growth and is important to maximize growth, important to control the temperature in algae studies (Raven and Geider, 1988). Temperature affects microalgae 's gross photosynthetic activity by undergoing cell division which in turn affects microalgae's biomass productivity. The cell division happens when enzymatic activities increase which connected with cycle Calvin. Several studies created a model for relating growth rate with temperature growth. the temperature increases with every 10 ℃, growth turn doubles until it reaches maximum temperature, then reached a stage to decrease. The decline in growth is due to the heat stress encountered by the algae, resulting in protein denaturation and inactivation of enzymes involved in the photosynthesis process (Danesi et al., 2011a)

Many lipid-related algae have a temperature range ranging from 15 to 40 ℃. Researchers have shown that many species of oil-producing algae grow best in the range of 25 to 30 ℃. The optimum temperature of growth varies by species and the optimal response of the algae. Yet regulating outdoor temperature is difficult and expensive. Not only the high temperature, but also media evaporation, outdoor overheating and cooling, and lipid composition are also major algal growth challenges. Higher temperature is responsible for accumulation of saturated lipids and lower temperature for accumulation of unsaturated lipids (Goncalves et al., 2018).

2.8.4 Light intensity

Light availability in autotrophic microalgae production is the most important factor influencing cell productivity and is one of the most difficult to control in outdoor cultures due to variations in solar radiation during the day and during seasonal changes (Fernandez et al., 1997). For photosynthesis, light energy is necessary to transform water and CO_2 into organic compounds, such as carbohydrates and proteins. Direct light exposure and intensity affect the process of photosynthesis which will directly influence the biochemical composition of microalgae and the production of biomass (Mandotra et al., 2016). When algae are cultivated photosynthetically, the efficiency of photosynthesis is a crucial determinant in their productivity since it affects the growth rate, biomass production, and lipid accumulation (Dasesi et al., 2004b). Effect of light intensity depends on depth of the culture medium and density of the algal biomass (Palanisamy et al., 2020). If the culture's depth and cell concentration is higher, light intensity must be increased in order to reach the medium. Otherwise, as a photo inhibitor, direct sunlight or high-density artificial light will work (Li et al., 2019). Alight/dark system is required for efficient microalgae photosynthesis as light is needed for the photochemical phase to produce ATP and NADPH and dark for biochemical phase to synthesize essential biomolecules for microbial growth (Yousuf, 2020). Although light is indispensable, some findings also suggest that for microalgae photosynthesis alternation of light and dark periods is desired as the synthesis of adenosine-5′-triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) requires light as this initiates the process of dark photosynthesis that produces carbon skeleton (Chew et al., 2017). Different species of algae have been reported to require different light intensities for optimum growth, and therefore experimentally determined light intensity and duration are needed to avoid photo-inhibition. However, most studies agreed that 16 h light with 8 h dark is the most appropriate condition for the growth of microalgae (Chew et al., 2017; Khoo et al., 2020).

2.8.5 Mixing

Mixing is another critical factor in the cultivation of microalgae, as it facilitates the homogeneous distribution of nutrients, temperature, pH and CO_2 in the crop system. This mixing method also helps prevent cell sedimentation, clumping and the occurrence of dead zones, thus promoting the distribution of light to each part of the algae (Khoo et al., 2020). Mixing provides intermittent light and dark cycle (L/D cycle or flashing-light

effect) that promotes photosynthesis reaction of microalgae as the algal cells rotate between the photographic zone and the dark zone (Show et al., 2017). Abu-Ghosh reported that, Microalgae photosynthesis process can be enhanced through flashing light effect to have significantly developed as well (Barreira et al., 2015). Microalgae were susceptible to shear force and so excessive mixing could damage the algal cells and even lysis. Therefore, appropriate mixing frequency and velocity must be applied to prevent undesirable lysis of cells. The productivity of microalgal biomass will be reduced without proper mixing, even when other requirements are generously met (Acien et al., 2017).

2.8.6 Predators

Invasive species and predators can be any kind of living organism that is totally unexpected in the field of microalgae cultivation as they inhibit the growth of microalgae, pollute the crop medium and nutrient deficit. Predators can be microalgae organisms, bacteria, insects, and even unwanted. Industrial algae production is mostly restricted to extremophile algae species, which can thrive in extreme environments where rival organisms are unable to survive, in order to avoid any potential problems from invasive and predator species. Open ponds are vulnerable to invasion by low oil-producing species of algae, although photobioreactors avoid this by holding the algae from the outside environment (Daissy et al., 2018)

Table 2.6 Growth factor of microalgae

Group of factors	Parameters
Environmental factors	pH, nutrients, & temperature
Processing parameters	Mixing & light intensity
Biotic factor	Invasive species and predators

Source: Yousuf (2020)

2.9 Extraction of lipid

Microalgae is a potential candidate for lipid-production. Microalgae cells can accumulate up to 20–50% of their cell dry weight (Araujo et al., 2013) and can be used as precursors in the development of biodiesel after a transesterification step (Kamaroddin et al., 2020). Algal lipids include polar, normally structural lipids such as phospholipids

and glycolipids, and neutral lipids. The main storage lipids are in mono-, di-, tri-acylglycerides (TAG) and sterols. TAG contain the desires metabolite components which is fatty acid after it removed from the glycerol frame. It transformed into fatty acid methyl ester through transesterification (Lakshmikandan et al., 2020). After the optimization process of microalgae cultivation in order gain more biomass which generate lipid rich cellular biomasses (Saliu et al., 2020). Significant amounts of lipids are trapped in the cytoplasm by the cell walls and membranes, so the efficiency of lipid extraction depends heavily on the technique of cell disruption and on the polarity of the solvents used to remove lipids from the cell water process (Ren et al., 2017). The yield is higher in the dry algal biomass extraction, but the associated costs are due to biomass drying is need to be considered.

2.9.1 Bligh and Dyer (1959)

The Bligh and Dyer method are one of the most popular lipid extraction methods, using chloroform/methanol (1:2 v/v) and separating the lipids in the chloroform process (Ranjithkumar et al., 2015). Several modifications were suggested to upgrade this system, one of which is the addition of 1 M NaCl to avoid denatured lipids. This method obtained extraction yields of 95% of the total lipid content, with the possibility to use it in both dry and wet algal biomass (Jacob, 2018).

2.9.2 Soxhlet extraction

Lipid extraction through Soxhlet technique has the advantage that the cells are in constant contact with fresh organic solvent, thereby preventing the constraint of balance found in solvent batch processes (Mubarak et al. 2015). It has been shown that using this method, nearly all microalgae lipids can be recovered as the reference method for comparison with other methods of extraction (Prommuak et al. 2012). De Jesus et al (2019), mentioned the steps on performing the method by transferring the dry biomass into a 25 × 80mm cellulose thimble and placed in extraction chamber with solvent. The solvent was heated under reflux to a temperature where reached the boiling points for 4-6 hours. After extraction, cooled down the solvent and evaporated in a rotary evaporator. The advantages of Soxhlet is increased interaction between solvent and biomass paste by periodic siphoning allows the liquid to refresh itself continuously, maintaining a gradient of oil concentration between solvent and sample. This allows the oil to be solubilised.

The advantage of immersion is that the solvent is more effective, because solvents with low polarity cannot remove all polar lipids when the method is applied for a short time (Ramola, et al., 2019). Solvent polarity affects specifically the extraction of lipids with nonpolar lipids, lower polarity, and higher selectivity (Lakshikandan et al., 2020).

2.9.3 Ultrasound-assisted extraction

Ultrasound-assisted is a unique approach that released high yield of quality extract and value-added biological compounds. This method is being more environmentally friendly, has many benefits such as simple operating conditions, reduced solvent usage, non-corrosive solvents, fast extraction time, overall low energy and temperature consumption ratio and inhibits thermo-labile compound degradation (Sosa-Hernandez et al., 2018). This approach is based on the breakup of the cell through the cavitation created by the ultrasound waves resulting from the collapse of microbubbles. Such bubbles are close to the cell wall and a shock wave is produced as they burst, fracturing the cell wall, releasing the microalgae's internal compounds (Ghasemiet-Naghdi et al., 2016). The ultrasonic waves create stable cavitation and transient because of the rapid compression occurring while the treatment. A cavitation implosion produces extremely localised heat shock waves, which disrupt the microalgal cells. Thus, sonication cracks the cell wall and membrane due to the cavitation effect (Ranjith-Kumar et al., 2015).

The advantages of the ultrasound-assisted that, it creates relatively low temperature when compared to microwave and autoclave. It does not need the addition of any chemicals, which have to be eliminated after the extraction process (Joven et al., 2020). The study of the ultrasound method has been combined with the use of organic solvents or solvent-free with the aim of has a clean and environmental process (Daissy., 2018). However, prolonged ultrasonication leads to the production of free radicals, which may be detrimental to the quality of the oil that is being extracted (Mason et al., 1996).

2.10 Transesterifications of lipid into fatty acid methyl ester

Previously, biodiesel production from microalgae oil has been demonstrated in the literature using the traditional method, which includes the extraction of lipids from the biomass of microalgae followed by its conversion to fatty acid methyl ester and glycerol. In order to obtain the essential fatty acid from the microalgae biomass, various

technique can be used to extract crude lipids. After the lipid extraction, the lipids need to convert into fatty acid methyl ester (FAME). The process of converting crude lipid to FAME is known as transesterification by using catalyst. The use of alkaline catalysts and virgin biomass oils with a free fatty acid (FFA) content of <0.5% w/w (based on oil weight) was the goal of the traditional transesterification scheme on biomass oils. Even alkali catalysts have been shown to be used in in-situ microalgae transesterification. A main advantage of alkali-catalysed transesterification is that it is a fast process compared to the acid one (Nagappan et al., 2019).

Hindryawati et al. (2017), stated that, Intact triacylglycerols and free fatty acids are not very volatile and are thus difficult to test with GC. For this reason, lipids are commonly derivatized to increase their stability before study. Triacylglycerols are first trans-esterified and then methylated which breaks them down to glycerol and free fatty acids. Trans esterification are decreases molecular weight, and polarity is decreased by methylation, both of which increase lipid volatility. The concentration of different methyl esters of volatile fatty acids (FAMEs) found in the sample is then analysed using GC. The FAME is dissolved in an effective organic solvent, which is then injected into a GC injection chamber (Maniam et al., 2015).

2.11 Analysis of fatty acid methyl ester by gas chromatography

The identification and quantification of fatty acid methyl ester or other compounds which extracted from microalgae lipid are usually performed by chromatographic techniques, primarily gas chromatography (GC) coupled with suitable detector, such as mass spectrometry (GC-MS) or flame ionization detector (GC-FID) (Berreira et al., 2015).

However, Vongsvivut et al. (2014), stated that, alternative methods that are able to determine accurately the total saturated and unsaturated fatty acids composition in crude lipid have successfully been established through mass spectroscopy, Raman spectroscopy, FTIR, and 1H-NMR. The following methodologies is shown to possess several advantages over chromatographic techniques including speed, cost effectiveness, eco-friendliness, and suitability for routine research and industrial purposes.

Nevertheless, for precise determination and purification of PUFA, chromatographic methods are much more reliable than spectroscopic analyses (Hindryawati et al., 2014).

Generally, GC-MS-based analytical methods for fatty acid analysis involved three steps. First of all, extraction of the fatty acids from the sample matrix. Then, derivatization of the fatty acids, and finally, GC–MS analysis. There are various well-established extraction protocols, and these extraction methods could be applied to different types of samples (Chiu and Kuo, 2020). However, to achieve the best performance for different target analytes, some process optimization is needed. Because several parameters such as instrumental settings will affect the method efficiency. A suitable column with good separation is essential for analysing isomeric mixtures of fatty acids. Many columns have been demonstrated to be effective for separating fatty acids with different chain lengths, degrees of saturation, double bond locations, and cis or trans isomers (Weatherly et al., 2016).

2.12 Overall summary

Microalga is well established as great promise feedstock for producing renewable energy based on biomass, providing profitable production of bioethanol, biogas and bioproducts. The microalgae species contain lipid content up to 77% of dry weight of microalgae cell. The higher biomass production, can extract the higher lipid content. However, the major challenge is cultivation of microalgae for commercial scale require larger amount of investment. With aim of reduce the cost in microalgae cultivation, use of palm oil mill effluent for cultivation of microalgae *Spirulina* sp. and enhance the growth rate by using different parameters. As a result, high biomass of *Spirulina* sp. can be obtained and extract various bioactive compounds especially lipid. By feeding POME to cultivate microalgae can reduce the accumulation of POME from environment. Less freshwater can be used, therefore reducing the cost of nutrients can be removed as microalgae assimilating toxins from the wastewater.

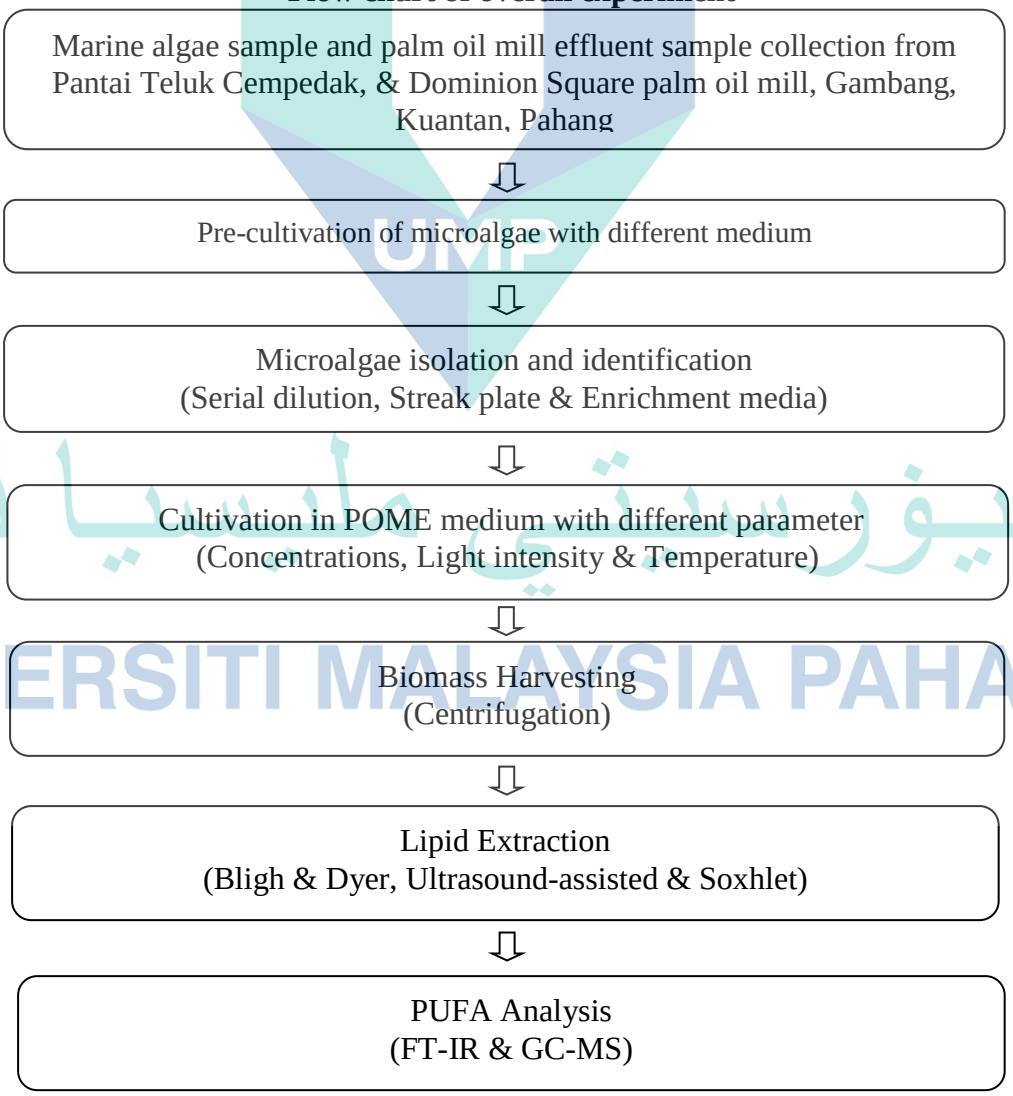
CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter includes the research methodology of the study according to the objectives. In this part, outlined the method of research, selection of sample, experiment techniques, process of research, methods of data and result collection, analysis of data, ethical considerations and the research limitations of the study.

Flow chart of overall experiment



3.2 Chemicals

Chemicals like Sodium nitrate ($NaNO_3$), Potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$), Magnesium sulphate ($MgSO_4.7H_2O$), Calcium chloride ($CaCl_2.2H_2O$), citric acid ($C_6H_8O_7.H_2O$), Disodium salt dihydrate (Na_2EDTA), Sodium bicarbonate (NaHCO3), Boric acid (H_3BO_3), Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), Manganese chloride ($MnCl_2.4H_2O$), Copper sulphate ($CuSO_4.5H_2O$), Zinc sulphate ($ZnSO_4.5H_2O$), Cobalt chloride ($CoCl_2.6H_2O$), Sodium molybdate dihydrate ($Na_2MoO4.2H_2O$), Thiamine HCI, Biotin, Sodium silicate (Na_2SiO_3), Potassium hydroxide (KOH), Hydrochloric acid (HCl), Hexane, methanol were of analytical grade and were purchased from Analisa Resources scientific resources Malaysia. The equipment's such as GC-MS analysis wax column CP9219 fused silica, Erlenmeyer flask (1000 and 2000 ml), Centrifuge tube (15 and 50ml), Eppendorf tube (1.5 and 2 ml), Philips Ecofit LED Tube G13 16W 765 T8 4FT cool white light were purchased from Nusantara Technologies Private limited, Shah Alam, Selangor.

3.3 Sample collection

3.3.1 Sampling collection of palm oil mill effluent

The POME was collected from the treated anaerobic ponds where the final discharge stage in Dominion Square palm oil mill (3.6197 N Latitude, 103.1604 E Longitute) Gambang, Pahang. 10 L of POME was filtered and eliminated the solid particles by using filter net. The brought sample were labelled with place and date. In laboratory placed in dark place to avoid degradation of substance. The samples were handled with proper personal protective equipment due to it hazard and bad odour. The sample was diluted by adding equivalent amount of distilled water (1:1 v/v) and reduced the concentration POME. The pH of POME medium was adjusted from 7.5±0.2 by using 1M of NaOH or added sodium bicarbonate 1.0±0.2 g per 1 L of filtered POME. The diluted POME was sterilized by using hirayama HV-85 automatic high-pressure autoclave at 121 ºC for 20 min. The characteristics of POME includes COD, BOD, Ammoniacal nitrogen, Total nitrogen, Total solids were determined by following American Public health Association (APHA) standard method. The analysis was carried out via spectrophotometer model DR 2800 and Hach standard reagent.

3.3.2 Sampling collection of marine microalgae

Marine algae samples containing various populations of microalgae species were collected from sea located in east coast region of the state of Pahang, Malaysia at Kuantan especially in Pantai Teluk Cempedak (3.8120° N Latitude, 103.3726° E Longitute) and Pantai Taman Gelora (3.8073° N Latitude, 103.3475° E Longitute) and Pantai Batu Hitam (3.8841° N, 103.3630° E) of Peninsular Malaysia. The plankton net with 600 µm mesh net was used to filter and collect the water sample in container. The samples were collected in 0.5-1.0 m depth of sea from the seashore. Each sample was labelled with date and place. The detail of water temperature (25±2 ℃), pH (7.0±0.2), salinity (3.5±0.2%) was recorded to ensure the compatible in laboratory scale. The water samples were centrifuged at 2000 g, 20 °C for 10 min to concentrate the algal cells and eliminate the solid particles. The concentrated algal samples were placed in refrigerator at -10 ℃ after discarding the supernatant to avoid degradation of microalgae.

Figure 3.1 The location of marine algae samples was collected from Kuantan costal region (Pantai Teluk Cempedak, Pahang)

3.4 Pre-cultivation of microalgae

The collected and filtered marine samples were enriched in media with mixture of 90% (v/v) algae water sample and 10% (v/v) of each conventional nutrient's medium such as BG-11 medium, Walne's, and Bold Basal Medium. The light intensity was maintained at 2000 lux by using cool fluorescent white light. Samples were aerated with filtered air by using air pump for 24 h continuously. They were cultured until visible signs of algae growth especially flask with full green (Schuelter et al., 2019). After two weeks,

enrichment microalgae growth was visually assessed using fluorescence microscope to confirm the presence of microalgae and others organism.

The bloomed microalgae samples were collected from BG-11 medium flask. The bloomed samples were serial diluted to 1×10^{-5}. In nutrient plates (100×15 mm), 500 µL of the diluted sample was streaked in 30 mL solid agar nutrient medium by using sterile metallic loop, under aseptic condition in laminar flow. The inoculated plates were sealed with parafilm and incubated under controlled temperature 26±2 °C and average continuous illumination of 2000 lux in a biological oxygen demand (BOD) incubator for a period of 15 to 18 days. After the incubation period, the grown colonies were picked and streaked again on a new agar plate in aseptic environment. This step was repeated several times for each colony showing various colour and morphology characterization (Lim et al., 2012. This process to minimise the microalgae population and to obtained several single colonies. Periodic testing on an optical microscope confirmed the microalgae colony 's purity (Prakash et al., 2010; Schuelter et al., 2019).

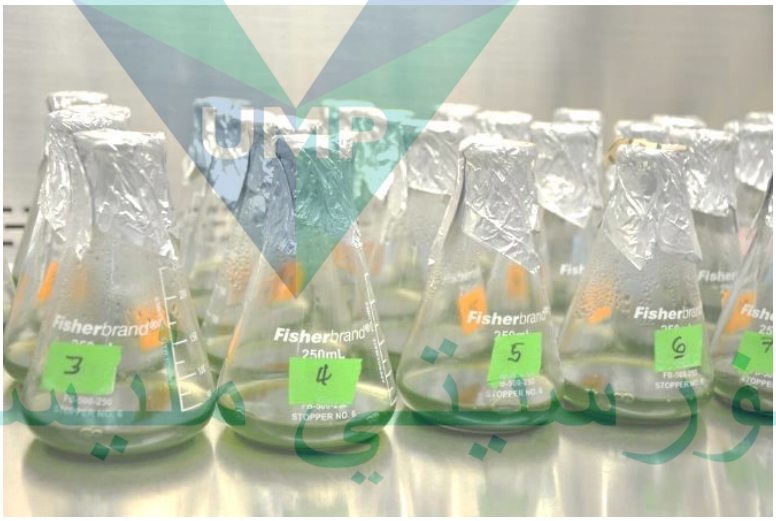


Figure 3.2 Enrichment of marine algae samples

3.5 Preparation of inoculums of isolated microalgae

Isolated green microalgae and brown microalgae from the marine algae samples were sub-cultured in the 50 ml of BG-11 and Walne's medium respectively in the 100 ml of Erlenmeyer flasks. The culture was incubated and allowed grow under light intensity of 2000 lux, 26±2 ℃ and aerated by filtered air from air pump for 18 days continuously. The cultured was examined under the fluorescence microscope regularly to ensure the purity of culture and prevent other contamination. The volume of culture keeps increased

until 2 L by adding medium for every four days under aseptic conditions. The flaks head part sealed with parafilm to avoid contamination.

3.5.1 Determination of isolates microalgae

The isolated individual colonies were examined under Olympus Bx53 fluorescence microscopy which equipped with a colour camera and a monochrome camera in Central Laboratory of Universiti Malaysia Pahang. The colonies morphological structures were observed with magnification of 50x, 100x, 400x, and 1000x and took pictures as evident with Olympus DP72 colour camera at 5Mpixel CCD with 2448×1920 pixels (3.45 mm pixel size). Green and brown microalgae colonies were observed in different size, shape colour intensity. Each colony was subculture in 2.0 mL of BG-11 and Walne's respectively in small glass tubes and the cultured in the laboratory at optimum environment. Examined again the single colony culture after 15 days then subculture in BG-11 and Walne's nutrient agar media. There were 16 species were observed. The morphological characterization of isolated microalgae was done based on their colour of scum, length of filaments, shape, length of spiral, breadth of trichome, presence of gas vacuole, pH and temperature tolerance. The examined species were listed in Table 4.1. The species of isolated microalgae name was identified by referring the algae manual based on the morphological structure.

3.5.2 Morphological identification

A 10 mL syringe connected to small opaque plastic container which was previously mounted a filter nucleopore 25 mm in diameter. 500 µL of culture was filtered by simple gravity to avoid damaging the cells. Second filter nucleopore was added and separated from the first by a silicone seal. The salt was removed by adding distilled water. The samples were dehydrated through in series of alcohol in increasing concentration (25%, 50%, 75% and 100% v/v). The dried material gone through the critical point process which helps to replace the ethanol with liquid CO_2 under controlled pressure and temperature condition. CO_2 evaporates without causing surface tension forces on the cell surface whiles the pressure reduced. The sample was dried in the atmosphere gases. A fine layer of platinum was given on samples for 70 secs with 10^{-1} vacuum mbar to enhanced the conductivity of sample while the strong electric field producing in order to stimulate electrons out of their atoms. The samples were placed on sample holder in the

chamber of SEM. The surfaces of the microalgae species were scanned in the computer monitor. The surfaces of samples were scanned from 100x magnification to 5000x magnification and observed the morphological surfaces of species.

3.6 Cultivation of isolated microalgae

Using standard microbiology agar streaking technique, colonies which formed on the agar plates were picked and re-streaked on fresh agar medium by using sterilised inoculation loop. After several repeated streaking on fresh agar media, pure isolates were screened and examined the colonies under microscopic. Five microalgae colonies were isolated from the nutrient agar media and cultured in the BG-11 and Walnes medium. The inoculums or seed culture prepared from single isolated colonies by culturing it with 10 mL of BG-11 and walne's medium in pre-sterilised 100 mL flasks. The flasks were incubated at 26±2 ℃, under cool fluorescent light of 2000 lux with continuous aeration. Then started to scaled up to 100 mL, 500 mL, 1000 mL and 2000 mL if Erlenmeyer flasks. The purity of culture examined regularly in order to maintain as monoculture. The cell density was measured by optical density (absorbance) at 680 nm towards all the algal isolates. Selected the strain with high potential growth rate and screened for lipid rich microalgae. Once the growth reached maximum, dilution and scale up the cultivation for increase the volume by transferring into new bigger flask (Lacroux et al., 2020).

3.6.1 Evaluate the performance of isolate microalgae

After the characterization of isolates microalgae which is obtained from the marine algae sample from East Coast region of peninsular Malaysia, Kuantan, Pahang. Evaluate the performance of six microalgae species which are *Amphora* sp., *Anaebanae* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Tetraselmis* sp. with diluted mix of BG-11 and Walnes with POME (5%) medium in small scale. First determine the growth rate by taking Optical density reading at 680 nm wavelength in UV-Vis Spectrophotometer. Then colour changes in the culture were regularly monitored. Lastly studied is settling, which is calculated by analysing which isolates settle more quickly than others by gravity, which is a significant advantage in order to enhance the harvesting process in large-scale applications and minimize harvesting costs. The growth rate of different monoculture in BG-11 + POME medium was determined, later on the experiment was set in 500 mL of Erlenmeyer flask with 50 mL of POME and 200 mL of

isolates microalgae. The culture was incubated in optimum growth condition such as light, temperature, and aeration, then growth of isolates was monitored for 18 days regularly until they reached the early stationary phase. The high growth of species in POME medium were selected for further experiment in this study.

3.7 Cultivation of *Spirulina* sp. in different parameters

The growth of isolated *Spirulina* sp. was investigated with different parameter such as POME concentration (v/v), light intensity (lux), and temperature (℃). In order to enhance the growth rate to optimised the biomass productivity (g/L) of *Spirulina* sp. Prior to the supply of gas into the medium, gas was filtered using a 0.22 µm pore size filter. Liquid samples were obtained regularly for biomass concentration analysis. The biomass productivity was determined by taking optical density (OD) value at 680 nm wavelength by using Spectrophotometer. The culture was grown in BG-11 medium as control. The growth of *Spirulina* sp. in different concentrations of POME was compared with the conventional medium.

3.7.1 Effect of POME concentration on growth of *Spirulina* sp.

The effect of POME concentration (10-50% v/v) on the growth of *Spirulina* sp. was determined separately. The pure 900 mL of culture of *Spirulina* sp. were added in 100 mL of prepared POME (10%, v/v) in 2 L of Erlenmeyer flask. and it followed for 20%, 30%, 40% and 50% v/v of POME with the culture and set up in 2 L Erlenmeyer flask. The experiment was conducted for 15 days whereas the culture is aerated with filtered air through 0.24 µm MCE syringe filter, illumination of 3000 lux, light intensity and maintain temperature 26±2 ℃ for 24 h continuously in laboratory. Every day the growth of culture was monitored by taking the optical density reading by using spectrophotometer at 680 nm wavelength and ensured the culture from any form of contamination and precautions steps were taken to avoid contamination in culture.

3.7.2 Effect of temperature on growth of *Spirulina* sp.

The effect on growth rate and biomass productivity was investigated at different temperature with 21±2 ℃, 26±2 ℃, 31±2 ℃ and 36±2 ℃. The culture was provided light intensity 3000 lux and aeration with filtered air through 0.24 µm MCE syringe filter

for 24 h continuously. The temperature of environment 21±2 ℃, 26±2 ℃ was adjusted by increasing and decreasing air condition and temperature 31±2 ℃ and 36±2 ℃ were adjusted without air conditions at room temperature. The temperature was keep monitored by using portable infrared thermometer device. The culture was grown for 15 days and recorded the absorbance reading via UV-Vis spectrophotometer at 680nm wavelength to measure the optical density (Gonzale-Camejo et al., 2019).

3.7.3 Effect of light intensities on growth of *Spirulina* sp.

To study the effect of various light intensity on the growth of *Spirulina* sp. Three different light intensities were used to optimized the biomass production. The *Spirulina* sp. was cultured in 30% of POME medium and temperature were maintained at 26±2 ℃. The culture was aerated by supplying filtered air to equalised the light intensities reached all over the culture in flasks. Different light intensities were provided such as 1000 lux, 2000 lux, 3000 lux and 4000 lux through cool fluorescent light to culture and study the effects of light intensities on the growth and biomass productivity of *Spirulina* sp. The light wave lengths were measured by using light meter in flux unit. Different wavelength of light intensities affects and stimulates the growth of *Spirulina* sp. under various light conditions (Ghasemi et al., 2016). The growth of cultures was monitored for 18 days and took necessary precaution steps to avoid contamination in the culture. The changes occurred in the all the flasks culture were recorded. This experiment was carried out triplicate to obtain valid results and data.

3.8 Growth measurement

The *Spirulina* sp. concentration was measured daily by optical density at 680nm with the use of a UV–Vis spectrophotometer. After scanning the visible wavelength range of the culture, was found highest peak at 680 nm and it turn up to reason for measure the absorbance of the culture. Before the culture taken in cuvette, ensure the cleanliness of the flask and swirl the flasks well for even mixture culture. Each time three samples with 2 ml of culture were collected daily from each Erlenmeyer flask and all measurement were performed in triplicates. In addition, samples taken from all cultures for absorbance determination were randomly subjected to microscopic examination at all stages of cultivation especially exponential and early stationary growth phase. The samples were taken from the flask under aseptic environment to prevent and minimize the

contamination from the external source. Through microscopic visualization confirm the purity of culture with no bacterial contamination has been found. Also, pigmentation of microalgae was brightly coloured, both macroscopically and under the microscope. By the end of each experiment, the overall productivity of biomass was measured as dry weight (g/l/d) after it harvested by process of centrifugation (Abd EI Baky et al., 2016).

3.8.1 Determination of biomass productivity

The biomass production was calculated using equation 1 in mg/l/d, DBWγ is final dry biomass weight in mg/L DBWδ is the initial dry biomass weight in mg/L and t is the cultivation period comprised between final and initial measured expressed in days.

$$BP = (DBW\gamma - DBW\delta) / t \quad 3.1$$

The specific growth rate expressed as μ as in equation 2 where d^{-1}, X_t represented the biomass concentration at the end of the exponential phase in mg L^{-1} and X_0 at the beginning, and $t\text{-}t_0$ symbolised the duration of the exponential phase in days (Pérez et al., 2017).

$$\mu = \ln \frac{X_t - X_o}{t - t_o} \quad 3.2$$

Finally, the doubling time (DT) expressed in days was determined as shown.

$$DT = \ln(2) / \mu_{max} \quad 3.3$$

where μ_{max}, is the highest specific growth rate during the exponential phase of the culture (Wang et al., 2014).

3.8.2 Determination of dry biomass and chlorophyll content

After centrifugation the harvested biomass was dried at 80 ℃ for 6 h. The empty petri plate was weighed before added the wet biomass and weighed again the same petri plate with yield after it dried. The difference between starting and final weight was recorded as the weight of dry *Spirulina* sp. and it mention in term of g/L. The harvested wet biomass was rinsed with distilled water and suspended in 10 mL of 95% methanol. The algal suspension was placed in water bath at 60 ℃ for 40 min. Intermittent shaking

of the tubes assured complete pigment extraction. To remove the cell debris, the tubes were removed from the water bath and centrifuged again. Clear supernatant containing the pigment was transferred to a volumetric flask, and methanol was added to the volume by up to 10 mL. The chlorophyll amount which is present in the biomass were measured from the absorbance reading at 645 nm and 665 nm of the methanolic extract. Methanol was used as blank (Prihantini et al., 2019).

3.9 Biomass harvesting via centrifugation

After the experiment completed, the culture of *Spirulina* sp. was transferred from Erlenmeyer flasks into 250 mL of falcon tubes on 18th day. The collected culture centrifuged at 8000 *g* at 4 ℃ for 10 min by using Kubota 5922 centrifuge. The supernatant was discarded and collected the wet biomass pellet in polystyrene antistatic weighing dish. The wet biomass was rinsed with distilled water and eliminated the solid particles, followed centrifuged again and collected the biomass was placed in oven at 80 ℃ from 6 h. The dried biomass was weighed and recorded the mass. The dried biomass were stored at -20 ℃ for further testing (Rajasekar et al., 2019).

3.10 Characterization of palm oil mill effluent

The collected supernatant was tested by water analysis and determined the compounds presents in POME before and after it treated with *Spirulina* sp. The characteristics of POME was tested before and after the cultivation of *Spirulina* sp. Metal analysis, total suspended solid, chemical oxygen demand, and nitrogen contents were tested in the POME medium before and after cultivation of *Spirulina* sp.

3.11 Lipid extraction technique

Extraction process can be separated into three phases like cell disruption, dehydration and recovery of desired metabolites (Ranjith Kumar et al., 2015). In this study Bligh and Dyer, ultrasound-assisted and Soxhlet methods were chosen to break the cell and released the lipid. These methods were studied to determine the high efficiency, effective and high yield collection of methods. Biomass needs to be handled more rapidly after algal cell walls have been broken, or it can be ruined within a few hours. Thus, dehydration is a procedure used before the desired metabolite is retrieved to increase the

algal biomass shelf-life (Zhang et al., 2018). The lipid yield or extraction yield was defined as the percentage of the lipid mass against the total mass of the raw microalgae sample, excluding the humidity. The calculation equation given below at equation 3.4.

$$\text{Yield of extraction} = \frac{\text{Mass of lipid (g)}}{\text{Mass of raw microalgae (g)}} \times 100\% \qquad 3.4$$

3.11.1 Bligh and Dyer (1959)

Weighted the 3.0 g of *Spirulina* sp. dry biomass and rehydrated with 2.5 mL of deionized water. 40 mL of hexane: methanol (1:1 v/v) which mixed with calcium carbonate was added into sample. The mixture leaves it overnight in the dark at room temperature. After that, the mixture was vortex for 10 sec and centrifuged at 8000 *g*, 5 min duration and 10 °C temp. The balance sample ensured with another Falcon tube which contain with approximately same volume. After centrifuged the supernatant fluid was collected into falcon tube. Then added 45 mL of hexane: methanol (1:1 v/v) mixture into *Spirulina* sp. pellet. The steps were repeated until the supernatant were become colourless. Collected supernatant transferred into multiple 50 mL tubes and each tube contained 15 mL of supernatant fluid. Then add additional 15 mL of deionized water into the falcon tube. And followed by 15 mL of hexane solvent was added with supernatant fluid. The supernatant mixture was vortex for 10 sec and centrifuged at 8000 *g*, 10 °C for 5 min. Two layers were formed in the falcon tubes mixture after the centrifugation process. The upper layer solution was collected in new falcon tube. Hexane solvent added again with lower layer to re-extract until the upper layer become colourless. The collected upper layer solution transferred into 500 mL of conical flask and placed in the fume hood. The hexane solvent was evaporated from the upper solvent for 6 h and collected the remaining product in falcon tube. The end product of crude oil was cap and store at -10 °C for further analysis (Ewald et al., 1998).

3.11.2 Ultrasound-assisted extraction

After drying process, the collected dry *Spirulina* sp. was grinded by using pestle and mortar. Ensure biomass turn completely to powder form. An empty breaker was weighed before adding the biomass and recorded. In falcon tube biomass added with organic solvent hexane: methanol (1:1 v/v). In 5 g of dried powder, add 2.5 mL of hexane solvent in 50 mL Falcon tube. The mixture was vortex for 15 sec. Then the mixture was

placed in ultrasonic water bath for 1 h at 60±5 °C to breakdown the microalgae cells at optimum temperature. The cavitation process where the bubbles induced by high frequency pressure (sound) waves to agitate a biomass mixture and this action penetrate a hole or crack the cells and release the lipid compounds. After 1 h, the mixture was centrifuged and collected the supernatant (Hindryawati et al., 2014). The first step was repeated with pellet biomass as recycling process. The total hexane mixture was collected and added with 1% of KOH methanolic. The mixture was vortexed for 20 sec and placed it in ultrasonic water bath at 60±5 °C for 15 min. The mixture was separated into two layers of solvents in the falcon tube and collected the top layer in 2 mL of centrifuge tube. Before the sample place in centrifuged, it weighed as empty. The collected upper layer solution placed in fume hood for 6 h to allowed hexane to be evaporated. A kind of sticky liquid which is known as lipid remain in the centrifuged tube (Bligh & Dyer, 1959). The yield of lipids determined by weighed it. The lipid samples were cap and store at 10 °C for further analysis.

3.11.3 Soxhlet extraction

Soxhlet extraction is an extensively used method for extracting lipids from microalgae in the mass production of biofuels. Hexane is used as a solvent, due to its non-polarity and high fat and oil selectivity. In the experiment, 5.2 g of dried sample was weighed and loaded in the thimble and it placed in the Soxhlet extractor. A 150 mL of round bottom flask took and clean it then filled with 90 mL of hexane. The whole setting was placed on a heating mantle and allowed the hexane to boil. The extraction process was carried out for 6 h. The refluxing started at 67±3 °C. The hexane solvent was vaporized, diluted, and repeatedly percolated through the dry microalgae samples until it exceeded the extraction was maximum. After extraction process was done for 6 h, the condensing unit was removed from the extraction unit and allowed the sample to cool down. Finally, it removed all the lipid. After the distillation all the solvents were collected. The sample was placed in the oven at 40 °C after removing it and placed in the desiccator. The sample weight was measured for further analysis (Kanda et al., 2020). Under reduced pressure the solvent was extracted from the extract by means of a rotary evaporator at 50 *g* and 50 °C. The residues and lipids were collected in a refrigerator and stored before further analysis (Hindryawati et al., 2017; Vandongen et al., 1993)

3.12 Transesterification of lipid

From the *Spirulina* sp. extract, the large macromolecules compound are breaks into micro molecules compounds by transesterification process. In 2 mL centrifuge tube, 0.5 g of *Spirulina* sp. lipid mixed with 0.5 g of methanol KOH and vortexed for 30 sec. Then the tubes were emerged three quadrants of tube into ultrasonicated water bath at 60±2 ℃ for 2 h. Then, 1.0 g of hexane was added with it and vortexed for 2 min continuously. The mixture turns into two separated layers of solvents. The upper layer solvent was collected in the 2 mL centrifuge tube and evaporated the hexane by placing it under fume hood for 30 min.

3.13 Analysis of lipid

3.13.1 Characterization of lipid via FTIR

The presents of functional groups in *Spirulina* sp. lipid were examined by using Perkin Elmer Spectrum 100 Fourier Transform Infrared (FT-IR) Spectrometer. The data collection can be processed by the software OPUS which was used and identified the functional molecular group. The samples were analysed between the ranges of 4000 cm^{-1} to 450 cm^{-1} with 32 scans. The background of the FT-IR was scanned completely to avoid unnecessary peaks before analysed the samples. The spectra were baseline corrected and characteristic peak area of lipid and recorded it in spectrum software. The fluctuation in peak absorbance was due to the environmental factor and inconsistent preparation of the samples. The relative lipid contents were evaluated to reduce those errors (Pohndorf et al., 2016; Shanthi et al., 2018).

3.13.2 Fatty acid profile via GC-MS

The gas chromatography mass spectrometry uses for the detection of fatty acid methyl esters content. Samples were analysed in Agilent 7890A gas chromatography system equipped with capillary mega wax MS column (30 m length × 0.32 mm diameter × 0.50 µm film thickness). The mass spectrometer detector used with helium as the carrier gas 1ml/min. The oven programmed with the following time–temperature program: 190 °C (2 min), 190–230 °C (5 °C/min), 230 °C (8 min). Peak areas were quantified with Chrome card for Windows software (Wang et al., 2014; Perez et al., 2017). The mass spectra were recorded at 70eV. Mass range was m/z 40-250. About 40 mg of methyl ester

sample was weighed in vial 1000 µL of internal standard dilute on octane 15 mg/ml concentration was added. In homogenous mixture form sample was injected into GC. The inlet temperature was maintained at 230 °C with a split ratio of 50:1. The injection volume was 1 µl, with a split ratio of 50:1. The variable quantification was performed on column based on their GC peak areas. By comparing mass spectra of the unknown peaks with those contained in the NIST 98/NBS 75 K GCMS library, the identification of separate free fatty acids was achieved by retention indices and mass spectrometry (Lim et al., 2009; Chen et al., 2018; Saengsawang et al., 2020).

3.13.3 Quantification of fatty acids

Morroson and Smith, (1964) method was used to identify and quantify the fatty acids in microalgae oil. The methylated fatty acids was analyse through gas chromatography with a polar capillary mega wax MS column (Lim et al., 2009; Manaf et al., 2018). Determination of ester content using methyl heptadecanoic acid, C_{17} as an internal standard. Peaks of methyl esters were identified by comparing them with their respective standards.

$$\text{Fatty acids methyl ester (wt.\%)} = \frac{(\sum Ai) - Ais}{Ais} \times \frac{Cis\ Vis}{m} \times 100 \qquad 3.5$$

Where

$\sum Ai$ is the sum of methyl esters peaks area

Ais is the area of internal standard

Cis is the concentration of internal standard in octane (mg/mL)

Vis is the volume of internal standard (mL)

M is the weight of sample (mg)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and characterization of microalgae species

The collected natural marine algae sample might contain zooplankters that feed upon the algae. These animals may eat or kill the algae after it concentrated. Through gentle filtration unwanted colonies algae and larger organisms were filtered out. Prefiltering of algae sample while collection was effective for these unwanted tiny organisms like bacteria. Though, care must be taken in order to prevent the target species from damaging or desiccating.

Sampling of local waterways based on intertidal rock pools, where the microclimate frequently switches between ideal conditions of growth and unfavourable conditions such as low/high light, temperature, low nutrients, micro-oxic conditions and quick changes in salinity. Sampling at such locations was considered advantageous as suboptimal conditions would require the algae to accumulate photo assimilates such as starch or lipids with important storage functions to survive, thus increasing the chances of obtaining high lipid content strains (Schenk et al., 2008). It was accompanied by an isolation method aimed at selecting microalgae species for high growth rate which could be induced to accumulate lipids under conditions deprived of nutrients.

The filtered algae samples were enriched by adding nutrient BG-11, Walne's and Bold Basal medium. The enrichment culture was incubated in a culture chamber, and the culture growth was monitored every few days for the target species. Among these nutrients medium, higher growth rate of microalgae population was found in BG-11 compared to other media.

The grown microalgae population was examined under fluorescence microscope in order to determine presence of microalgae species and other small organisms. Microscopes helps to observe the microalgae species with detailed image of targeted cell and contamination. In Table 4.1 listed out the presence of microalgae species from the

enriched culture. These 16 different indigenous species were observed and characterized based morphological feature as colour, shape, size and its structures. The observed species found in different structure spherical, ovoid, barrel, long thin filament, spirilla, cylindrical and ellipsoidal shape under fluorescent microscope. The collected marine microalgae samples were expected to adapted to the laboratory environment by tolerating with conventional medium like nutrient concentrations, pH, temperature and light

Green microalgae which grown well in the BG-11 enrichment media was centrifuged to concentrated to the culture and streaked on the BG-11 nutrient agar plate medium under aseptic conditions. A sterilised bacterial loop was loaded with concentrated algae was used to spread across the agar with several pattern. Serial dilution techniques also been used to minimise the microalgae population from the enriched culture and obtained axenic culture under aseptic environment. But streaking plate technique much ease to isolate single colonies. To prevent the growth of fungal and bacteria in plates 0.2 g/L of antibiotic was added in the media and further plates was sealed with parafilm. The streaking plate technique was easy to perform and effective to obtain the dominant single colonies from the nutrient agar plate. In addition, axenic cultures can often be directly established without further treatment.

All the safety precautions were taking to avoid contaminations. After 15 days of incubation period, found the green microalgae colonies were appeared on agar plates as shown in Figure 4.1. Normally, the root of the streak has so many cells that are not isolated, but rather as the distance from the source single cells are beginning to grow separately. After several re streaking process from the previous plates single colonies were isolated. Normally the effective of streaking were in the range of 6-9 times in order to obtain pure colonies very easily and can observed through fluorescence microscope.

Although variations in the shape, size and colour of the colony were considered when making selections, such differences were assumed different species did not automatically guarantee these. Morphological structures of isolated colonies were examined by using fluorescence microscope again to confirm the purity and prevent contamination of other unwanted organisms. The isolated species were classified based on the morphological appearance, and the isolated colonies' microscopic cellular appearance.

Several single colonies with minimum population of microalgae were found grown well and healthy conditions. From these colonies randomly 5 green microalgae colonies and one brown microalgae colony (diatom) were isolated from the plate and inoculated each colony in the 10ml of semi-liquid medium separately. Green microalgae colonies were inoculated in BG-11 media and brown microalgae colony were inoculated in Walne's medium.

These 6 isolated colonies were examined under fluorescence microscope in order to confirm the purity of single colonies and observed the morphological structure of species to identify the family of species and characterization as well. Based on the species morphological structures and thallus, the name of species was identified by referring algae manual book (Carmela., 1997; Hendey, 1951; Desikachary, 1989). Table 4.2 shown the 6 isolated microalgae species with characterization of *Amphora* sp., *Anabaena* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp. and *Tetraselmis* sp.

From the similar location of Kuantan port, Pahang, Liow et al (2019) reported that, total six dinoflagellate genera, eight diatom genera, and one genus of raphidophyte (*Chattonella*) were reported with eleven species of potentially harmful dinoflagellates: *Alexandrium* spp, *Prorocentrum micans, Psigmoides, Dinophysis acuminate, D.caudata, D.miles, Tripos furca, T.fusus, Akashiwo sanguinea, Nictiluca scintillans* and *chattonella* sp. Other studies mentioned that, marine algae *Oscillatoria* sp. was identified as reported in (Mansor et al., 2013). Phang et al. (2015) reported that, from East Coastal region of Peninsular Malaysia, Kelantan had highest was identified 43 taxa phytoplankton. The samples were dominated by the Euglenophytes, Chlorophytes (*Pediastrum, Chlorella*) and Cyanophytes (*Oscillatoria*). From straits of Malacca, especially in Aman Island, Penang, marine microalgae such as *Chaetoceros* sp., *Skeletonema* sp., and *Thalassiosira* sp. were identified among total 58 taxa phytoplankton which been reported by Razali et al. (2015).

Serial dilutions should also be chosen for increasingly growing strains that eventually rule a society. Particular attention must be paid to ensuring that a single rapidly growing strain does not overpower other potentially high lipid content strains which may have a slower growth rate. Precaution steps requires to implement before streaking on nutrient agar plate (Rahayu et al., 2015). Agar also can support the growth of bacteria and

fungus. Marine microalgae samples from field with major fungal contamination. It cannot prove because the fungus grows quickly, producing sporangia and spore that contaminate algae isolation attempts. This contamination can be minimized by using filters and organic substrate.

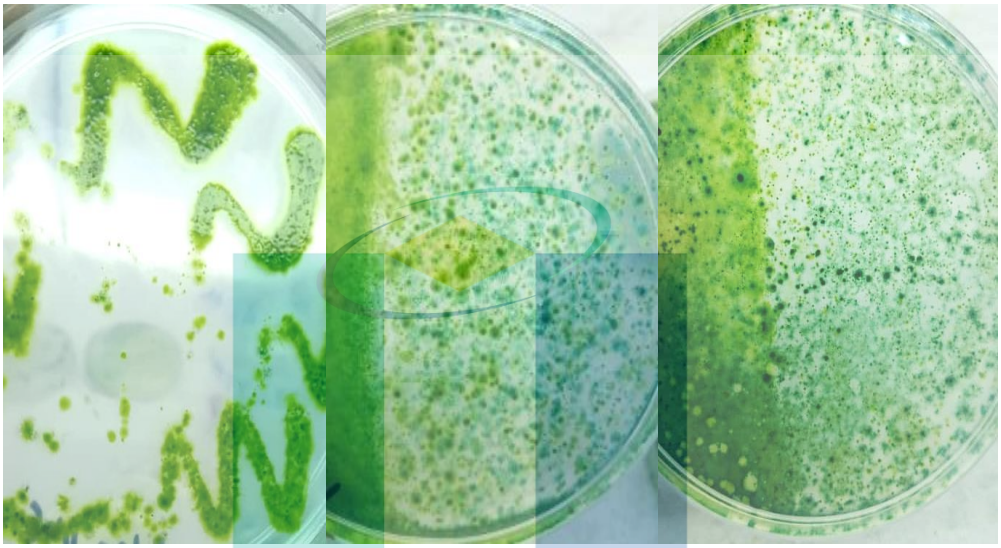
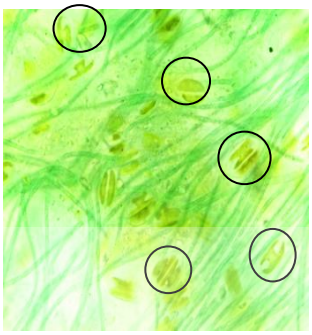
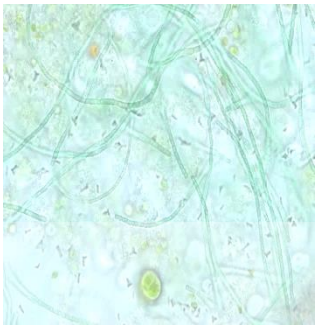


Figure 4.1 Formation of microalgae colonies on the nutrient agar plate

Table 4.1 List of identified marine microalgae species from coastal region of Kuantan, Pahang

Microalgae species	**Microalgae species**
Amphiprora sp.	*Nannochloropsis* sp.
Amphora sp.	*Parachlorella* sp.
Anabaena sp.	*Rhodomonas* sp.
Chlorella sp.	*Scenedesmus* sp.
Chlorococcum sp.	*Skeletonema* sp.
Gyrosigma sp.	*Spirulina* sp.
Mamiellagilva sp.	*Tetraselmis* sp..
Merismopedia sp.	*Thalassiosira* sp.

Table 4.2 Morphological characteristics of the isolated microalgae species

Species	***Amphora* sp.**	***Anabaena* sp.**	***Chlorella* sp.**
Image			
Characteristics	Valve semi-lanceolate, sub-capitate ends, dorsal margin convex, flattened in the center, with straight ventral margin, 16-22µm long, 4-6µm wide.	Straight and solitary trichomes, vegetative cells are cylindrical, without aerotopes and apical cells are rounded Heterocysts are long rounded and intercalary and single.	Green, Spherical cells, cup-shaped chloroplast, 2-10 µm in diameter, single cells, non-motile
References	Wachnicka et al., 2007	Galhano et al., 2011	Ru et al., 2020
Image			
Characteristics	Consist of 4 cells arranged in a row, feature bristles, green algal cell, has a central vacuole, a two layered cellulose and pectin cell wall	Blue-green, Unicellular, long, 8 µm in diameter, no flagella, motile, cylindrical cell arranged to form unbranched	Light green, Cup shaped chloroplast, 10-25µm long, 7-20µm broad and 6.5-18 µm thick, cell shape to be oval with posterior part wider than the anterior
References	Gour et al., 2016	Vonshak, 1997	Arora et al., 2013

4.2 The growth assessment of the isolate's microalgae species with POME

The selected microalgae species were cultivated in 5% v/v of POME as side-by-side cultures to determine and compare growth rates and doubling times. *Amphora* sp., *Anabaena* sp., *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Tetraselmis* sp. were highly adapted for culturing in the laboratory at optimum conditions. Five green microalgae species were cultured in mixture medium of BG-11 with 5% v/v of POME and *Amphora* sp. was a diatom and it cultured in mixture of Walne's and 5% v/v of POME medium for 15 days.

From the result shown in Figure 4.2, all flask cultures were at lag phase initially after inoculation. These indicating the culture were require time to adapt to the POME environment before it begins to double the populations. Each species performed variously in the POME environment. Among these species only *Spirulina* sp. and *Chlorella* sp. were shown high positive response compared to other species with high growth rate as shown in Figure 4.5. This indicated that *Spirulina* sp. and Chlorella sp. have potential for growing in POME environment and produce more biomass.

The present of nutrient compounds in POME support the growth of *Spirulina* sp. and *Chlorella* sp. Other species unable to survive in POME environment due to the sensitivity of species and lacking micronutrient for consumption. If compared the biomass productivity between *Spirulina* sp. (959.2±112.3 mg/L) higher than *Chlorella* sp. (785.7±102.1 mg/L) in POME medium. *Spirulina* sp. with higher growth rate produced higher amount biomass at early of stationary phase.

The growth rate of *Amphora* sp. was declined and these species were not adapting to the POME environment. The light penetration in the diatoms culture were inhibited by the dark POME. As a result, photosynthesis reaction unable to carried out by the species. Therefore, the growth level and productivity of biomass reduced automatically. Apart from that, diatom species requires silicate compounds for boost up the growth. But in POME medium are lacking of Silicate compounds.

Therefore, species were unable to perform growth activity. Silicate compounds strengthen the cell wall and cell membrane of microalgae. Resdi (2016) reported that, *Spirulina platensis* shown high biomass productivity 9.8 g/l/d whereas *Chlorella* sp. was only 0.058 g/l/d while cultivated in POME. POME support the growth of microalgae. However, excess nutrients can resist the microalgae growth and not all nutrients can have

utilized by all microalgae. The excess nutrients in POME may inhibit the growth and converted it into toxic compounds (Nur et al., 2015; Resdi et al., 2016).

Therefore, Microalgae *Spirulina* sp. been selected for further experimental in order to achieve second and third objectives of the study. Figure 4.3 shown the morphological structure under fluorescence microscopy at magnification of 400x and 1000x. where us the Figure 4.4 shown the observance of *Spirulina* sp. under scanning electron microscope at magnifications of 5000x and 2500x.

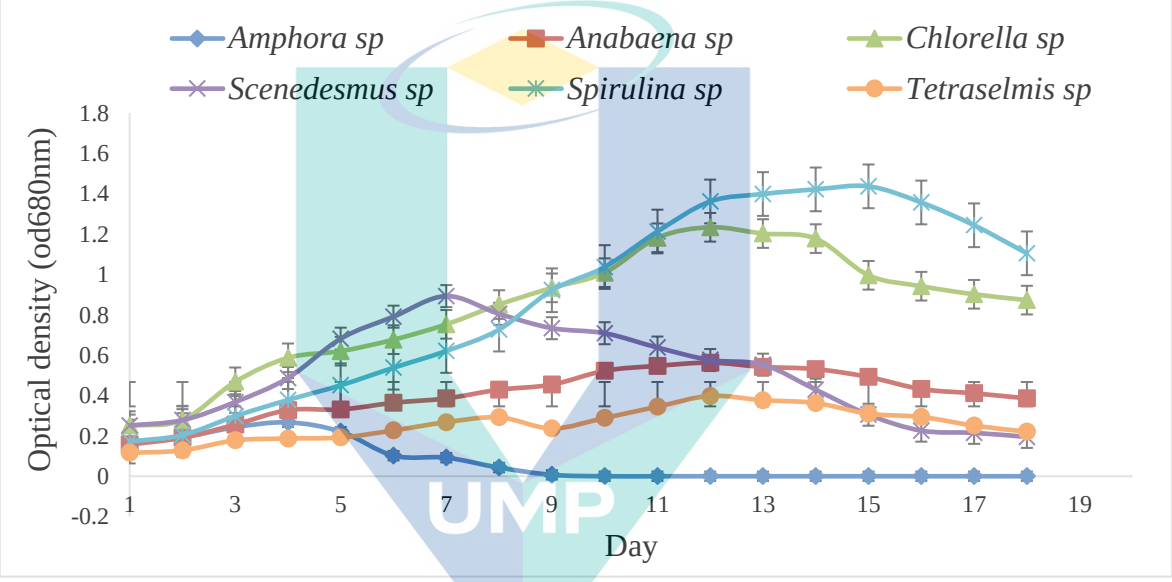


Figure 4.2 Optical density value of isolated species

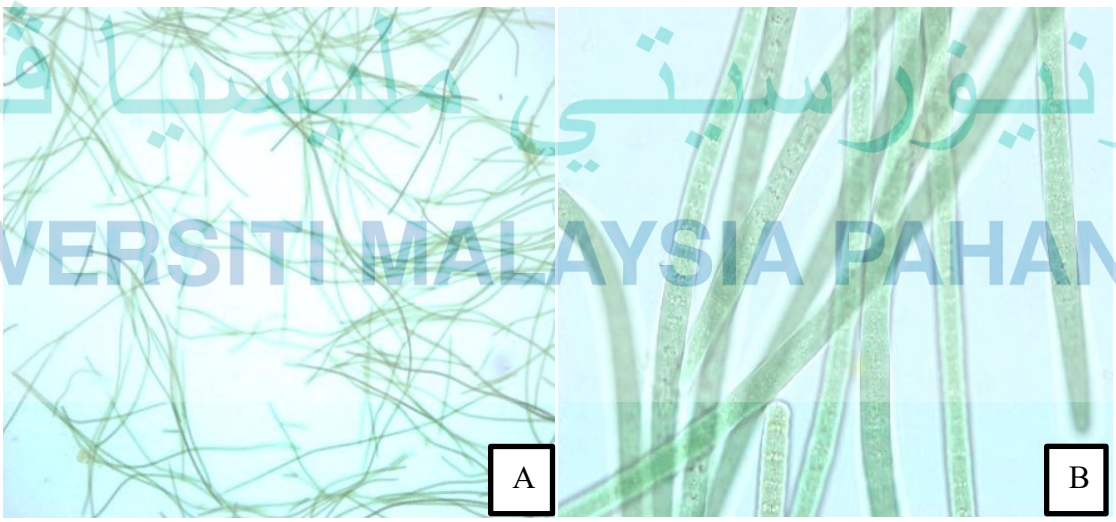


Figure 4.3 Fluorescence micrographs of microalgae *Spirulina* sp. under (A) magnification of 400x and (B) 1000x

Figure 4.4 Scanning electron micrographs of microalgae *Spirulina* sp. at magnification of 500x (A) and 2500x (B) magnifications

4.3 Cultivation of *Spirulina* sp. for stock culture

The microalgae *Spirulina* sp. culture conditions rely on the species to be cultivated and the experiments planned. Various factors, such as the culture medium composition, temperature, relative humidity, air flow, concentration of CO_2, lighting, among others, affecting *Spirulina* sp. cultivation. At starting, the *Spirulina* sp. suspensions are kept in test tubes of 10 mL at 26±2 ℃ with 2000 lux light intensity. While the culture's cell density increases, it transferred to 100, 500, 1000 and 2000 mL of Erlenmeyer flasks. The culture was agitated by supplying continues filtered air through motor pump. The culture was examined every two days once under fluorescence microscope to ensure the purity of *Spirulina* sp.

4.3.1 Cultivation of *Spirulina* sp. in POME

Figure 4.5 shows the growth of *Spirulina* sp. in five different concentration of POME and in BG-11 media as control. The culture gone through the lag phase in the first three days in all the experimental flask. Increasing in palm oil mill effluent concentration from 10-30% (v/v) increasing the biomass concentration mg/L. After 6 days of cultivation of *Spirulina* sp. in 30% (v/v) of POME the growth rate was more intense than others. The highest biomass of *Spirulina* sp. was obtained at 30% (v/v) of POME medium was 1118.4±134.2 mg/L on day 12. *Spirulina* sp. shown similar biomass concentration in both BG-11 medium and 20% (v/v) of POME throughout experiment whereas culture in 10%

(v/v) POME end up in stationery and death phase after day 12. The maximum biomass concentration in 40% of POME reached on day 9 (483.9±62.90 mg/L) than declined. However, *Spirulina* sp. in 50% (v/v) of POME does not grow and it decreasing slowly after day 3. Raw POME contains high concentrations of nitrate, orthophosphate and BOD_3 concentrations. Such inorganic elements are intended to be used as nutrients for microalgae cultivation. Because of the POME 's appearance as a waste of freshwater and its dark color the correct percentage to be included in the formulation for optimal growth of marine *Spirulina* sp. was at 30% (v/v) of POME. The raw POME was diluted and modified the pH for the utilization by the microalgae.

The similar result was reported that, between 5 to 30% (v/v) POME on *Spirulina platensis* biomass productivity was significantly enhanced ($P< 0.05$). Increasing POME concentration significantly enhanced the concentration of productivity due to presents of more nutrient components. However, above 30% of POME did not enhance the biomass productivity in the medium (Nur et al., 2019). Hadiyanto, (2012) found slightly different result that, the cultivation of *Spirulina* sp. in 30% (v/v) of POME with synthetic nutrient 50% reduction, given 5.685 g/L total biomass in the first 8 days of cultivation. When continues the cultivation by adding POME at 40% (v/v) without synthetic nutrients in *Spirulina* sp. obtained 9.8 g/L at day 14. Cheah et al., (2018) found that, the maximal biomass concentration obtained at 5% and 20% (v/v) of POME while culturing of microalgae *Chlorella vulgaris* 3.46 g/L and *Chlorella sorokiniana* 3.30 g/L. The high concentration of POME did not support the microalgae growth and production (Sukumaran et al., 2014).

The growth of microalgae was resisted by the characteristics of POME which is in dark liquor form. In high concentration of POME medium, light penetration was inhibited (Cheah et al., 2018). As a result, growth rate was inhibited due to lacking of photosynthetic reaction in the culture. This is same as reported by Nur et al (2019), that culture requires longer time to adapt due to limited light penetration in the lag process. Longer lag phase can affect in longer exponential and stationary phase, reducing the overall growth of microalgae cells. Sukumaran (2014) reported that, maximum biomass microalgae *Spirulina platensis* was obtained was 1.79±0.028 g/L by culturing at 20% of fresh POME medium within 7 days. There is similar biomass production approximately, but period of time was double time more. This is because of the adaptation period microalgae with POME environment and POME composition mixture. The effectiveness

of using POME for cultivation of microalgae has not been determined only by the concentration of biomass but also the lipid content. The higher biomass production the greater lipid content in the biomass while harvesting (Puteri et al., 2011).

Figure 4.6 demonstrates the graphic of chlorophyll of *Spirulina* sp. in different media. It shows the highest chlorophyll content 12.17±0.6 µg/mL found in the culture from 30% of POME. It is in correlation with biomass concentration (1118.4±134.2 mg/L) which highest among different POME concentrations. Higher biomass concentration produced higher lipid content in the *Spirulina* sp. biomass. This helps to acquire the desirable content from the species.

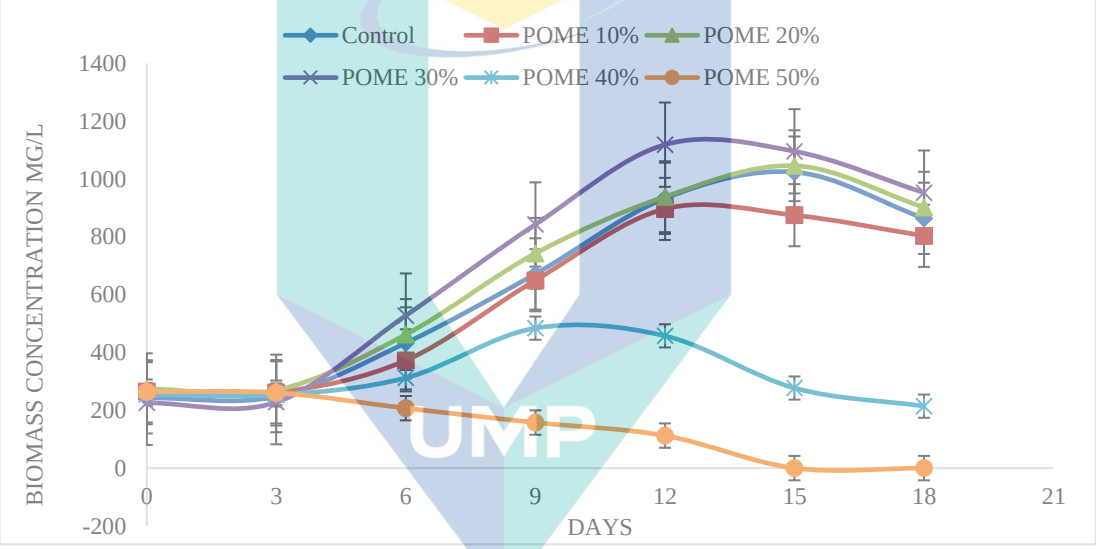


Figure 4.5 Biomass concentration of *Spirulina* sp. in different POME concentration

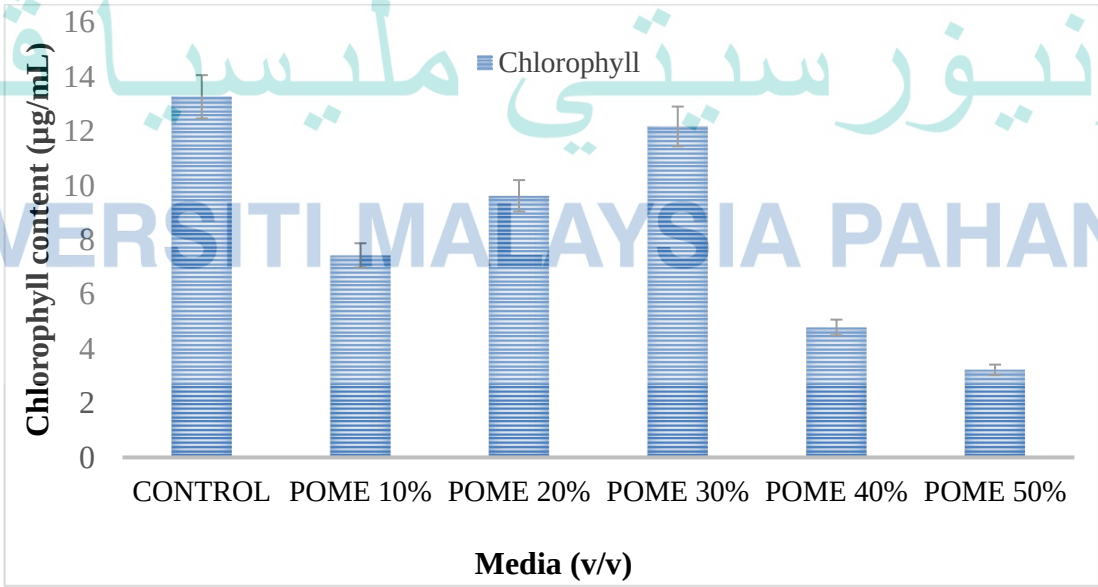


Figure 4.6 Comparison of chlorophyll content of *Spirulina* sp. from different POME concentration

4.3.2 Effectiveness of light intensity on *Spirulina* sp.

The time period, light intensity and quality of light playing vital role in photosynthesis of microalgae *Spirulina* sp. The biomass concentration and cell components were influenced by the light intensity. Based on the Figure 4.7, increase of light intensity cause increases the biomass concentration of *Spirulina* sp. The highest biomass concentration was achieved at 4000 lux light intensity (991.5±128.8 mg/L) at day 9. The maximal growth and the initial stationary phase reached by *Spirulina* sp. in all the light intensities was at day 9. Moreover, the growth *Spirulina* sp. was found that it attached to the inner surface of bottle where the source of light at 3000 lux where available more. This indicating that, growth of *Spirulina* sp. required sufficient quantity of lights for it to grow.

Sorokin et al., (1965) reported that, had it been stated that an increase in light intensity initially enhanced the cell division, an increase in light intensity inhibited cell division after optimum light intensity was achieved. At initial stage or lag phase of cultivation, *Spirulina* sp. required low light intensity to prevent damage of cell but photolysis (Nzayisenga et al., 2020), due to that moderate light intensity were much preferable as compared to high light intensity because it also might cause photolysis (Dubey et al., 2006). In addition, Ajayan (2012) reported that, maximum *Spirulina platensis* biomass were achieved at 4800-6000 lux with 6.9 mg/L. Increase in light intensity enhance the cell division of microalgae until it reaches optimal light intensity.

Apart from this, researchers mention that, white LED could give a higher biomass concentration, biomass productivity and CO_2 fixation rate than using red and blue LED lights, indicating that the growth of *Spirulina platensis* requires a wider range of light wavelengths (Ho et al., 2018). It is also noted that the growth efficiency associated with CO_2 fixation capability under white LED (410–610 nm) is marginally higher than with the same light intensity fluorescent lamp (400–700 nm).

This phenomenon shows that while white LEDs lack the spectrum of red-light wavelengths, their light distribution is still more suited to *Spirulina platensis* production when compared to traditional fluorescent lamp. Since the *Spirulina* sp. cells are sensitive to light spectrum. The red LED give the highest biomass than blue LED because chlorophyll have the potential to absorb the red light very strongly. Impact of light intensity on microalgae reported that, the growth of *Spirulina platensis* had significant impact by light intensity and growth increased up to 3 days. In addition, light intensity effect on the *Spirulina platensis* photosystem was noticed much more favourable at LED white source due to the wavelength (Kim et al., 2013).

Figure 4.8 showing the Chlorophyll content of *Spirulina* sp. at different light intensity. The maximal content was 10.23±0.71 µg/ml which was achieved at 3000 lux intensity. The chlorophyll content in *Spirulina* sp. was reducing at 4000 lux which is 7.71±0.37 µg/mL. The maximal cell concentration of *Spirulina* sp. was found at intermediate light intensity of 3000 lux 968.5±125.9 mg/L. Greater chlorophyll content represents the higher biomass concentration which indirectly produced higher lipid content in the *Spirulina* sp. biomass. This indicating that, intermediate light intensity enough to support maximum cell growth. Danasei et al., (2011) mentioned that, High light intensity did not have increase the cell growth likely because a shadow effect that can be responsible for growth interruption.

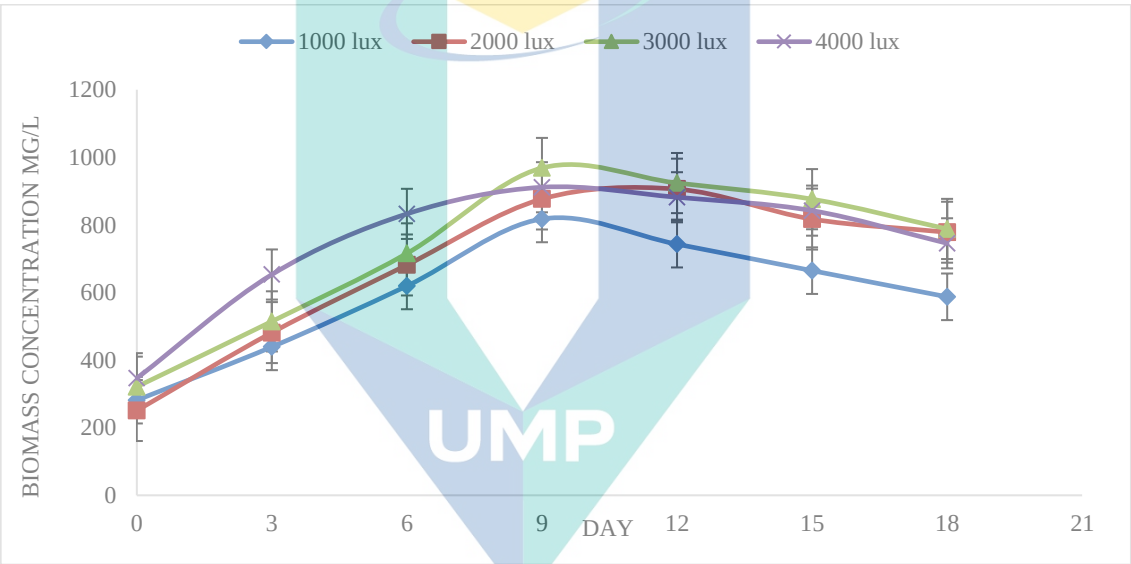


Figure 4.7 Biomass concentration of *Spirulina* sp. in different light intensity

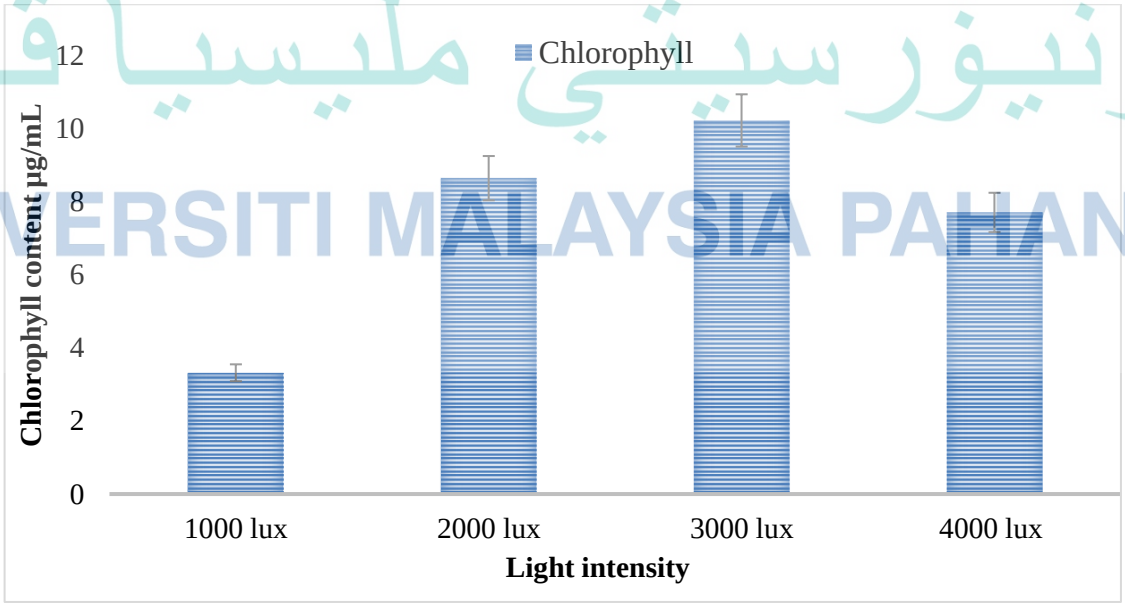


Figure 4.8 Chlorophyll content of *Spirulina* sp. in different light intensity

4.3.3 Effectiveness of temperature on *Spirulina* sp.

Growth analysis of microalgae *Spirulina* sp. at different temperatures showed different growth pattern. Maximum growth rate and concentration of biomass have been observed in the culture with temperature of 30±2 °C. *Spirulina* sp. has wide range of temperature tolerance from 21-36 ℃ based on Figure 4.9. The biomass concentration increases with increase in temperature and noticed that 31±2 ℃ is the optimum temperature for *Spirulina* sp. However, the higher temperature inhibits the growth and biomass concentration of *Spirulina* sp. Figure 4.10 shown the chlorophyll content of *Spirulina* sp. at different temperature. At 31±2 ℃, *Spirulina* sp. contains highest amount of chlorophyll as compared to other temperature. This temperature which is optimized and support the mechanism of chlorophyll synthesis. Greater chlorophyll content represents the higher biomass concentration which indirectly produced higher lipid content in the *Spirulina* sp. biomass. This indicating that, intermediate light intensity enough to support maximum cell growth.

The similar resulted reported that, the optimum temperature for *Spirulina* sp. biomass production was 30 ℃ (Ogbonda et al., 2007). An optimum growth temperature of 35–37 °C was recorded for *Spirulina* under laboratory conditions. Outdoor organism cultivation has an optimum temperature for growth of around 39 °C (Richmond, 1986). *Spirulina* thermo-tolerant strains were cultivated at 35 to 40 °C where an estate has the benefit of eliminating microbial mesophiles (Vonshak et al., 1982). In addition, kumar (2011) reported that, *Spirulina platensis* growth rate increased with temperature rise and was found to be maximum at 35 °C. Increased *Spirulina platensis* growth rate was due to a drop in the doubling time. Later it declines due to reduced chlorophyll and other pigments with more rise in temperature. For all living organisms, temperature is the most important factor as it influences metabolic processes and cell biochemical composition. The optimum temperature of growth and resistance to the extreme values usually differ from strain to strain. Kim et al., (2015) reported that, temperature and algae growth were exponential or linear which depending on the environment variable. Rising temperatures are correlated with improved photosynthesis and high nutrient rates assimilation.

Grobbelaar and Soeder (1985) stated that the loss of biomass due to dark breathing in Coelastrum, grown both indoors and under field conditions, depended on the daytime temperature and light background to which the algae were exposed. Soni et al (2019) reported that *Spirulina* has given maximum content of biomass at the maximum

temperature 25-34 ℃. The solar radiation event was very high in during summers, Bhopal. The elevated temperature is attributed to scope that does not permit any growth of the contamination of the microorganism during the summer season. There the optimum temperature of this study for the growth of *Spirulina* sp. prevents the contamination, support and enhanced the growth rate of *Spirulina* sp. for biomass production.

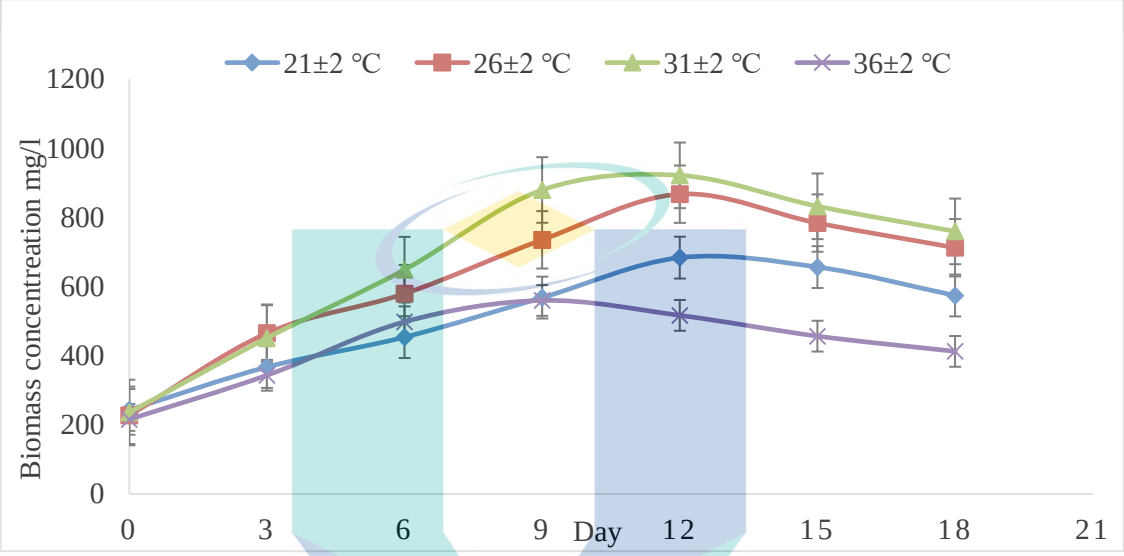


Figure 4.9 Biomass concentration of *Spirulina* sp. at different temperature

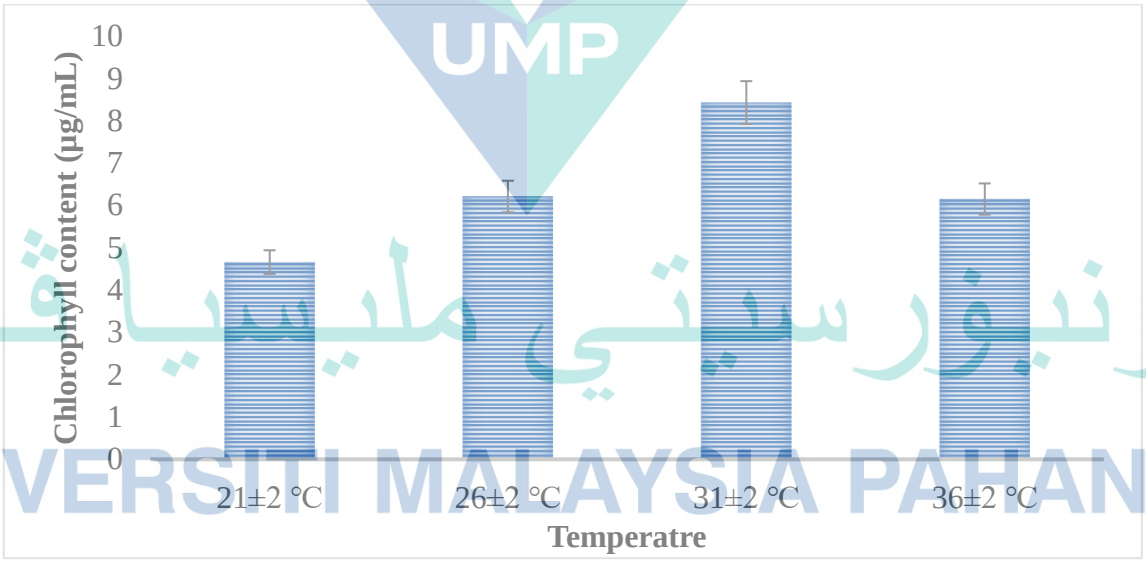


Figure 4.10 Chlorophyll content of *Spirulina* sp. at different temperature

4.4 Cultivation of *Spirulina* sp. at optimum conditions

Biomass productivity of *Spirulina* sp. was evaluated at optimum conditions for 18 days. The culture was grown in 30% (v/v) of POME medium, under light intensity of 3000 lux, and temperature at 31±2 ℃ to maximize the yield of *Spirulina* sp. The culture

was harvested on the early stationary phase. As shown in Figure 4.11 the maximum dry biomass was obtained on day 15 which is 1546.9±110.2 mg/L. The biomass production increases from day 3 after the culture was adapted to the environment in first 2 days. Nutrient deficiency started occurred at day 18 as result, biomass productivity decreases. Apart from that, high density of culture reduced the light penetration into the culture of *Spirulina* sp. Evidently, the effect of illumination on cell growth showed a strong association between cell concentration and light intensity. Indeed, once the nutrient and temperature requirements have been met, so that growth is enhanced to maximum level. The light intensity and its length, temperature and nutrients determine the growth rate and the yield of biomass production.

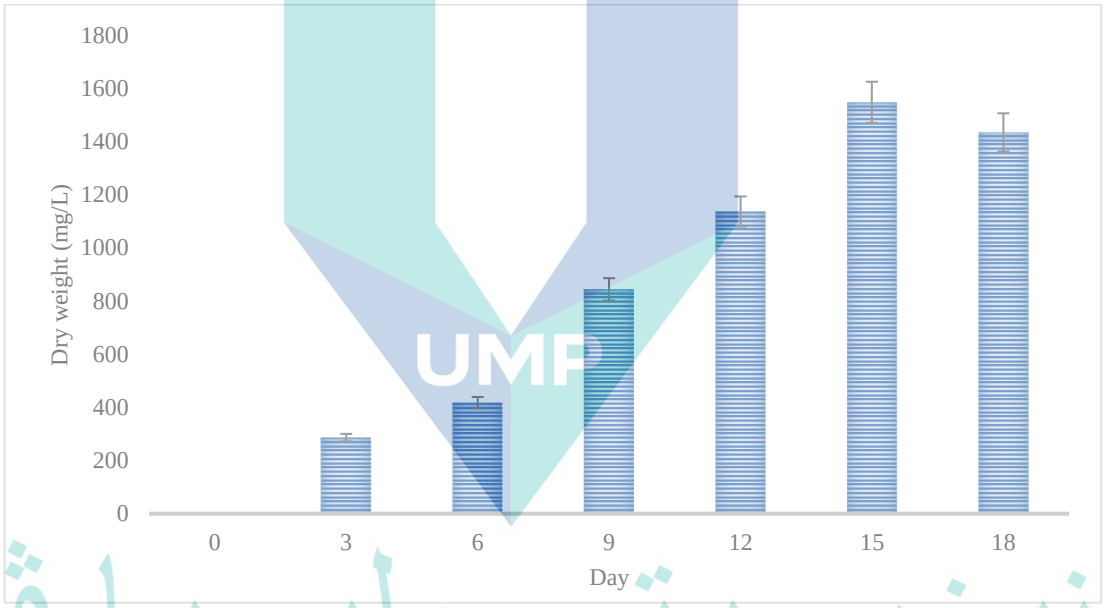


Figure 4.11 Biomass productivity of *Spirulina* sp. at optimized conditions

4.5 Characterization of treated palm oil mill effluent

The characteristics of POME such as BOD, COD, TN and TP were tested as mention in chapter 2.1. Reduction of pollutants was measured after the cultivation of *Spirulina* sp. Table 4.3 shown the characterization of POME. The highest removal efficiencies obtained were 80.0% for TN, 67.76% for COD, 61.25% for BOD, attained in pretreated 30% (v/v) POME. These results shown better pollutant removal performance than in non-pretreated 30% (v/v) POME. Cheah et al. (2018) mention that, the highest removal efficiencies obtained were 62.07% for total nitrogen, 47.09% for chemical oxygen deamd, and 30.77% for total phosphate after preteated in 30% (v/v) POME.

Although the pollutant removal efficiencies of organics were lower than nitrogen, it was degraded during the cultivation cycle due to COD concentration for 247 mg/L in COD (Salama et al., 2017).

In the analysis, nitrogen tended to be optimal for assimilation as opposed to phosphorus. Nitrogen source is vital to the growth and regulation of microalgae while phosphorus and ribosomal RNA synthesis are important. Occurding to the report of Kamyab et al (2016). COD values marginally decreased during the trial, with the lowest value reached at the end of experiment. The microalgae *Chlamydomonas* sp. Consume the COD as a backup substrate by itself. This species eliminate about 68.2%, 67.3%, 43.2% and 34.1% of COD for 0 mg/L, 250 mg/L 500 mg/L and 1000 mg/L of POME concentrations respectively.

A maximum value of 0 mg/L was achieved as the regulation without the use of POME. *Chlamydomonas* sp. Archieve optimum growth without POME, this indicates that POME may have an inhibitory effect towards the growth of species. Sleman et al. (2015) concluded that, the growth of Spirulinaplatensis was limited by ratio of C:N:P, and the organic and inorganic carbon source concentration. Coconut milk skim effluent (CMSE) provided a possible medium for microalgae growth, generating biomass by dry weight up to 0.206 g/L. White color and oil content should therefore be in CMSE treated before microalgae can be used as a medium.

Table 4.3 Pollutants removal in POME before and after cultivation

Parameter	Before (mg/L)	After (mg/L)
pH	7.3	7.8
COD	515	166
BOD	620	205
Ammoniacal nitrogen	1.3	1.5
Total nitrogen	200	40
Total solids	4,968	16,496

4.6 Extraction of lipid from *Spirulina* sp. biomass

Nature of the solvent, lipid size, type of microalgae, temperature solvent ratio, time period and extraction techniques contribute for yield of lipid. In this study, mainly three techniques were investigated to obtain high lipid yield such as Bligh and dyer, ultrasound-assisted and Soxhlet extraction were compared, adapted and evaluated prior to selecting a method suitable for rapid and simple procedure for simultaneous extraction of *Spirulina* sp. biomass. Microalgae extraction in the solvent was heavily dependent on the penetrability of the microalgae cell membrane and the solubility of microalgae lipids in organic solvents.

Therefore, after evaluating the efficiency of single solvent as solvent-lipid interaction, mixed acceptable proportions of polar and non-polar solvent to achieve higher lipid extraction yield when the Hildebrand Solubility Parameter (HSP) value of both solvent and microalgae lipid was nearby. Therefore, solvent mixture of hexane/methanol (v/v 3:1) was used to extract the lipid from *Spirulina* sp. biomass in all the techniques. Organic solvent playing important role in extracting the metabolite compounds from the biomass. Solvent mixture enhanced the desolation of the lipid and separated from the biomass. Furthermore, methanol has excellent ability to penetrate the cell membrane of microalgae whereas hexane has a much higher solubility in neutral lipids than methanol. The ratio of solvent mixtures to biomass at 15:1 (v/w) and the extraction duration was investigated from 1-5 h.

4.6.1 Bligh and Dyer technique

Based on the Figure 4.12, released of lipid yield from *Spirulina* sp. biomass increases as time increase. The longer time of biomass cell contact with solvent cause breaking of cell wall and cell membrane. As a result, more lipids were released from the biomass. The maximum lipid yield obtained from Bligh and Dyer technique is 16.6% after 5 h of time duration. Averagely 11.3±6.0% of lipid was yielded from the biomass by using hexane /methanol (2:1). Similar result reported by Yang et al., (2019) that, lipid content in *Spirulina platensis* was 11.52±0.11% of dry cellular weight after extracted by Bligh and Dyer method. Mata et al., (2016) reported that, maximum lipid content obtained from *Spirulina platensis* was 15.4% which grown under mixotrophic environment. Extractions using wet biomass yield a smaller percentage of lipids than dry biomass extractions. Extractions with conventional solvents have better results, especially when

applying the Bligh and Dyer. With respect to green solvents, the 2-MeTHF: isoamyl alcohol: water formulation used in the Bligh and Dyer process succeeds in producing the highest total fatty acid content (Wu et al., 2017).

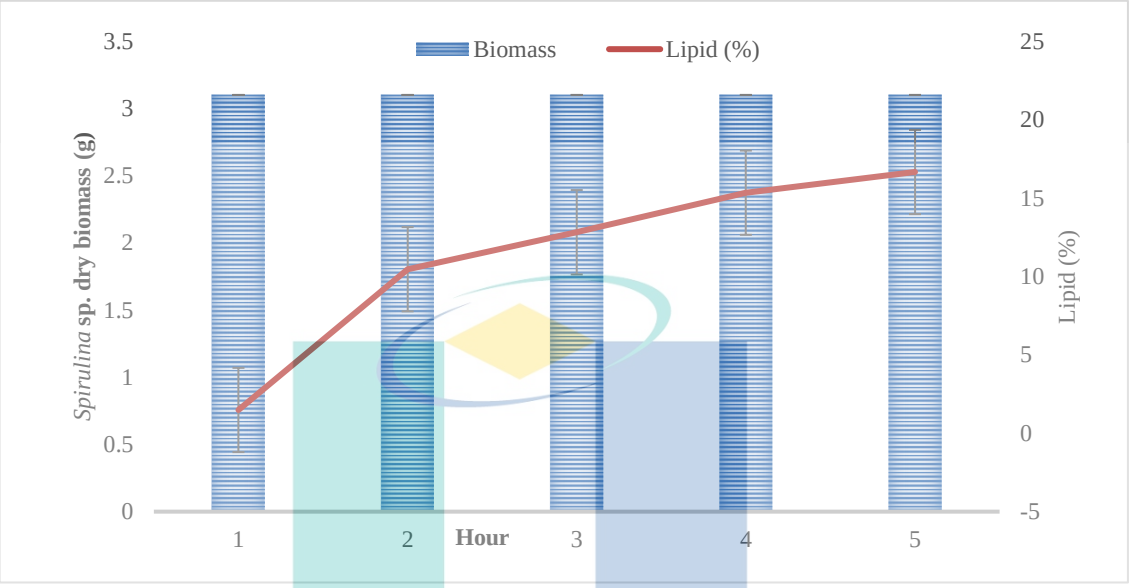


Figure 4.12 Extraction of lipid by Bligh and Dyer

4.6.2 Ultrasound-assisted technique

The percentage of lipid (%) from *Spirulina* sp. dry biomass shows exponential increase of yield lipid in first one hour. As time goes, from hour 2 until 5 it became constant as shown in Figure 4.13. The maximum lipid obtained was 31.79±5.1%. The longer time contributes to greater yield of lipid. The similar result reported that, at 20 min of extraction there was gradual increase of yield lipid until 35 min, later it became constant. This increase in yield lipid indicates that the amount of cell disturbances during the extraction cycle is influenced by ultrasound irradiation. The formation of micro-bubbles due to the cavitation effect which gave the surrounding biomass a large amount of energy which caused disruption of the cell wall. Cell wall disturbance can release lipid from the cell of microalgae. The amount of extraction time will increase the mass transfer rate, but on the other hand, constant ultrasonic radiation will cause the solvent to become saturated and therefore the lipid yield decreases (El-Shimi et al., 2013). Purkan et al., (2019) stated that, ultrasound-assisted extraction by cavitation and some mechanical effects may improve extraction efficiency.

Conversely, Nogueira et al., (2018) reported that, obtained 24.6±1.3% lipid from *Chaetoceros calcitrans* and explained that, decreases in the lipid recovery from microalgae due to increase in exposure time in chemical and ultrasound methods. Viswanathan et al (2012), studied the effects of three methods of cell disruption (autoclave, ultrasound and high-pressure homogenization) to obtain lipids from microalgae (*Chlorella mnutissima, Chlamydomonas globose* and *Scenedesmus bijuga*). The result found that, ultrasound technique increases the lipid content from 10.78% to 12.22%.

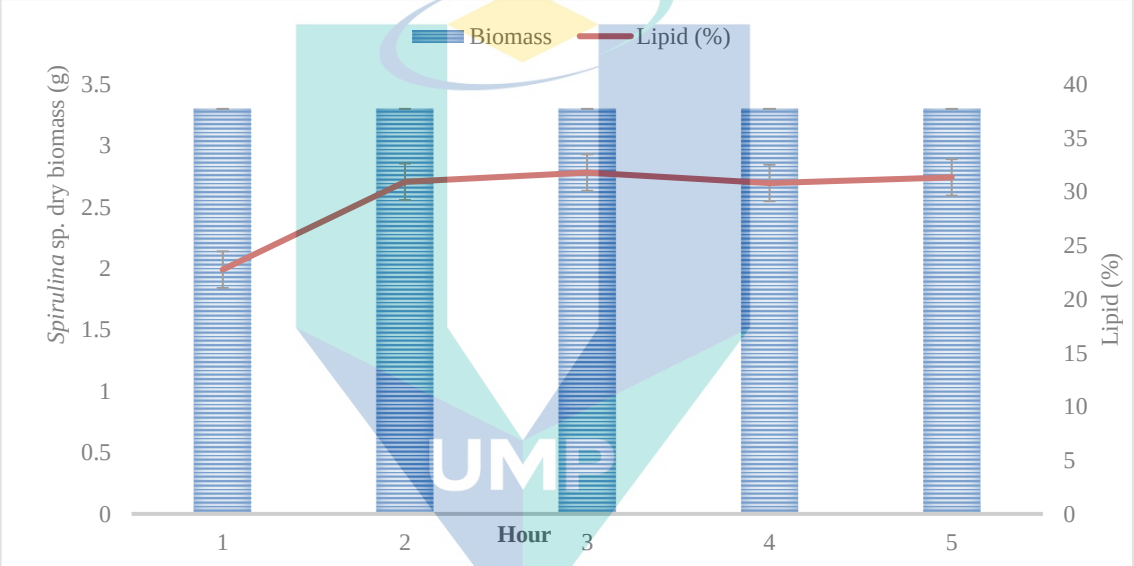


Figure 4.13 Extraction of lipid by ultrasound-assisted

4.6.3 Soxhlet extraction technique

This method is the best way to obtain large yields with a small number of solvents, a faster extraction time, and high yield collection as replicated. A notable increase in lipid extraction was confirmed in extractions using the Soxhlet process while the time increase as shown in Figure 4.14. The maximal lipid yield extracted from *Spirulina* sp. biomass is 51.25% at 5 h. As time increases, the lipid productivity increases from the biomass. The increased interaction between solvent and biomass paste by periodic siphoning allows the liquid to refresh itself continuously, maintaining a gradient of oil concentration between solvent and sample. It allows the oil to be solubilized.

The advantage of immersion is that the solvent is more effective, because solvents with low polarity cannot remove all polar lipids when the method is applied for a short time. Solvent polarity affects specifically the extraction of lipids with nonpolar lipids,

lower polarity, and higher selectivity. The greater number of yields was obtained with little amount of hexane solvent. The time duration for each cycle to complete and reuse the solvent back was 12±2 min.

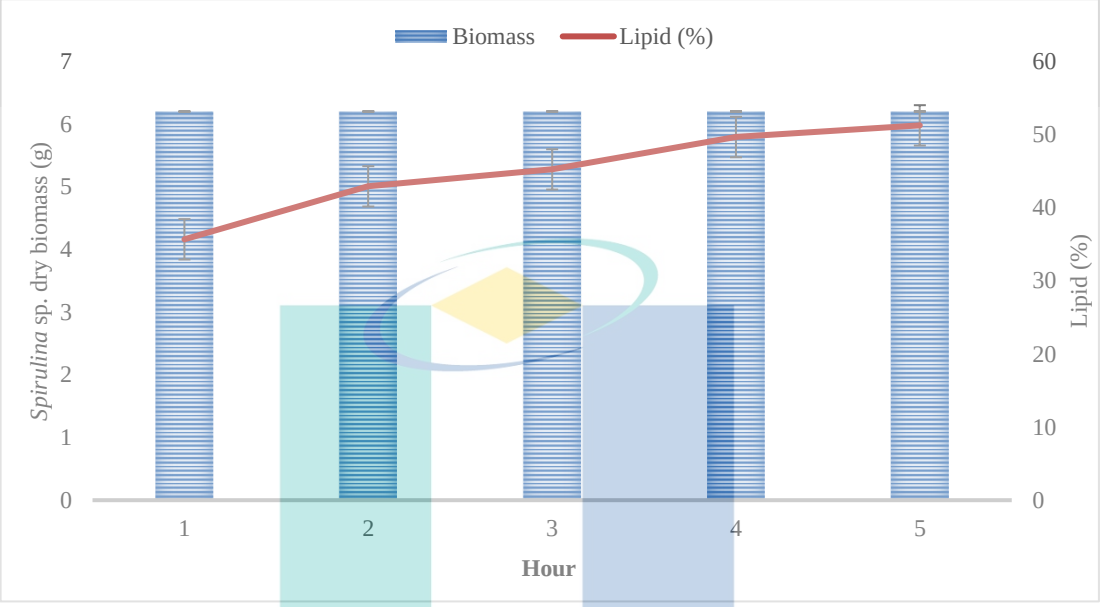


Figure 4.14 Extraction of lipid by Soxhlet extractor

4.6.4 Comparison of lipid extraction techniques

The influence of Bligh and Dyer, Ultrasound-assisted and Soxhlet methods on the lipid yield recovered from *Spirulina* sp. by using hexane: methanol (1:1). As shown in Figure 4.15. Soxhlet technique had significantly better efficiency than other techniques for extracting *Spirulina* sp. lipids. The maximal lipid yield was obtained from Soxhlet extraction is (44.94±6.16%) as compared to ultrasound-assisted (32.32±6.52%) and Bligh and Dyer technique (12.33±4.08%).

Solana et al., (2014) investigated the lipid extraction from *Scenedesmus obliquus* by comparing Supercritical carbon dioxide extraction and conventional Soxhlet extraction. The yield for extraction of *Scenedesmus obliquus* obtained from Soxhlet extraction was 29.0%, higher than the supercritical technology recovery of the crude. However, only 51.1% of free fatty acids was contained in the oil extracted from Soxhlet, compared to 73.6% of the free fatty acids obtained from supercritical extraction. Therefore, the overall lipid yield obtained by Soxhlet extraction was less than that obtained by super critical CO_2.

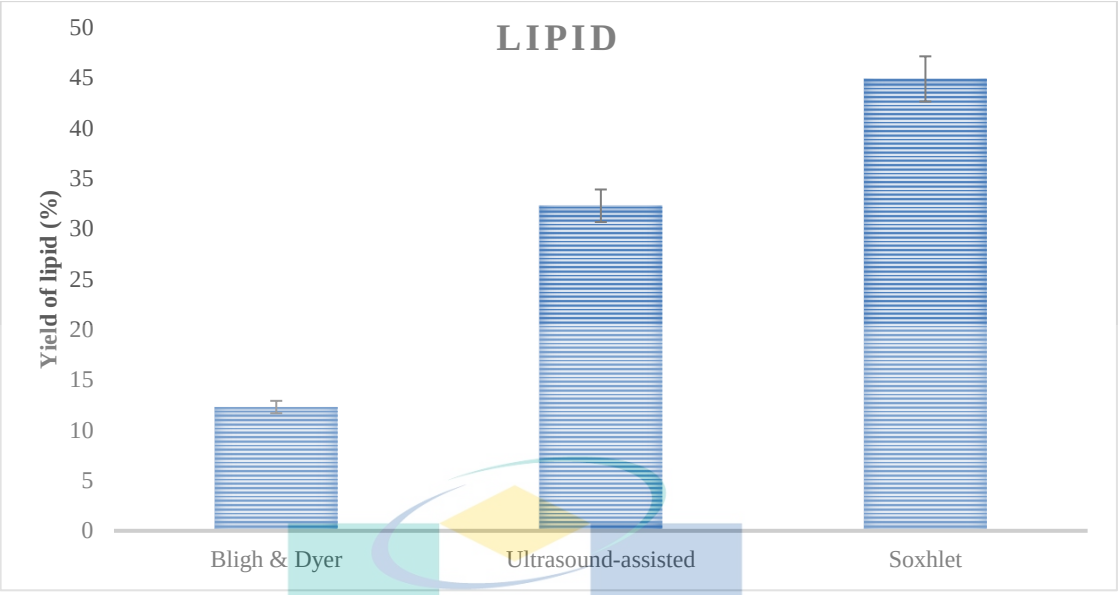


Figure 4.15 Comparison of lipid extraction by different techniques

Organic solvent playing crucial role for extraction of lipid. In this study, hexane solvent was selected for extracting lipid since it has great efficiency. The cell disruption by Soxhlet was more effective for lipids extraction than the Bligh and Dyer, ultrasound-assisted. Various results were obtained from different techniques even though all are best methods for extraction. But it probably correlated with another extraction procedure, the effectiveness of a lipid extraction method can also depend on the microalgae species used. The period of conductivity between biomass and organic solvent lead for lipid production where its breakdown the cell wall of species. Temperature plays vital role in cell disruption of species. Soxhlet extraction where have the continuous high temperature during the process as compared to other extraction methods.

4.7 FTIR analysis of lipid

Fourier transform infrared spectroscopy (FT-IR) spectra of *Spirulina* sp. showed eight distinct absorption bands over the wavelength range 4000-400 cm^{-1}. The bands were given to specific molecular groups on the basis of biochemical standard and published studies.

The peaks shown in the Figure 4.16 represent the functional groups presents in the lipid content of *Spirulina* sp. extract. It was identified by the peaks related to C-H stretching vibration (C-H stretch at 2925.93 cm^{-1} and 2855.22 cm^{-1}) which represent asymmetric and symmetric hydrocarbon stretches of lipids (CH_3) and (CH_2) from the

alkane group. O-H bending found at 1419.02 cm^{-1} which represent carboxylic acids groups. C-H in plane bending at 1402.01 cm^{-1} and 1376.93 cm^{-1} which represents the alkenes groups. At peak 1239.82 cm^{-1} found C-C(O)-C stretching which represents the carbonyl stretches of fatty acid esters. Ketones groups represent by C-C stretch at 1159.55 cm^{-1} and 1118.23 cm^{-1}. This resulted in the absolute hydrocarbon and triacyl glycerides (TAG) content being calculated. As the second derivative continuum itself is normalised, the absolute quality is defined by the combined peak field.

The similar peaks areas result was reported that for microalgae species *Monoraphidium contortum*, *Pseudomuriella* sp. and *Chlamydomonas* sp. lipid content. The bands found in the 3050 to 2800 cm^{-1} region belong to asymmetric and symmetric hydrocarbon stretches of lipids (CH_3) and (CH_2) and the band at approximately 1745 cm^{-1} was attributed to carbonyl stretches of fatty acid esters, mostly referred to as TAG (Sosa-Hernández et al., 2018). The monitoring of lipid accumulation in microalgal species in these two regions is significant (Poojary et al., 2016; Grace et al., 2020).

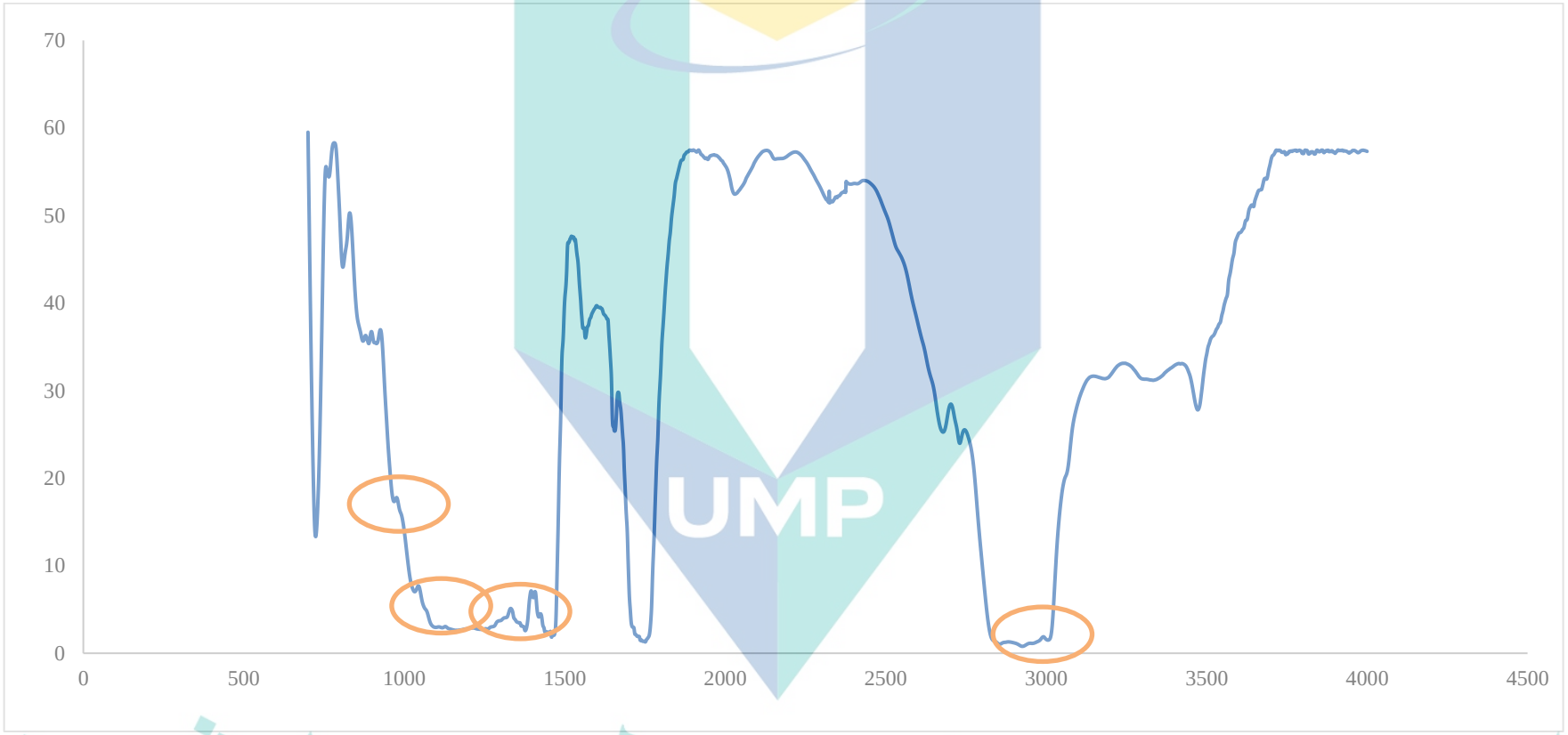


Figure 4.16 Infrared spectra of *Spirulina* sp. extracted lipid

Table 4.4 Functional group compounds present in the *Spirulina* sp. lipid

Band position (cm^{-1})	Molecular motion	Functional groups
3472.49	N-H stretch	amines
3333.60	O-H stretch	alcohol
3005.69	C-H stretch	aromatics
2925.93	C-H stretch	alkanes
2855.22	C-H stretch	alkanes
2730.83	C-H aldehyde stretch	aldehyde
1419.02	O-H bend	Carboxylic acids
1402.01	C-H in plane	alkenes
1376.93	C-H in plane bend	alkene
1239.82	C-C(O)-C stretch	esters
1159.55	C-C stretch	ketones
1118.23	C-C stretch	ketones
1032.52	C-N stretch (alkyl)	Amines
968.40	P-H stretch	Phosphines
913.51	P-H stretch	Phosphines
889.24	S=O stretch	sulfonates
764.98	C-Cl stretch	Alkyl halides
723.80	C-Cl stretch	Alkyl halides

4.8 Fatty acid profile of *Spirulina* sp.

Generally, in order to analyse and quantify lipid composition, the extracted lipid need to be trans esterified. The very purpose of this transesterification is to break the macromolecules of triglycerides (molecular weight in the range of 820-870) to smaller molecules of about 260-280 molecular weight. The smaller fatty acids molecules then can be easily analysed using polar capillary column in gas chromatography. By using an internal standard (methyl heptadecanoate, C_{17}), those fatty acids can be analysed both qualitatively and quantitatively.

Fatty acid composition of *Spirulina* sp. shown in Table 4.5. The capillary mega wax MS column provide clear separation of fatty acids methyl ester as shown in Figure 4.17. Total 13 fatty acids were identified in the *Spirulina* sp. lipid. Sample contains oleic acid, palmitoleic acid, linoleic acid, stearic acid, linolelaidic and paullinic acid as most dominant fatty acids and the rest fatty acids with low quantity. Identified fatty acids were classified into saturated and unsaturated fatty acids. The major fatty acid component comprised the saturated fatty acid (SFA) is palmitic acid, methyl ester (43.97%). Unsaturated fatty acids including monounsaturated fatty acids (MUFA) with major component is elaidic acid, methyl ester (6.76%) and polyunsaturated fatty acids (PUFA) with major component is linoleic acid, methyl ester (30.62%). It can observe that the proportion of MUFA (84.10 %) and PUFA (10.91%) are higher than SFA (4.99%) which obtained from *Spirulina* sp. lipid.

The similar result reported that, through microwave-assisted process, the proportion of saturated fatty acids extracted was higher, while superior extraction of unsaturated fatty acids was achieved using ultrasound and Soxhlet extraction methods (e Silva et al., 2017). Muhling et al., (2005) stated that, in the *Spirulina* strain found predominant acids such as linoleic, palmitic and γ-linolenic acids are combined together formed 88-92% total fatty acids. However, the relative proportions of the two polyunsaturated C_{18} acids, linoleic acid and γ-linolenic acid, ranging differently from 13.1% to 31.5% and 12.9% to 29.4%, respectively. e Silva et al., 2017 obtained three saturated fatty acid such as myristic (19.9%), palmitic (34.7%) and stearic (6.7%) acid respectively and unsaturated acids as oleic (39.3%) and erucic (36.0%) acid from *Spirulina platensis* extract.

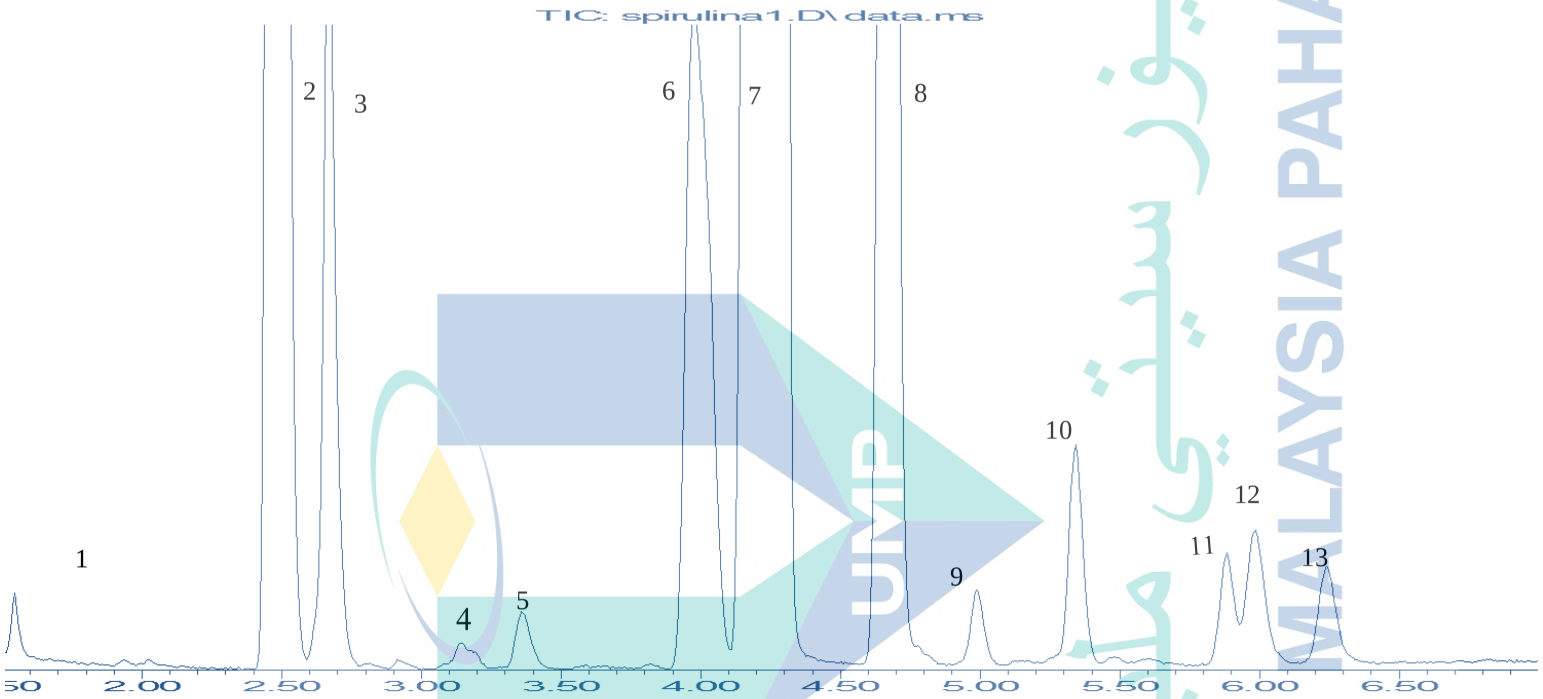


Figure 4.17 Chromatogram of fatty acid profile of *Spirulina* sp.

(1) Myristoleic acid, methyl ester (2) Palmitoleic acid, methyl ester, (3) *Internal standard: Heptadecanoic acid*, methyl ester, (4) cis-10-heptadecenoic acid, methyl ester, (5) Elaidic acid, methyl ester (6) Stearic acid, methyl ester (7) Oleic acid (8) Linoleic acid, methyl ester (9) γ-linolenic acid, methyl ester, (10) Linolenic acid, methyl ester, (11) Eicosanoic, methyl ester, (12) Paullinic acid, methyl acid, (13) Docosanoic acid, methyl ester

Table 4.5 Fatty acid profile of *Spirulina* sp.

Fatty acids	**Class of amino acids**	**Composition (%)**
Saturated fatty acid		
Docosanoic acid, methyl ester	Non-essential	0.27
Stearic acid, methyl ester	Non-essential	2.11
Eicosanoic acid, methyl ester,	Non-essential	1.34
Heptadecanoic acid, methyl ester	Non-essential	1.27
∑ *Saturated fatty acid*		4.99
Monounsaturated fatty acid		
Myristoleic acid, methyl ester	Essential	0.09
Palmitoleic acid, methyl ester	Essential	13.07
Oleic acid, methyl ester	Essential	70.16
Elaidic acid, methyl ester	Essential	0.31
Cis-10-heptadecenoic acid, methyl ester	Essential	0.23
11-Eicosenoic acid, methyl ester		0.24
∑ *Monounsaturated fatty acid*		84.10
Polyunsaturated fatty acid		
γ-linolenic acid, methyl ester	Essential	0.43
Linolelaidic acid, methyl ester	Essential	1.86
Linoleic acid, methyl ester	Essential	8.62
∑ *Polyunsaturated fatty acid*		10.91

CHAPTER 5

CONCLUSION

5.1 Conclusion

Based on the results of study, potential microalgae species were isolated by serial dilution and streaking technique from the sample which collected at east costal region of Kuantan, Pahang. Among six isolated species, *Spirulina* sp. have great potential to adapt, survived and grow in palm oil mill effluent at different concentration especially 30%, (v/v). The effect of light intensity (3000 lux) and temperature at (31±2 ℃) increased the biomass productivity at optimum conditions. Under the optimized conditions, highest biomass of *Spirulina* sp. was obtained 1.54±0.11 g/l after cultivated for 18 days. *Spirulina* sp. were separated from the culture by centrifugation at 6000 g to obtain maximum biomass. Among lipid extraction techniques, Soxhlet extraction (44.9±6.1%) was found highest lipid yield as compared to ultrasound-assisted (32.3±6.5%) and Bligh and Dyer (12.3±4.0%). Total 13 fatty acids found in fatty acid methyl ester and oleic acid (70.16%), palmitoleic acid (13.07%), linoleic acid (8.62%), and stearic acid (2.11%) and γ-linolenic acid (0.43%) as dominant fatty acids after it analysed through GC-MS. There essential fatty acid product can be obtaining through *Spirulina* sp. biomass by cultivating in POME with lower cost. Moreover, accumulation of POME can be reduced from the environment. *Spirulina* sp. lipid have great potential to be use in nutraceutical product due to its composition of essential fatty acids.

5.2 Recommendations

The study has investigated a series of experiments on microalgae *Spirulina* sp. cultivation in POME in particular time frame and obtained the best results from various experiments which helps to complete the study. In order to improve the study, few recommendations are proposed to extend the depth of the research.

i. Using different type of wastewater from industries such as coconut oil mill effluent and rubber mill effluent for cultivation of *Spirulina* sp.

ii. Isolating different indigenous microalgae species with contains higher lipid content for commercial production.

iii. Study on the cell morphology and life cycles of marine algae species.

iv. Study on molecular biology of *Spirulina* sp. with higher metabolite content.

v. Cultivation of commercial value microalgae species for synthesis of organic compounds.

REFERENCES

Acién, F.G., Molina, E., Reis, A., Torzillo, G., Zittelli, G.C., Sepúlveda, C., & Masojídek, J. (2017). Photobioreactors for the production of microalgae. In *Microalgae-based Biofuels and Bioproducts*. Woodhead Publishing. 1-44.

Ahmad, S., Ab Kadir, M.Z.A., & Shafie, S. (2011). Current perspective of the renewable energy development in Malaysia. *Renewable and Sustainable Energy Reviews*, *15*(2), 897-904.

Ajayan, K.V., Selvaraju, M., & Thirugnanamoorthy. (2012). Enrichment of chlorophyll and phycobiliproteins in *Spirulina platensis* by the use of reflector light and nitrogen sources: An *in vitro* study. *Biomass and Bioenergy*, 47, 436-441.

Akpor, O.B., Otohinoyi, D.A., Olaolu, D.T., & Aderiye, B.I. (2014). Pollutants in wastewater effluents: impacts and remediation processes. *International Journal of Environmental Research and Earth Science*, *3*(3), 050-059.

Alhusseiny, S.M., & El-Beshbishi, S.N. (2020). Omega polyunsaturated fatty acids and parasitic infections: An overview. *Acta Tropica*, 207, 105466.

Altunoz, M., Allesina, G., Pedrazzi, S., & Guidetti, E. (2020). Integration of biological waste conversion and wastewater treatment plants by microalgae cultivation. *Process Biochemistry*, *91*, 158-164.

Andiappan, V., Ng, D.K.S., & Tan, R.R. (2018). Cooperative game theory analysis for implementing green technologies in palm oil milling processes. In: Foo DCY, Tun Abdul Aziz MK, editors. Green technologies for the oil palm industry. Singapore: *Springer* Singapore, 173-190.

Araujo, G.S., Matos, L.J., Fernandes, J.O., Cartaxo, S.J., Gonçalves, L.R., Fernandes, F.A., & Farias, W.R. (2013). Extraction of lipids from microalgae by ultrasound application: Prospection of the optimal extraction method. *Ultrasonics Sonochemistry*, *20*(1), 95-98.

Arora, M., Anil, A.C., Leliaert, F., Delany, J., & Mesbahi, E. (2013). *Tetraselmis indica* (Chlorodendrophyceae, Chlorophyta), a new species isolated from salt pans in Goa,India. *European Journal of Phycology*, *48*(1), 61-78.

Nur, A.M.M., & Irawan, M.A. (2015). Utilization of Coconut Milk Skim Effluent (CMSE) as Medium Growth for *Spirulina platensis*. *Procedia Environmental Sciences*, 23, 72-77.

Banakar, V., Alam, Q., Rajendra, S.V., Pandit, A., Cladious, A., & Gnanaprakash, K. (2020). *Spirulina*, The Boon of Nature. *International Journal of Research in Pharmaceutical Sciences*, *11*(1), 57-62.

Barreira, L., Pereira, H., Gangadhar, K.N., Custodio, L., & Varela, J. (2015). Medicinal effects of microalgae-derived fatty acids. In: Kim, S-K. (Eds), Handbook of Marine Microalgae Biotechnology Advances. Busan, *Academic Press*, 209-231.

Bhatia, S.K., Bhatia, R.K., & Yang, Y.H. (2017). An overview of microdiesel a sustainable future source of renewable energy. *Renewable and Sustainable Energy Reviews, 79*, 1078-1090.

Bhuvana, P., Sangeetha, P., Anuradha, V., & Ali, M.S. (2019). Spectral characterization of bioactive compounds from microalgae: *N. Oculata* and *C. Vulgaris*. *Biocatalysis and Agricultural Biotechnology, 19*, 101094.

Bhuyar, P., Yusoff, M.M., Rahim, M.H.A., Sundararaju, S., Maniam, G.P., & Govindan, N. (2020). Effect of plant hormones on the production of biomass and lipid extraction for biodiesel production from microalgae *Chlorella* sp. *Journal of Microbiology, Biotechnology and Food Sciences, 9*(4), 671-674.

Boone, D.R., & Castenholz, R.W. (2001). *Bergey's Manual of Systematic Bacteriology*. Brown, M.R., & Miller, K.A. (1992). The ascorbic acid content of eleven species of microalgae used in mariculture. *Journal of Applied Phycology, 4*(3), 205-215.

Brown, M.R., & Jeffrey, S.W. (1992). Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. *Journal of Experimental Marine Biology and Ecology, 161*(1), 91-113.

Brown, M.R., & Miller, K.A. (1992). The ascorbic acid content of eleven species of microalgae used in mariculture. *Journal of Applied Phycology, 4*(3), 205-215.

Butler, E., Hung, Y.T., Al Ahmad, M.S., Yeh, R.Y.L., Liu, R.L. H., & Fu, Y.P. (2017). Oxidation pond for municipal wastewater treatment. *Applied Water Science, 7*(1), 31-51.

Chaisutyakorn, P., Praiboon, J., & Kaewsuralikhit, C. (2018). The effect of temperature on growth and lipid and fatty acid composition on marine microalgae used for biodiesel production. *Journal of Applied Phycology, 30*(1), 37-45.

Cheah, W.Y., Show, P.L., Juan, J.C., Chang, J.S., & Ling, T.C. (2018). Microalgae cultivation in palm oil mill effluent (POME) for lipid production and pollutants removal. *Energy Conversion and Management, 174*, 430-438.

Chew, K.W., Chia, S.R., Show, P.L., Yap, Y.J., Ling, T.C., & Chang, J.S. (2018). Effects of water culture medium, cultivation systems and growth modes for microalgae cultivation: a review. *Journal of the Taiwan Institute of Chemical Engineers, 91*, 332-344.

Chew, K.W., Yap, J.Y., Show, P.L., Suan, N.H., Juan, J.C., Ling, T.C., & Chang, J.S. (2017). Microalgae biorefinery: high value products perspectives. *Bioresource Technology, 229*, 53-62.

Chin, Y.C., Jing, C.Y., Kheang, L.S., Vimala, S.C., Chin, S.A., Fong, C.M., & Keong, L.L. (2020). Comparison of different industrial scale palm oil mill effluent anaerobic systems in degradation of organic contaminants and kinetic performance. *Journal of Cleaner Production*, 262, 121361.

Chinnasamy, S., Bhatnagar, A., Hunt, R.W., & Das, K.C. (2010). Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technology, 101*(9), 3097-3105.

Chiu, H.H., & Kuo, C.H. (2020). Gas chromatography-mass spectrometry-based analytical strategies for fatty acid analysis in biological samples. *Journal of Food and Drug Analysis*, *28*(1), 60-73.

Christi, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, 25, 294-306.

Christenson, L., & Sims, R. (2011). Production and harvesting of microalgae for wastewater treatment biofuels and bioproducts. *Biotechnology Advances*, *29*(6), 686-702.

Chye, I.L., & Wahidul, B. (2019). Sustainability assessment for crude palm oil production in Malaysia using the palm oil sustainability assessment framework. *Sustainable Development*, 27(3), 253-269.

Cruz, Y.R., Aranda, D.A., Seidl, P.R., Diaz, G.C., Carliz, R.G., Fortes, M.M., & de Paula, R.C.B (2018). Cultivation systems of microalgae for the production of biofuels. *Biofuels-State of Development, Krzysztof Biernat, IntechOpen*.

Vaz, B.D.S., Moreira, J.B., de Morais, M.G., & Costa, J.A.V. (2016). Microalgae as a new source of bioactive compounds in food supplements. *Current Opinion in Food Science*, *7*, 73-77.

Daneshvar, E., Zarrinmehr, M.J., Koutra, E., Kornaros, M., Farhadian, O., & Bhatnagar, A. (2019). Sequential cultivation of microalgae in raw and recycled dairy wastewater: microalgal growth, wastewater treatment and biochemical composition. *Bioresource Technology, 273*, 556-564.

Danesi, E.D.G., Rangel-Yagui, C.O., Sato, S., & Carvalho, J.C.M.D. (2011). Growth and content of *Spirulina platensis* biomass chlorophyll cultivated at different values of light intensity and temperature using different nitrogen sources. *Brazilian Journal of Microbiology*, *42*(1), 362-373.

Danesi, E.D.G., Rangel-Yagui, C.O., Carvalho, & Sato, S. (2004). Effect of reducing the light intensity on the growth and production of chlorophyll by *Spirulina platensis*. *Biomass and Bioenergy*, 26(4), 329-335.

De Jesus Raposo, M.F., de Morais, R.M.S.C., & de Morais, A.M.M.B. (2013). Health applications of bioactive compounds from marine microalgae. *Life Sciences*, *93*(15), 479-486.

De Jesus, S.S., Ferreira, G.F., Moreira, L.S., Maciel, M.R.W., & Maciel Filho, R. (2019). Comparison of several methods for effective lipid extraction from wet microalgae using green solvents. *Renewable Energy*, *143*, 130-141.

De Lorgeril, M., & Salen, P. (2012). New insights into the health effects of dietary saturated and omega-6 and omega-3 polyunsaturated fatty acids. *BMC medicine*, *10*(1), 1-5.

De Meester, F., Watson, R.R., & Zibadi, S. (Eds.). (2012). *Omega-6/3 fatty acids: functions, sustainability strategies and perspectives*. Springer Science & Business Media.

De Morais, M.G., Vaz, B.D.S., de Morais, E.G., & Costa, J.A.V. (2015). Biologically active metabolites synthesized by microalgae. *BioMed Research International*, *2015*.

De Oliveira, M.R., Nabavi, S.F., Nabavi, S.M., & Jardim, F.R. (2017). Omega-3 polyunsaturated fatty acids and mitochondria, back to the future. *Trends in Food Science & Technology*, *67*, 76-92.

DOE (1999). Industrial processes and the environment (Handbook no.3): Crude Palm Oil Industry, Kuala Lumpur: Department of Environment Malaysia, 47-56.

Dubey, R.C., (2006). A textbook of Biotechnology. Fourth revised and enlarge edition, S. Chand and company limited, 419-421.

E Silva, A.D.S., Moreira, L.M., de Magalhães, W.T., Farias, W.L., Rocha, M.V.P., & Bastos, A.K.P. (2017). Extraction of biomolecules from *Spirulina platensis* using non-conventional processes and harmless solvents. *Journal of Environmental Chemical Engineering*, *5*(3), 2101-2106.

Elagizi, A., Lavie, C.J., Marshall, K., Dinicolantonio, J.J., O'Keefe, J.H., & Milani, R.V. (2018). Omega-3 polyunsaturated fatty acids and cardiovascular health: a comprehensive review. *Progress in Cardiovascular Diseases*, *61*(1), 76-85.

El-Shimi, H.I., Attia, N.K., El-Sheltawy, S.T. and El-Diwani, G.I. (2013) Biodiesel Production from *Spirulina-Platensis* Microalgae by in-situ Transesterification Process. *Journal of Sustainable Bioenergy Systems*, 3, 224-233.

Ercolano, G., De Cicco, P., & Ianaro, A. (2019). New drugs from the sea: Pro apoptotic activity of sponges and algae derived compounds. *Marine Drugs*, *17*(1), 31.

Feng, J., Long, S., Zhang, H. J., Wu, S. G., Qi, G. H., & Wang, J. (2020). Comparative effects of dietary microalgae oil and fish oil on fatty acid composition and sensory quality of table eggs. *Poultry Science*, *99*(3), 1734-1743.

Fernandez, F.G.A., Camacho, A.C., Pérez, J.A.S., Sevilla, J.M.F., & Grima, E.M. (1997). A model for light distribution and average solar irradiance inside outdoor tubular photobioreactors for the microalgal mass culture. *Biotechnology and Bioengineering*, 55, 701–714.

Galhano, V., De Figueiredo, D. R., Alves, A., Correia, A., Pereira, M. J., Gomes-Laranjo, J., & Peixoto, F. (2011). Morphological, biochemical and molecular characterization of *Anabaena*, *Aphanizomenon* and *Nostoc* strains (*Cyanobacteria, Nostocales*) isolated from Portuguese freshwater habitats. *Hydrobiologia*, *663*(1), 187-203.

Gao, F., Yang, H.L., Li, C., Peng, Y.Y., Lu, M.M., Jin, W.H., Bao, J.J., Guo, Y.M. (2019). Effect of organic carbon to nitrogen ratio in wastewater on growth, nutrient 449 uptake and lipid accumulation of a mixotrophic microalgae *Chlorella* sp. *Bioresource Technology*, 282, 118-124.

Ghasemi, N. F., González González, L.M., Chan, W., & Schenk, P. M. (2016). Progress on lipid extraction from wet algal biomass for biodiesel production. *Microbial Biotechnology*, *9*(6), 718-726.

Gershwin, M. E., & Belay, A. (Eds.). (2007). *Spirulina in human nutrition and health*. CRC Press.213-275.

Gertsakis, J., & Lewis H. (2003). Sustainability and the waste management hierarchy. *Eco Recycle Victoria*, 30, 2008.

Goldstein, R. E. (2015). Green algae as model organisms for biological fluid dynamics. *Annual Review of Fluid Mechanics*, *47*, 343-375.

Gour, R. S., Chawla, A., Singh, H., Chauhan, R. S., & Kant, A. (2016). Characterization and screening of native *Scenedesmus* sp. isolates suitable for biofuel feedstock. *PloS one*, *11*(5), e0155321.

Grace, C.E.E., Lakshmi, P.K., Meenakshi, S., Vaidyanathan, S., Srisudha, S., & Mary, M.B. (2020). Biomolecular transitions and lipid accumulation in green microalgae monitored by FTIR and Raman analysis. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *224*, 117382.

Greenwell, H.C., Laurens, L.M.L., Shields, R.J., Lovitt, R.W., & Flynn, K.J. (2010). Placing microalgae on the biofuel priority list: a review of the technological challenges. *Journal of the Royal Society Interface*, *7*(46), 703-726.

Grobbelaar, J.U., & Soeder, C.J. (1985). Respiration losses in planktonic green algae cultivated in raceway ponds. *Journal of plankton research*, *7*(4), 497-506.

Guiry, M.D., 2012. How many species of algae are there? *Journal of Phycology* 48, 1057–1063.

Gupta, P.L., Choi, H.J., Pawar, R.R., Jung, S.P., & Lee, S.M. (2016). Enhanced biomass production through optimization of carbon source and utilization of wastewater as a nutrient source. *Journal of Environmental Management*, *184*, 585-595.

Hadiyanto, H. Nur, M.M.A., & Hartano, G.D. (2012). Enhancement of biomass production from *Spirulina* sp. cultivated in pome medium. *Proceeding of International Conference on Chemical and Material Engineering 2012*.

Hamilton, M.L., Warwick, J., Terry, A., Allen, M.J., Napier, J.A., & Sayanova, O. (2015). Towards the industrial production of omega-3 long chain polyunsaturated fatty acids from a genetically modified diatom *Phaeodactylum tricornutum*. *PloS One*, *10*(12), e0144054.

Halim, R., Danquah, M.K., & Webley, P.A. (2012). Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology Advances*, *30*(3), 709-732.

Han, P., Lu, Q., Fan, L., & Zhou, W. (2019). A review on the use of microalgae for sustainable aquaculture. *Applied Sciences*, *9*(11), 2377.

Harwood, J.L. (2019). Algae: Critical sources of very long-chain polyunsaturated fatty acids. *Biomolecules*, *9*(11), 708.

Hasanudin, U., Sugiharto, R., Haryanto, A., Setiadi, T., & Fujie, K. (2015). Palm oil mill effluent treatment and utilization to ensure the sustainability of palm oil industries. *Water Science and Technology*, 72(7), 1089-1095.

Heimann, K., & Huerlimann, R. (2015). Microalgal classification: major classes and genera of commercial microalgal species. In *Handbook of marine microalgae*, *Academic Press*, 25-41.

Hindryawati, N., Daniel, D., Erwin, E., & Maniam, G.P. (2017). Preparation of Spent Bleaching Earth-Supported Calcium from Limestone as Catalyst in Transesterification of Waste Frying Oil. *Jurnal Bahan Alam Terbarukan*, *6*(1), 68-75.

Hindryawati, N., Maniam, G.P., Karim, M.R., & Chong, K.F. (2014). Transesterification of used cooking oil over alkali metal (Li, Na, K) supported rice husk silica as potential solid base catalyst. *Engineering Science and Technology, an International Journal*, *17*(2), 95-103.

Ho, C.C., Tan, Y.K., & Wang, C.W., (1984). The distribution of chemical constituents between the soluble and the particulate fractions of palm oil mill effluent and its significance on its utilisation/treatment. *Agricultural Wastes*, 11(1), 61–71.

Ho, S.H., Liao, J.F., Chen, C.Y., & Chang, J.S. (2018). Combining light strategies with recycled medium to enhance the economic feasibility of phycocyanin production with *Spirulina platensis*. *Bioresource Technology*, 247, 669-675.

Huang, G., Chen, F., Kuang, Y., He, H., & Qin, A. (2016). Current techniques of growing algae using flue gas from exhaust gas industry: a review. *Applied Biochemistry and Biotechnology*, *178*(6), 1220-1238.

Huang, G., Chen, F., Wei, D., Zhang, X., & Chen, G. (2010). Biodiesel production by microalgal biotechnology. *Applied Energy*, *87*(1), 38-46.

Huerlimann, R., De Nys, R., & Heimann, K. (2010). Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnology and Bioengineering*, *107*(2), 245-257.

Hupfauf, B., Süß, M., Dumfort, A., & Fuessl-Le, H. (2016). Cultivation of microalgae in municipal wastewater and conversion by hydrothermal carbonization: a review. *ChemBioEng Reviews*, *3*(4), 186-200.

Hwang, S.W., Choi, H.I., & Sim, S.J. (2019). Acidic cultivation of *Haematococcus pluvialis* for improved astaxanthin production in the presence of a lethal fungus. *Bioresource Technology*, *278*, 138-144.

Jaiswal, K.K., Kumar, V., Vlaskin, M.S., Sharma, N., Rautela, I., Nanda, M., & Chauhan, P.K. (2020). Microalgae fuel cell for wastewater treatment: Recent advances and challenges. *Journal of Water Process Engineering*, *38*, 101549.

Jayakumar, S., Yusoff, M.M., Rahim, M.H.A., Maniam, G.P., & Govindan, N. (2017). The prospect of microalgal biodiesel using agro-industrial and industrial wastes in Malaysia. *Renewable and Sustainable Energy Reviews*, *72*, 33-47.

Ji, X.J., & Ledesma-Amaro, R. (2020). Microbial Lipid Biotechnology to Produce Polyunsaturated Fatty Acids. *Trends in Biotechnology*, 38(8), 832-834.

Joven, J.M.O., Gadian Jr, J.T., Perez, M.A., Caingles, J.G., Mansalaynon, A.P., Ido, A.L., & Arazo, R.O. (2020). Optimized ultrasonic-assisted oil extraction and biodiesel production from the seeds of *Maesopsis eminii*. *Industrial Crops and Products*, *155*, 112772.

Kamaroddin, M.F., Rahaman, A., Gilmour, D.J., & Zimmerman, W.B. (2020). Optimization and cost estimation of microalgal lipid extraction using ozone-rich microbubbles for biodiesel production. *Biocatalysis and Agricultural Biotechnology*, *23*, 101462.

Kamyab, H., Din, M.F.M., Keyvanfar, A., Abd Majid, M.Z., Talaiekhozani, A., Shafaghat, A., & Ismail, H.H. (2015). Efficiency of microalgae Chlamydomonas on the removal of pollutants from palm oil mill effluent (POME). *Energy Procedia*, *75*, 2400-2408.

Kamyab, H., Din, M.F.M., Ghoshal, S.K., Lee, C.T., Keyvanfar, A., Bavafa, A.A., & Lim, J.S. (2016). *Chlorella pyrenoidosa* mediated lipid production using Malaysian agricultural wastewater: effects of photon and carbon. *Waste and Biomass Valorization*, *7*(4), 779-788.

Kamyab, H., Chelliapan, S., Din, M.F.M., Rezania, S., Khademi, T., & Kumar, A. (2018). Palm oil mill effluent as an environmental pollutant. *Palm Oil*, *13*.

Kaplan, D., Richmond, A.E., Dubinsky, Z., & Aaronson, S. (2017). Algal nutrition. In *Handbook of Microalgal Mass Culture (1986)* CRC Press, 147-198.

Khan, M.I., Shin, J.H., & Kim, J.D. (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial Cell Factories*, *17*(1), 36.

Khatun, R., Reza, M.I.H., Moniruzzaman, M., & Yaakob, Z. (2017). Sustainable oil palm industry: The possibilities. *Renewable and Sustainable Energy Reviews*, 76, 608–619.

Khoo, K.S., Chew, K.W., Yew, G.Y., Leong, W.H., Chai, Y.H., Show, P.L., & Chen, W.H. (2020). Recent advances in downstream processing of microalgae lipid recovery for biofuel production. *Bioresource Technology*, *304*, 122996.

Kim, K., Hoh, D., Ji, Y., Do, H., Lee, B., & Holzapfel, W. (2013). Impact of light intensity, CO_2 concentration and bubble size on growth and fatty acid composition of *Arthrospira (Spirulina) platensis* KMMCC CY-007. *Biomass and Bioenergy*, 49, 181-187.

Kim, S.K. (Ed.). (2015). Handbook of marine microalgae: Biotechnology advanc*es*. *Academic Press.*45-76.

Koyande, A.K., Chew, K.W., Rambabu, K., Tao, Y., Chu, D.T., & Show, P.L. (2019). Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness*, *8*(1), 16-24.

Kumar, B.R., Deviram, G., Mathimani, T., Duc, P.A., & Pugazhendhi, A. (2019). Microalgae as rich source of polyunsaturated fatty acids. *Biocatalysis and Agricultural Biotechnology*, *17*, 583-588.

Kumar, K., Ghosh, S., Angelidaki, I., Holdt, S.L., Karakashev, D.B., Morales, M.A., & Das, D. (2016). Recent developments on biofuels production from microalgae and macroalgae. *Renewable and Sustainable Energy Reviews*, *65*, 235-249.

Kumar, M., Kulshreshtha, J., & Singh, G.P. (2011). Growth and biopigment accumulation of cyanobacterium Spirulina platensis at different light intensities and temperature. *Brazilian Journal of Microbiology*, 42(3), 1128-1135.

Kumar, P., Kuppusamy, S., Yusop, H.M., & Alwi, S.I.S. (2011). POME treatment using *Spirulina Platensis* Geitler. *International Journal of Current Science*, 1, 11-13.

Lakshmikandan, M., Murugesan, A.G., Wang, S., Abomohra, A.E.F., Jovita, P.A., & Kiruthiga, S. (2020). Sustainable biomass production under CO_2 conditions and effective wet microalgae lipid extraction for biodiesel production. *Journal of Cleaner Production*, *247*, 119398.

Laye, S., Nadjar, A., Joffre, C., & Bazinet, R.P. (2018). Anti-inflammatory effects of omega-3 fatty acids in the brain: physiological mechanisms and relevance to pharmacology. *Pharmacological Reviews*, *70*(1), 12-38.

Levasseur, W., Perré, P., & Pozzobon, V. (2020). A review of high value-added molecules production by microalgae in light of the classification. *Biotechnology Advances*, 4, 107545.

Lim, C. I., & Biswas, W. (2019). Sustainability assessment for crude palm oil production in Malaysia using the palm oil sustainability assessment framework. *Sustainable Development*, *27*(3), 253-269.

Lim, D.K., Garg, S., Timmins, M., Zhang, E.S., Thomas-Hall, S.R., Schuhmann, H., & Schenk, P.M. (2012). Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters. *PLoS One*, *7*(7), e40751.

Liow, G.R., Lau, W.L. S., Law, K., Hii, K.S., Noor, N.M., Leaw, C.P., & Lim, P.T. (2019). Phytoplankton community changes in Kuantan Port (Malaysia), with emphasis on the paralytic-shellfish toxin-producing dinoflagellate Alexandrium tamiyavanichii. *Regional Studies in Marine Science*, *26*, 100504.

Long, S.F., Kang, S., Wang, Q.Q., Xu, Y.T., Pan, L., Hu, J.X., & Piao, X.S. (2018). Dietary supplementation with DHA-rich microalgae improves performance, serum composition, carcass trait, antioxidant status, and fatty acid profile of broilers. *Poultry Science*, *97*(6), 1881-1890.

Lupette, J., & Benning, C. (2020). Human health benefits of very-long-chain polyunsaturated fatty acids from microalgae. *Biochimie*. In Press. 53-79.

Lupette, J., & Maréchal, E. (2018). Phytoplankton Glycerolipids: Challenging but promising Prospects from Biomedicine to Green Chemistry and Biofuels. *Blue Biotechnology: Production and Use of Marine Molecules*, 1, 191-215.

Madaki, Y.S., & Seng, L. (2013). Palm oil mill effluent (POME) from Malaysia palm oil mills:waste or resource. *International Journal of Science, Environment and Technology*, *2*(6), 1138-1155.

Malcata, F.X., Pinto, I.S., & Guedes, A.C. (Eds.). (2018). Marine Macro-and Microalgae: An Overview. *CRC Press*, 56-78.

Manaf, I.S.A, Rahim, M.H.A., Govindan, N., & Maniam, G. P. (2018). A first report on biodiesel production from Aglaia korthalsii seed oil using waste marine barnacle as a solid catalyst. *Industrial Crops and Products*, 125, 395-400.

Mandotra, S.K., Kumar, P., Suseela, M.R., Nayaka, S., & Ramteke, P.W. (2016). Evaluation of fatty acid profile and biodiesel properties of microalga Scenedesmus abundans under the influence of phosphorus, pH and light intensities. *Bioresource Technology*, 201, 222-229.

Maniam, G.P., Hindryawati, N., Nurfitri, I., Abd Manaf, I.S., Ramachandran, N., & Rahim,M.H.A. (2015). Utilization of waste fat from catfish (Pangasius) in methyl esters preparation using CaO derived from waste marine barnacle and bivalve clam as solid catalysts. *Journal of the Taiwan Institute of Chemical Engineers*, 49, 58-66.

Mason, T.J., Paniwnyk, L., & Lorimer, J.P. (1996). The uses of ultrasound in food technology. *Ultrasonics Sonochemistry*, *3*(3), S253-S260.

Mata, T.M., Martins, A.A., & Caetano, N.S. (2010). Microalgae for biodiesel production and other applications: a review. *Renewable and Sustainable Energy Reviews, 14*(1), 217-232.

Mathimani, T., Baldinelli, A., Rajendran, K., Prabakar, D., Matheswaran, M., van Leeuwen, R.P., & Pugazhendhi, A. (2019). Review on cultivation and thermochemical conversion of microalgae to fuels and chemicals: process evaluation and knowledge gaps. *Journal of Cleaner Production*, *208*, 1053-1064.

Mathimani, T., & Mallick, N. (2018). A comprehensive review on harvesting of microalgae for biodiesel–Key challenges and future directions. *Renewable and Sustainable Energy Reviews*, *91*, 1103-1120.

Mishra, P., Thakur, S., Singh, L., Krishnan, S., Sakinah, M., & Wahid, Z.A. (2017). Fermentative hydrogen production from indigenous mesophilic strain Bacillus anthracis PUNAJAN 1 newly isolated from palm oil mill effluent. *International Journal of Hydrogen Energy*, 42(25), 16054-16063.

Molino, A., Mehariya, S., Di Sanzo, G., Larocca, V., Martino, M., Leone, G.P., & Musmarra, D. (2020). Recent developments in supercritical fluid extraction of bioactive compounds from microalgae: Role of key parameters, technological achievements and challenges. *Journal of CO_2 Utilization*, *36*, 196-209.

Mühling, M., Belay, A., & Whitton, B. A. (2005). Variation in fatty acid composition of *Arthrospira (Spirulina)* strains. *Journal of Applied Phycology*, *17*(2), 137-146.

Nagappan, S., Devendran, S., Tsai, P.C., Dinakaran, S., Dahms, H.U., & Ponnusamy, V.K. (2019). Passive cell disruption lipid extraction methods of microalgae for biofuel production–a review. *Fuel*, *252*, 699-709.

Ng, D.K.S., Ng, W., Chong, M.F., & Lim, D.L.K. (2015). Waste recovery and regeneration (REGEN) system for palm oil industry. *Chemical Engineering Transactions*, 45, 1315–1320.

Ng, W.J., Goh, A.C.C., & Tay, J.H., (1987). Palm oil mill effluent (POME) treatment-an assessment of coagulants used to aid liquid-solid separation. *Biological Wastes*, 21(4), 237-248.

Niangoran, U., Tian, F., Canale, L., Haba, C.T., Buso, D., & Zissis, G. (2018, June). Study of the LEDs spectrums influence on the *Spirulina* platensis growth in batch culture. In 2018 IEEE *International Conference on Environment and Electrical Engineering and 2018 IEEE Industrial and Commercial Power Systems Europe (EEEIC/I&CPS Europe)*, 1-4.

Nur, M.A., Garcia, G.M., Boelen, P., & Buma, A.G.J. (2019). Enhancement of C-phycocyanin productivity by *Arthrospira platensis* when growing on palm oil mill effluent in a two-stage semi-continuous cultivation mode. *Journal of Applied Phycology*, *31*(5), 2855-2867.

Nzayisenga, J.C., Farge, X., Groll, S.L., & Sellstedt, A. (2020). Effects of light intensity on growth and lipid production in microalgae grown in wastewater. *Biotechnology for Biofuels,* 13 (4).

Ogbonda, K.H., Aminigo, R.E., & Abu, G.O. (2007). Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina* sp. *Bioresource Technology*, *98*(11), 2207-2211.

Osman, N.A., Ujang, F.A., Roslan, A.M., Ibrahim, M.F., & Hassan, M.A. (2020). The effect of palm oil mill effluent final discharge on the characteristics of *Pennisetum purpureum*. *Scientific Reports* 10, 6613.

Oswald, W.J., & Gotaas, H.B. (1957). Photosynthesis in sewage treatment. *Transactions: American. Society of Civil Engineers*, *122*(1), 73-105.

Ötleş, S., & Pire, R. (2001). Fatty acid composition of Chlorella and Spirulina microalgae species. *Journal of AOAC international*, *84*(6), 1708-1714.

Palanisamy, K.M., Paramasivam, P., Maniam, G.P., Rahim, M.H.A., Govindan, N., & Chisti, Y. (2021). Production of lipids by *Chaetoceros affinis* in media based on palm oil mill effluent, *Journal of Biotechnology*, 327, 86-96.

Palanisamy, K.M., Paramasivam, P., Maniam, G.P., Rahim, M.H.A., Ramaraj, R., & Govindan, N. (2020). Palm oil mill effluent as a potential medium for microalgae *Chlorella* sp. cultivation for lipid production. *Maejo International Journal of Energy and Enviromental Communication, 2-2, 1-7.*

Patil, L., & Kaliwal, B B. (2019). Microalga Scenedesmus bajacalifornicus BBKLP-07, a new source of bioactive compounds with in vitro pharmacological applications. *Bioprocess and Biosystems Engineering*, *42*(6), 979-994.

Phang, S.M., Mustafa, E.M., Ambati, R.R., Sulaiman, N.M.N., Lim, P.E., Majid, N.A., & Liew, K.E. (2015). Checklist of microalgae collected from different habitats in peninsular Malaysia for selection of algal biofuel feedstocks. *Malaysian Journal of Science*, *34*(2), 141-167.

Poojary, M.M., Barba, F.J., Aliakbarian, B., Donsì, F., Pataro, G., Dias, D.A., & Juliano, P. (2016). Innovative alternative technologies to extract carotenoids from microalgae and seaweeds. *Marine Drugs*, *14*(11), 214.

Purkan, P., Nidianti, E., Abdulloh, A., Safa, A., Retnowati, W., Soemarjati, W., & Kim, S.W. (2019). Biodiesel production by lipids from Indonesian strain of microalgae *Chlorella vulgaris*. *Open Chemistry*, *17*(1), 919-926.

Puteri, E.V., Din, M.F.M., Ahmed, Z., Jamaluddin, H., & Chelliapan, S. (2011). Investigation of microalgae for high lipid content using palm oil mill effluent (POME) as carbon source. In: International conference on environment and industrial innovation, IPCBEE.

Rahayu, A.S., Karsiwulan, D., Yuwono, H., Trisnawati, I., Mulyasari. S., Rahardjo. S., Hokermin. S., & Paramita. V. (2015). Handbook Pome-to-Biogas Project Development in Indonesia; *Winrock International: Little Rock*.212-254.

Rahmawaty, S., & Meyer, B.J. (2020). Stunting is a recognized problem: Evidence for the potential benefits of ω-3 long-chain polyunsaturated fatty acids. *Nutrition*, *73*, 110564.

Ramola, B., Kumar, V., Nanda, M., Mishra, Y., Tyagi, T., Gupta, A., & Sharma, N. (2019). Evaluation, comparison of different solvent extraction, cell disruption methods and hydrothermal liquefaction of Oedogonium macroalgae for biofuel production. *Biotechnology Reports*, *22*, e00340.

Ranjith Kumar, R., Hanumantha Rao, P., & Arumugam, M. (2015). Lipid extraction methods from microalgae: a comprehensive review. *Frontiers in Energy Research*, *2*, 61.

Ran, Z., Kong, F., Xu, J., Liao, K., Xu, X., Shi, P., & Yan, X. (2020). Fad and Elovl expressions, fatty acid compositions, and feed effects of three representative microalgae in Sinonovacula constricta (Lamarck 1818) at early developmental stages. *Aquaculture*, *521*, 735101.

Raven, J.A., and Geider, R.J. (1988). Temperature and algal growth. *New Phytologist*. 110, 441.

Ren, X., Zhao, X., Turcotte, F., Deschênes, J.S., Tremblay, R., & Jolicoeur, M. (2017). Current lipid extraction methods are significantly enhanced adding a water treatment step in *Chlorella protothecoides*. *Microbial Cell Factories*, *16*(1), 1-13.

Prommuak, C., Pavasant, P., Quitain, A.T., Goto, M., & Shotipruk, A. (2012). Microalgal lipid extraction and evaluation of single-step biodiesel production. *Engineering Journal*, *16*(5), 157-166.

Richmond, A. (1986). *Spirulina*. Borowitzka, M., & Borowitzka, L. (Eds.), MicroalgalBiotechnology, Cambridge University Press, London, UK. 87-121.

Rosenfeldt, F. (1999). Omega-3 polyunsaturated fatty acids: fishing for new medical strategies. *The Asia Pacific Heart Journal*, *8*(1), 17-18.

Ru, I.T.K., Sung, Y.Y., Jusoh, M., Wahid, M.E.A., & Nagappan, T. (2020). *Chlorella vulgaris*: a perspective on its potential for combining high biomass with high value bioproducts. *Applied Phycology*, *1*(1), 2-11.

Rupani, P.F., Singh, R., Ibrahim, M., & Esa, N. (2010). Review of current palm oil mill effluent (POME) treatment methods: Vermicomposting as a sustainable practice. *World Applied Science Journal*, 10(10), 1190–1201.

Salama, E.S., Kurade, M.B., Abou-Shanab, R.A., El-Dalatony, M.M., Yang, I.S., Min, B., & Jeon, B.H. (2017). Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation. *Renewable and Sustainable Energy Reviews*, *79*, 1189-1211.

Saliu, F., Magoni, C., Torelli, A., Cozza, R., Lasagni, M., & Labra, M. (2020). Omega-3 rich oils from microalgae: A chitosan mediated in situ transesterification method. *Food Chemistry*, *337*, 127745.

Sanghamitra, S., Deshmukh, S., & Narayan, K.P. (2020). Effects of alternate nutrient medium on microalgae biomass and lipid production as a bioenergy source for fuel production. *Materials Today: Proceedings*.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., & Hankamer, B. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research*, *1*(1), 20-43.

Seghiri, R., Kharbach, M., & Essamri, A. (2019). Functional composition, nutritional properties, and biological activities of Moroccan *Spirulina* microalga. *Journal of Food Quality*, 2019.

Seyidoglu, N., Inan, S., & Aydin, C. (2017). A prominent superfood: *Spirulina platensis*. *Superfood and Functional Food the Development of Superfoods and Their Roles as Medicine*, 1-27.

Shahidi, F., & Ambigaipalan, P. (2018). Omega-3 polyunsaturated fatty acids and their health benefits. *Annual Review of Food Science and Technology*, *9*, 345-381.

Show, P.L., Tang, M.S., Nagarajan, D., Ling, T.C., Ooi, C.W., & Chang, J.S. (2017). A holistic approach to managing microalgae for biofuel applications. *International Journal of Molecular Sciences*, *18*(1), 215.

Singh, J., & Gu, S. (2010). Commercialization potential of microalgae for biofuels production. *Renewable and Sustainable Energy Reviews*, *14*(9), 2596-2610.

Smibert, R.M., Buchanan, R.E., & Gibbons, N.E. (1974). Bergey's manual of determinative bacteriology. *The Williams and Wilkins Co., Baltimore*, *208*.

Solana, M., Rizza, C. S., & Bertucco, A. (2014). Exploiting microalgae as a source of essential fatty acids by supercritical fluid extraction of lipids: Comparison between *Scenedesmus obliquus*, *Chlorella protothecoides* and *Nannochloropsis salina*. *The Journal of Supercritical Fluids*, *92*, 311-318.

Soni, R. A., Sudhakar, K., & Rana, R. S. (2019). Comparative study on the growth performance of *Spirulina platensis* on modifying culture media. *Energy Reports*, *5*, 327-336.

Sorokin, C., and Kranss, R.W. (1965). The dependence of the cell division in Chlorella on temperature and light intensity. *American Journal of Botany*, 52(4), 331-339.

Sosa-Hernández, J.E., Escobedo-Avellaneda, Z., Iqbal, H., & Welti-Chanes, J. (2018). State-of-the-art extraction methodologies for bioactive compounds from algal biome to meet bio-economy challenges and opportunities. *Molecules*, *23*(11), 2953.

Stokes, J., Tu, R., Peters, M., Yadav, G., Fabiano, L.A., & Seider, W.D. (2020). Omega-3 fatty acids from algae produced biodiesel. *Algal Research*, *51*, 102047.

Sukumaran, P., Nulit, R., Zulkifly, S., Halimoon, N., Omar, H., & Ismail, A. (2014). Potential of fresh POME as a growth medium in mass production of *Arthrospira platensis*. *International Journal of Current Microbiology and Applied Sciences*, *3*(4), 235-250.

Swanson, D., Block, R., & Mousa, S. A. (2012). Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Advances in nutrition*, *3*(1), 1-7.

Udaiyappan, A.F.M., Hasan, H.A., Takriff, M.S., Abdullah, S.R.S., Maeda, T., Mustapha, N.A., & Hakimi, N.I.N.M. (2020). Microalgae-bacteria interaction in palm oil mill effluent treatment. *Journal of Water Process Engineering*, *35*, 101203.

Vandongen, R., Mori, T.A., Burke, V., Beilin, L.J., Morris, J., & Ritchie, J. (1993). Effects on blood pressure of omega 3 fats in subjects at increased risk of cardiovascular disease. *Hypertension*, *22*(3), 371-379.

Venkatesan, J., Manivasagan, P., & Kim, S.K. (2015). Chapter 1-Marine microalgae biotechnology: present trends and future advances. *Handbook of Marine Microalgae*, 1-9.

Vidyashankar, S., VenuGopal, K.S., Swarnalatha, G.V., Kavitha, M.D., Chauhan, V. S., Ravi, R., & Sarada, R. (2015). Characterization of fatty acids and hydrocarbons of chlorophycean microalgae towards their use as biofuel source. *Biomass and Bioenergy*, *77*, 75-91.

Viswanathan, T., Mani, S., Das, K.C., Chinnasamy, S., Bhatnagar, A., Singh, R.K., & Singh, M. (2012). Effect of cell rupturing methods on the drying characteristics and lipid compositions of microalgae. *Bioresource Technology*, *126*, 131-136.

Vongsvivut, J., Miller, M.R., McNaughton, D., Heraud, P., & Barrow, C.J. (2014). Rapid discrimination and determination of polyunsaturated fatty acid composition in marine oils by FTIR spectroscopy and multivariate data analysis. *Food and Bioprocess Technology*, *7*(8), 2410-2422.

Vonshak, A., Abeliovich, A., Boussiba, S., Arad, S., & Richmond, A. (1982). Production of Spirulina biomass: effects of environmental factors and population density. *Biomass*, *2*(3), 175-185.

Vonshak, A. (Ed.). (1997). *Spirulina platensis arthrospira: physiology, cell-biology and biotechnology*. CRC Press, 220-234.

Wachnicka, A.H., & Gaiser, E.E. (2007). Characterization of *Amphora* and *Seminavis* from south Florida, USA. *Diatom Research*, *22*(2), 387-455.

Weatherly, C.A., Zhang, Y., Smuts, J.P., Fan, H., Xu, C., Schug, K.A., & Armstrong, D.W. (2016). Analysis of long-chain unsaturated fatty acids by ionic liquid gas chromatography. *Journal of Agricultural and Food Chemistry*, *64*(6), 1422-1432.

Wu, J., Alam, M. A., Pan, Y., Huang, D., Wang, Z., & Wang, T. (2017). Enhanced extraction of lipids from microalgae with eco-friendly mixture of methanol and ethyl acetate for biodiesel production. *Journal of the Taiwan Institute of Chemical Engineers*, 71, 323-329.

Yousuf, A. (Ed.). (2019). *Microalgae Cultivation for Biofuels Production*. *Academic Press.*213-267.

Yue, D.T., & Lim, J.S. (2019). Feasibility of palm oil mill effluent elimination towards sustainable Malaysian palm oil industry. *Renewable and Sustainable Energy Reviews*, 111, 507-522.

APPENDICES

APPENDIX A: CONVENTIONAL MEDIA

Conventional media used for cultivation of microalgae isolates

Constituent	Quantity (g)
Solution A (10ml per litre of medium)	
Na_2Mg EDTA	0.10
Ferric ammonium citrate	0.60
Citric acid. $1H_2O$	0.60
$CaCl_2.2H_2O$	3.60
Make up to 1 litre with fresh water	
Solution B (10ml per litre of medium)	
$MgSO_4.7H_2O$	7.50
Make up to 1 litre with fresh water	
Solution C (10ml per litre of medium)	
$K_2HPO_4.3H_2O$	4.00
Solution D (1ml per litre of medium)	
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$COCl_2.6H_2O$	0.050
$CuSO_4.5H_2O$	0.079
$NaMoO_4.2H_2O$	0.391
Na_2CO_3 (per litre of medium)	0.02
$NaNO_3$ (per litre of medium)	1.50

The chemical composition of BG-11 medium (Rippka et al., 1979).

The composition of Walne's medium (Laing, 1991)

Constituent	Quantity (g)
Solution A (at 1ml per litre of culture)	
Ferric chloride ($FeCl_3$)	0.80
Manganous chloride ($MnCl_2.4H_2O$)	0.40
Boric acid (H_3BO_3)	33.60
EDTA, di-sodium salt	45.00
Sodium di-hydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	20.00
Sodium nitrate ($NaNO_3$)	100.00
Solution B	1.00 ml
Make up to 1 litre with fresh water (Heatto dissolve)	
Solution B	
Zinc chloride ($ZnCl_2$)	2.10
Cobaltous chloride ($CoCl_2.6H_2O$)	2.00
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	0.90
Cupric sulphate ($CuSO_4.5H_2O$)	2.00
Concentrated HCl	10.00 ml
Make up to 100ml fresh water (Heatto dissolve)	
Solution C (at 0.1 ml per litre of culture)	
Vitamin B_1	0.20
Solution E	25.00 ml
Make up to 200 ml with fresh water	
Solution D (for culture of diatoms-used in addition to solutions A and C, at 2 ml per litre of culture)	
Sodium metasilicate ($Na_2SiO_3.5H_2O$)	40.00
Make up to 1 litre with fresh water	
Solution E	
Vitamin B_{12}	0.10
Make up to 250 ml with fresh water	
*35g of sea salt added in 1L of medium	

For preparation of media 35 g of sea salt was dissolved and added 1ml of solution A&D and 0.1 ml of solution C and in 1,000 ml of distilled water. Place the media in hot plate for complete dissolved of sea salt in the mixture.

The composition of Bold Basal Medium (Azizul et al., 2018)

Stocks of chemicals	Quantity (g/L)
Solution A	
$NaNO_3$	25.00
$MgSO_4.7H_2O$	7.50
NaCl	2.50
K_2HPO_4	7.50
KH_2PO_4	17.50
$CaCl_2.2H_2O$	2.50
Solution B-Trace elements	
$ZnSO_4.7H_2O$	4.42
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co\ (NO_3)_2.6H_2O$	0.49
Solution C	
H_3BO_3	11.40
Na_2-EDTA	50.00
KOH	31.00
$FeSO_4.7H2O$ with 1.0ml concentrated H_2SO_4	4.98

APPENDIX B: EXPERIMENT PROCEDURE

Figure 1. Collection of marine algae samples

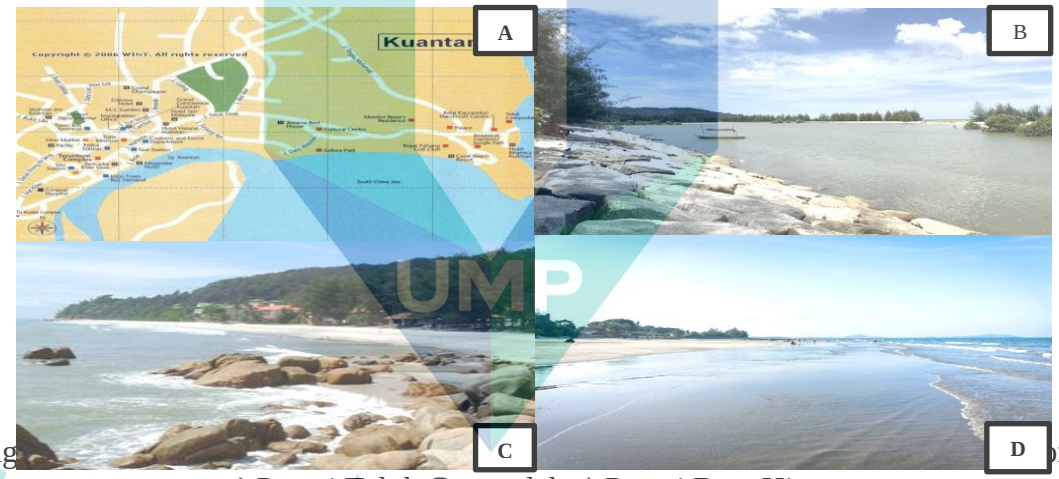


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Figure 3. Palm oil mill effluent and BG-11 medium

Figure 4. Cultivation of isolated microalgae species from marine algae sample

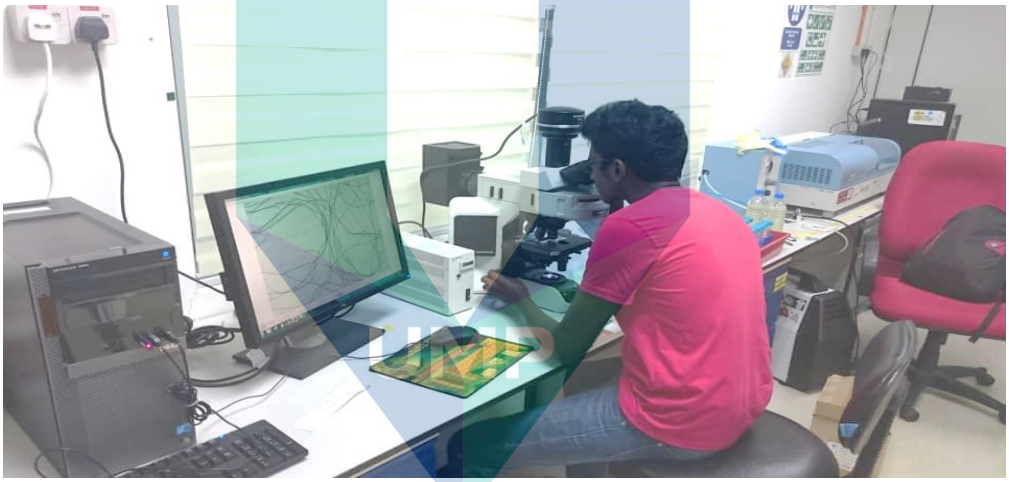

Figure 5. Morphological identification of algae under fluorescence microscope

Figure 6. Isolated species of microalgae from Kuantan coastal area

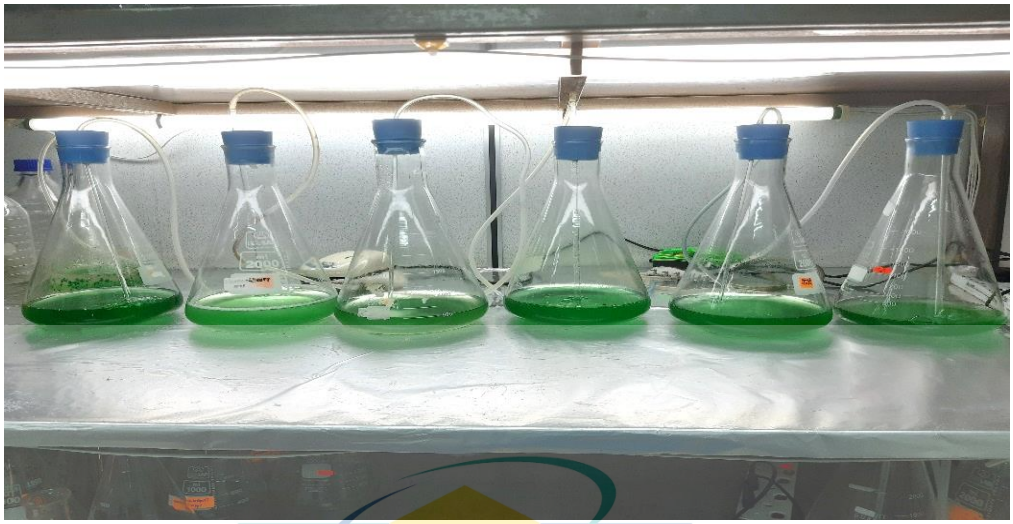

Figure 7. Cultivation of *Spirulina* sp. in Erlenmeyer flask

Figure 8. Cultivation of *Spirulina* sp. in 5L bottles as stock culture

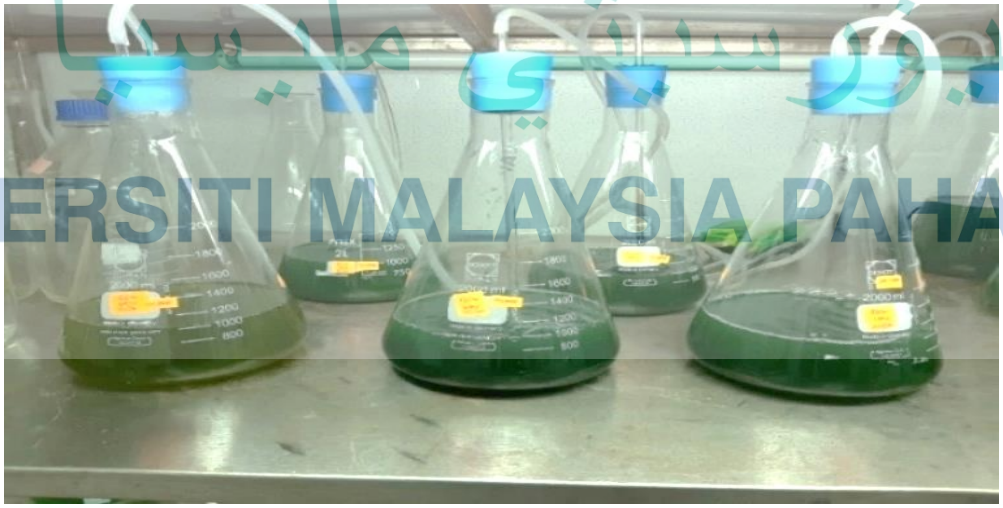

Figure 9. *Spirulina* sp. cultured in different concentrations of POME medium

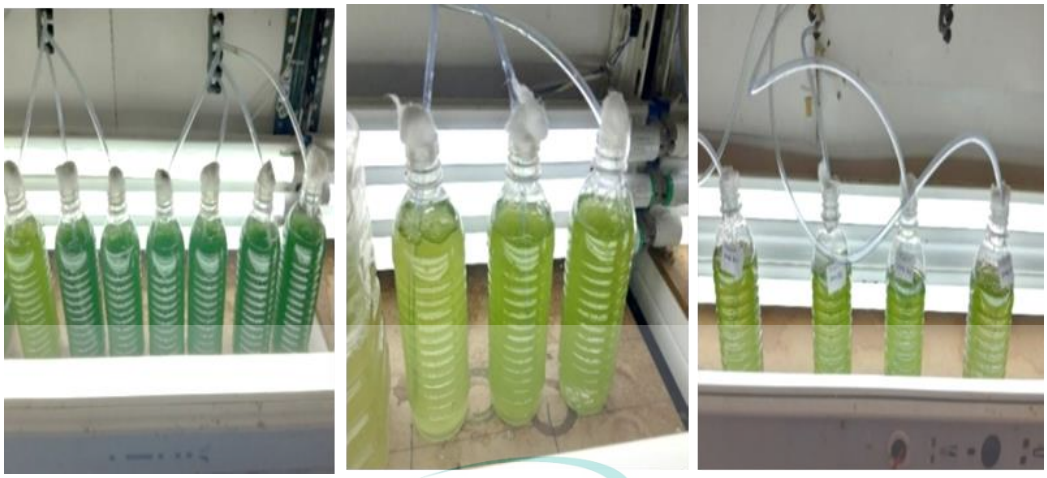

Figure 10. Culture grown in bottles at different temperature

Figure 11. Growth of *Spirulina* sp. under light intensity

Figure 12. cultivation of *Spirulina* sp. at different temperature

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Figure 13. Centrifugation method for harvesting *Spirulina* sp. culture

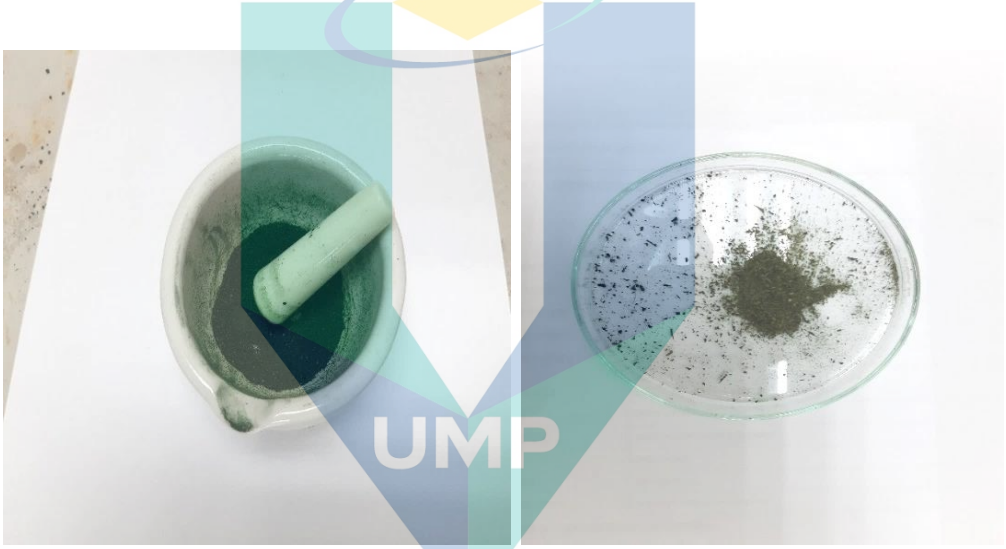

Figure 14. Harvested *Spirulina* sp. biomass from POME cultivation

Figure 15. The collected *Spirulina* sp. biomass

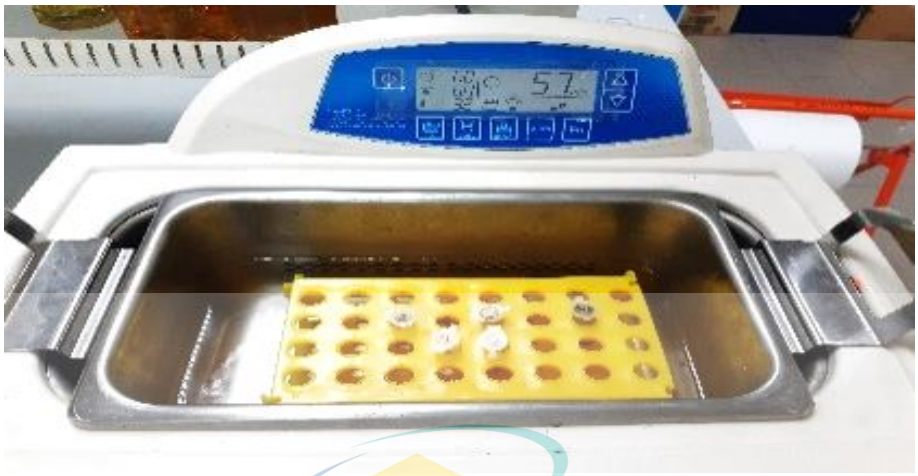

Figure 16. Trans-esterification of lipid by ultrasonicated assisted

Figure 17. Soxhlet extraction method for lipid collection from *Spirulina* sp.

Figure 18. Extracted crude lipid and trans esterified fatty acid methyl esters

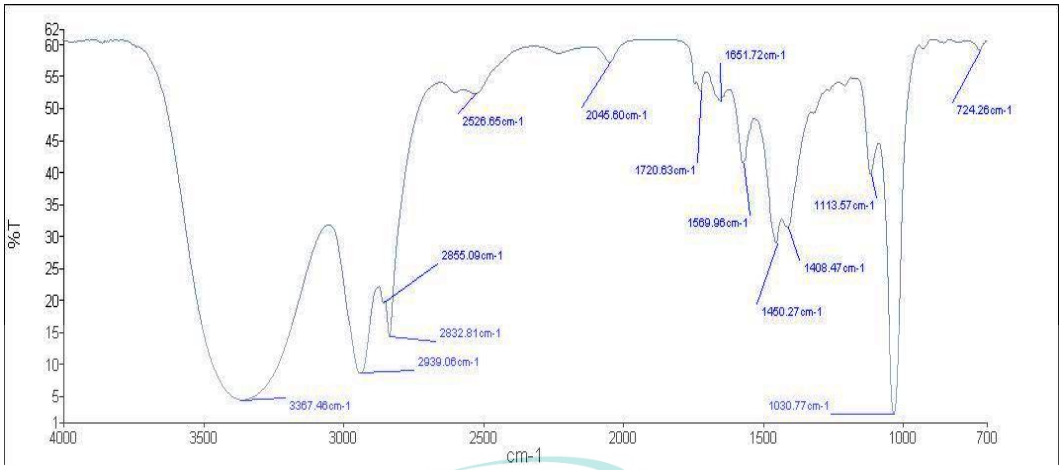

Figure 19. Infrared spectra of *Spirulina* sp. extracted lipid

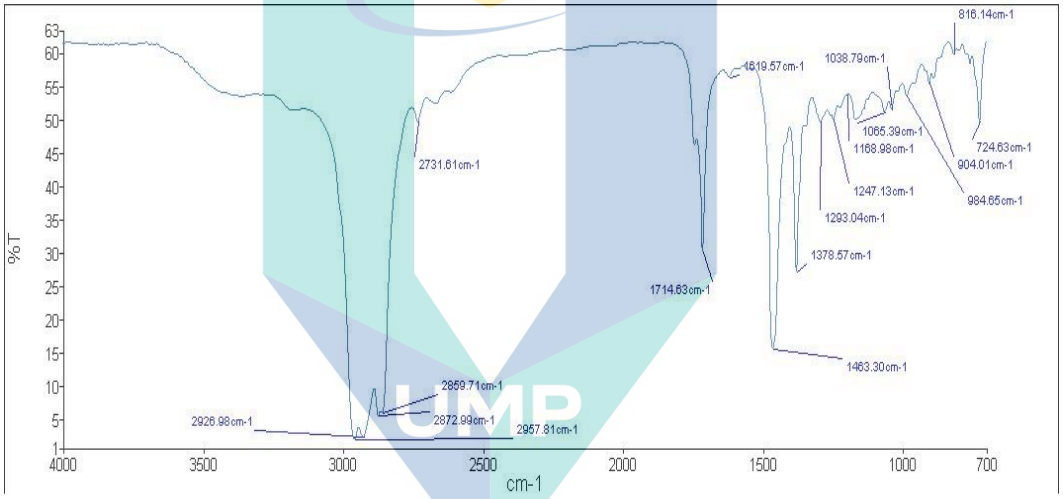

Figure 20. FTIR analysis of *Spirulina* sp. lipid from POME medium

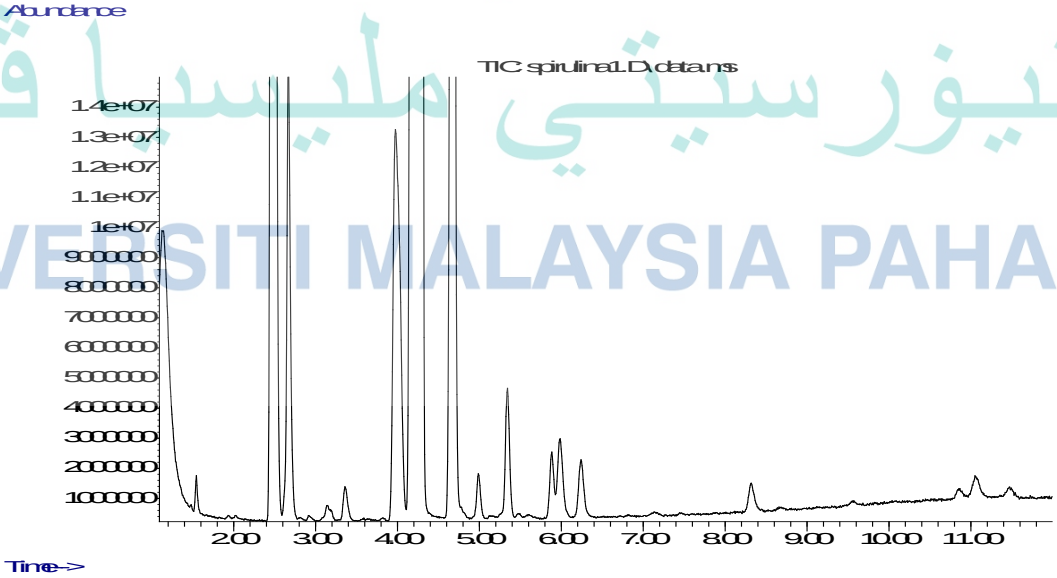

Figure 21. Chromatogram of fatty acid methyl ester from GCMS

APPENDIX C: LIST OF PUBLICATIONS

PUBLICATIONS

1. **Palanisamy, K.M**., Paramasivam, P., Maniam, G.P., Rahim, M.H.A, Govindan, N., & Chisti, Y. Production of lipids by *Chaetoceros affinis* in media based on palm oil mill effluent. *Journal of Biotechnology*, 327, 86-96.

2. **Palanisamy, K.M.,** Paramasivam, P., Maniam, G.P., Rahim, M.H.A., Ramaraj, R., Govindan, N. Palm oil mill effluent as a potential medium for microalgae *Chlorella* sp. cultivation for lipid production. *Maejo International Journal of Energy and Enviromental Communication, 2-2, 1-7.*

3. **Palanisamy, K.M.,** Paramasivam, P., Jayakumar, S., Maniam, G.P., Rahim, M.H.A., Govindan, N. Economical cultivation system of microalgae *Spirulina platensis* for lipid production. *Proceedings of International Conference of Sustainable Earth Resources Engineering 2020 (SERIES 2020)*- In press.

CONFERENCE

1. International Conference of Sustainable Earth Resources Engineering 2020 (SERIES 2020) “Exploring Future Sustainable Engineering Technology” on 28 April 2020.

2. International Webinar on “Environmental Conservation with Sustainable Renewable Energy Resources” on 10 August 2020.