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Academic Session : SEMESTER 2 2020/2021

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MASS CULTIVATION AND LIPID PRODUCTION OF MICROALGAE *NANNOCHLOROPSIS* SP. USING 10L PHOTOBIOREACTOR FOR BIODIESEL PRODUCTION

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Master of Science

Faculty of Industrial Sciences and Technology
UNIVERSITI MALAYSIA PAHANG

JUNE 2021

ACKNOWLEDGEMENTS

My heartfelt gratitude to God for giving me the ultimate strength through mental and physical health in order for me to complete this thesis successfully. May the peace and blessings be upon to God. Special extended thanks to Assoc. Prof. Dr. Gaanty Pragas Maniam, who had provided a significant contribution by providing all the guidance, support, ideas and knowledge needed throughout my study. Again thankful and grateful to him for the continues guidance throughout my master's journey.

I would like to also express my appreciation to my co-supervisor, Dr. Natanamurugaraj Govindan for his efficient support and encouragement throughout this research. I wish to thank Science Officers at Central Laboratory along with Faculty of Industrial Sciences and Technology Laboratory Assistants and Science Officers for allowing me to utilize the laboratory and for their valuable assistance. Credit also goes to the Dean of Faculty of Industrial Sciences and Technology and to the Institute of Postgraduate Studies (IPS) for their support. I am highly grateful to Universiti Malaysia Pahang for financial support for this work through Flagship Research Grant (RDU182205).

I would also like to thank my dearest parents, my sister and my brother in law for their prayers, encouragement, love and support to pursue my dream without any hesitation. Finally, my foremost gratitude to my laboratory colleague, Mr. Karthick Murugan Palanisamy and Mr. Muhammad Nor Fazli bin Abdul Malek for helping me throughout my research.

ABSTRAK

Kekurangan bahan bakar fosil berkait rapat dengan kemerosotan persekitaran diramalkan akan menjadi masalah terbesar di masa hadapan. Pembakaran bahan bakar fosil menghasilkan pencemaran udara yang boleh membahayakan ekosistem. Biodiesel menjadi salah satu alternatif yang paling sesuai untuk menggantikan minyak diesel berasaskan bahan bakar fosil. Transformasi tanaman berasaskan makanan kepada biodiesel secara besar-besaran dapat mengakibatkan defisit bekalan makanan global. Sebagai alternatif, penghasilan mikroalga secara besar-besaran mampu menghasilkan hasil lipid yang tinggi. Dalam kajian ini, mikroalga dari Teluk Cempedak, Kuantan yang mempunyai kandungan lipid yang tinggi diasing dan dikultur untuk penghasilan biodiesel. Di antara sampel yang dikumpulkan, tujuh mikroalga disaring dan pemeriksaan awal dengan kajian morfologi, mikroalga hijau *Nannochloropsis* sp. diasing dan dicirikan dengan mikroskop pendarfluor dan mikroskop imbasan elektron. *Nannochloropsis* sp. yang telah dikultur dalam medium Conway, didapati sebagai mikroalga yang paling sesuai dengan kadar pertumbuhan yang tinggi dan kandungan lipid yang banyak. Parameter yang mempengaruhi penghasilan lipid intraselular juga dikaji. Faktor cahaya, kandungan garam dan nilai pH dikaji untuk menentukan nilai optimum bagi pertumbuhan dan peningkatan lipid mikroalga *Nannochloropsis* sp. Dibawah keadaan optimum, *Nannochloropsis* sp. yang dikultur dalam 10 L fotobioreaktor menghasilkan kandungan lipid tertinggi iaitu 64.8%. Pengekstrakan lipid dilakukan dengan menggunakan teknik Bligh & Dyer, ultrasonik dan Soxhlet. Antara teknik-teknik ini, didapati kaedah pengekstrakan Soxhlet menghasilkan kandungan lipid yang tertinggi iaitu sebanyak 64.8% dalam tempoh pengekstrakan 3 jam. Lipid yang diekstrak kemudiannya ditransesterifikasi dengan metanol untuk menghasilkan metil ester (biodiesel) dalam masa 1.5 jam, dimana KOH digunakan sebagai mangkin homogen. Kromatografi lapisan tipis (TLC) dilakukan untuk memastikan penukaran minyak *Nannochloropsis* sp. ke biodiesel. Kromatogram GC-MS mencirikan metil ester yang terhasil merangkumi asid oleik ($C_{18:1}$) 72.6%, asid palmitik ($C_{16:0}$) 13.35%, asid linolenik ($C_{18:3}$) 8.86%, asid stearik($C_{18:0}$) 3.07 %, asid palmitoleik ($C_{16:1}$) 1.20%, asid eikosanoik ($C_{20:0}$) 0.44% dan asid gadolik ($C_{20:1}$) 0.28%. Indeks biasan (nD 20 °C) metil ester ialah 1.4472, memenuhi spesifikasi untuk biodiesel. Lipid *Nannochloropsis* sp. berpotensi besar untuk digunakan dalam industri biodiesel.

ABSTRACT

The depletion of fossil fuel is closely associated with environmental degradation is predicted to become the biggest problem in the future. Burning fossil fuel generates air pollutants that can harm the ecosystem. Biodiesel has been one of the most promising alternative to substitute fossil fuel based diesel oil. The biodiesel content of edible feedstock is less renewable and lower in quantity. The transformation of these crops into large-scale production of biodiesel oil could lead to a global food supply deficit and global could face a food versus fuel dilemma immediately as a result. Fortunately, microalgae capable to grow in large scale and able to produce high lipid yield. In this study, attempts have been made to isolate and mass cultivate high lipid content microalgae from Teluk Cempedak, Kuantan coast, for biodiesel production. Among the collected samples, seven microalgae were screened and upon preliminary screening for morphological studies, green microalgae *Nannochloropsis* sp. was identified using fluorescence microscope and scanning electron microscope. *Nannochloropsis* sp. was found to be the most suitable microalgae with high growth rate and abundant of lipid content that has been cultivated under Conway medium. Stress factors influencing the intracellular lipid body were investigated. The effect of different light conditions, salinity and pH range were examined to determine the optimum factor for *Nannochloropsis* sp. growth and lipid enhancement. The combined optimized stress factors of *Nannochloropsis* sp. produced the highest lipid content of 64.8% which was cultivated in a 10-L photobioreactor. Lipid extraction was carried out using Bligh & Dyer, ultrasound and Soxhlet techniques. Among them, Soxhlet extraction method yielded the highest lipid content of 64.8% in 3 h extraction duration. The extracted lipid then transesterified with methanol to produce methyl esters (biodiesel) in 1.5 hours, where KOH was used as a homogenous catalyst. Thin layer chromatography (TLC) was done to ensure the conversion of *Nannochloropsis* sp. oil to biodiesel. GC-MS chromatogram depicts potential fatty acid methyl esters that include oleic acid ($C_{18:1}$) 72.6%, palmitic acid ($C_{16:0}$) 13.35%, linolenic acid ($C_{18:3}$) 8.86%, stearic acid ($C_{18:0}$) 3.07%, palmitoleic acid ($C_{16:1}$) 1.20%, eicosanoic acid ($C_{20:0}$) 0.44% and gadoleic acid ($C_{20:1}$) 0.28%. The physical property of FAME was evaluated for refractive index (nD 20 °C) as 1.4472, met the specification for biodiesel. *Nannochloropsis* sp. lipid have great potential to be used in biodiesel industry due to its composition of fatty acid methyl ester.

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LIST OF SYMBOLS

%	Percentage
USD	United States Dollar
µm	Micrometre
°C	Celcius

LIST OF ABBREVIATIONS

A	Absorbance
ASTM	American Society for Testing and Materials
EIA	Energy Information Administration
EN	European Standard
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric agents
FAME	Fatty Acid Methyl Ester
PBR	Photobioreactor
FFA	Free fatty acids
g	Gram
GC-MS	Gas Chromatography- Mass Spectrometry
GM	Genetically modified
Kg	Kilogram
kHz	Kilohertz
kWh/m^3	Kilowatt hour per cubic meter
L/ha	Liter per hectare
LED	Light-emitting diode
M	Molarity
mm	Millimeter
MUFA	Monounsaturated Fatty Acid
ppt	Parts per trillion
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid
TAG	Triacylglyceride
UAE	Ultrasound Assisted Extraction
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The biosphere prioritizes efforts to find renewable and sustainable energy sources that could significantly help to reduce the Earth's carbon footprint. The greenhouse effects are compounded by carbon monoxide, carbon dioxide, sulphur dioxide and nitrogen dioxide gasses released from the burning of fossil fuels into the atmosphere, contributing to global warming, thus damaging the balance of the global environment (Yin et al., 2020). The present carbon dioxide level is 394.5 ppmv and is predicted to exceed 500 ppmv by 2050 if emissions are not reduced. Meanwhile, we consume the equivalent of more than 11 billion tonnes of fossil fuel oil globally last year (Mathimani & Mallick, 2018). At a rate of 4 billion tonnes a year, crude oil reserves are decreasing. If this rates continue to arise, Energy Information Administration (EIA) statistics predicted that worldwide fossil fuel reserves will be depleted in under 50 years (Plant and Floorspace, 2010).

Meanwhile, researchers found that biodiesel to be the best alternative source to substitute conventional fuel as it has similar combustion properties with petroleum diesel. Based on the Energy Information Administration (EIA), it has been reported that in the last few years, biodiesel production has increased rapidly. From 2000 until 2011, the production of biodiesel increased from 806 to 21400 million litres. Meanwhile, the global production of biodiesel increased substantially at 80,000 million litres in 2020. Inaddition, relative to vehicle gasoline, biodiesel is more environmentally friendly becauseit emits less sulphur dioxide than conventional fuel. Governments in different countries, including the United States, Italy, China, Germany and India, have been financing biofuelprojects these days. For example, the United States itself has proposed a USD100 billion investment plan to develop clean, alternate, and sustainable technologies. Incentives and subsidies for vendors that produce biofuels have also been implemented (Mallick et al., 2016). Recent findings have shown that this renewable fuel could be used as a

replacement for diesel supplies to cars, ships or even aviation (Biello, 2020; Jesus et al., 2020).

Biodiesel-powered cars with microalgae have been successfully developed by manufacturers from United States (Solazyme) and Japan (Euglena) respectively. In addition, another two of the aviation manufacturer including Sapphire Energy and Continental Airlines used a blend of 50 percent petroleum jet fuel and 50 percent renewable biofuel. The biofuel which derived from renewable sources (Jatropha and microalgae) were utilised to perform a 2-hour flight (Biello, 2020; Technocrazed, 2003; ScientificAmerican, 2009). Instead of becoming an export product, biodiesel is already acting as a domestic energy source, which has recently begun in Malaysia (Loy et al., 2019). On the other hand, Malaysian Biodiesel Association predicted that the overall production and export will rise up to 55% to 1.4 million tonnes in 2019 (The Star Online, 2019).

Malaysia has a total land area of 32.90 million ha, natural forests of 20.1 million ha (61 percent) and farms of 4.89 million ha (14.9 percent). However, due to the area sources and also energy demands, biodiesel had been announced as the most reliable and required renewable energy source. Malaysia was rated as one of the top 10 biodiesel producer among all the countries in the world (Ashnani et al., 2014). On 12th December 2018, the Malaysian Cabinet had decided on the implementation of the use of B10 biodiesel programme (blend of 10% methyl ester and 90% of diesel) for the subsidised sector in stages beginning of February 2019 (The Star Online, 2018). The president of the Malaysian Biodiesel Association had indicated on 24th April 2020 that the directive to manufacture biofuel with a 20% palm oil component known as B20 for the transport sector was first rolled out in January 2020 was set to be fully implemented across the country by mid of June 2021. Malaysia is also looking into a biodiesel stabilisation for biofuel prices. This effort has been taken in order to make biofuel more attractive for consumption.

Biodiesel is formed through long-fatty acid triglycerides in the form of mono-alkyl esters that undergo transesterification or alcoholysis using methanol (Chisti, 2007). Biodiesel which has similar characteristics to petroleum diesel able to fulfill the ever-growing demand for energy use and is branded as carbon neutral and green (Markeb et al., 2019). Biodiesel is currently obtained from edible feedstocks (soybeans, rapeseed,

corn, palm). In comparison, due to the food versus fuel conflicts, constant biodiesel production from edible oil is not renewable. Consequently, microalgae have appeared as the most promising candidate to resolve the limitations of edible oil in biodiesel output (Sati et al., 2019). The cultivation of microalgae in large scale has many advantages towards transportation sector. Algae is a plant that develops rapidly where their doubling time is every few hours once thus, they can be harvested on daily basis. Microalgae able to produce ten times as much of oil equally as a typical terrestrial oleaginous crop (Sajjadi et al., 2018). Microalgae are capable of fixing atmospheric carbon dioxide, facilitating a decrease in the amount of atmospheric carbon dioxide, which is now becoming a worldwide issue (Deshmukh et al., 2019). In addition, biomass microalgae production can influence the biofixation of major carbon dioxide waste greenhouse gas emissions (1kg of microalgae biomass needs 1.8 kg of CO_2) (Rodolfi et al., 2009).

In addition, microalgae can be cultivated in different environment (fresh, blackish or saline water) which are incompatible for conventional agriculture. Furthermore, these can also be grown on farmland or in bioreactors, (Kumar & Singh, 2019). Due to this non-selective production, microalgae generate a superior yield per hectare with enhanced ecological efficiency. Most of the marine microalgae usually consists of oil levels between 20% to 50% by dry cell weight but it has the tendency to achieve higher productivity. The common doubling time of microalgae by dry biomass is within 24 hours. However, during exponential phase their biomass tend to produce within 3.5 hours (Deshmukh et al., 2019). Among all the marine microalgae, *Nannochloropsis* sp. has been one of the most potential candidate for biodiesel production and aids in tropical country for developing into bio-based products (Yustinadiar et al., 2020). It has been reported that this species has shown the best combination of biomass productivity (~$0.2 g^{-1}L^{-1}d^{-1}$) and lipid production (~68wt%) (Allen et al., 2018).

Large-scale industrial growth systems use open ponds with a paddle wheel to grow microalgae to disperse cells of microalgae, nutrients and water while constantly being exposed to the atmosphere. Nonetheless, in order to reduce the risk of contamination and to ease parameter monitoring for higher efficiency, closed systems such as photobioreactor of various designs are favoured. The harvested microalgae biomass converted into biodiesel through a process called transesterification. Transesterification is the most common method used to transform crude oils into methyl ester. It is some consecutive reaction in the presence of a catalyst between vegetable oils

and alcohol. Triglycerides are converted into monoglycerides through the reactions (Mofijur et al., 2019).

1.2 Problem Statement

Microalgae is now becoming the world's primary source of biodiesel production. They are considered among those that could be used for biodiesel production as safer, non-competitive and rapidly growing species. They have the potential to expand on waste nutrients without much treatment. Microalgae currently being the best source of biodiesel production is considered because other sources can cause food problems, since they mostly include plants which is used for food production. In addition, the biodiesel content of crops compared to algae is less renewable and lower in quantity. Other edible feedstocks have historically produced biodiesel, but these edible feedstocks have had a significant effect on worldwide markets and food safety. For instance, palm and soy are crops whose oils are an important element of human food. The transformation of these crops into large-scale production of biodiesel oil could lead to a global food supply deficit and the global could face a 'food versus fuel' dilemma immediately as a result (Ahmad et al., 2011).

In some cases, this scenario seems to have been excessive. However, the ability of these oils to meet the production objectives of biodiesel is limited and their use as a biofuel will lead to competition with the demand for edible oil, while rising both the price of edible oils and the price of biodiesel. The production of biodiesel from edible oil also has a negative effect on the environment as it enables much of the arable land available to be used for biodiesel production. In order to grow biodiesel crops for the first generation. Very large portions of land were needed to make a major contribution to the world's demand for fuel, which created severe environmental imbalances, such as for plantation purposes, and countries around the world started to cut down forests. In tropical countries such as Malaysia and Indonesia, which account for about 80 percent of the world's palm oil supply, the use of these feedstocks could therefore affect biodiversity which can also lead to deforestation (Sajjadi et al., 2018).

This trend has shown how large-scale deforestation has been triggered by the expansion of oil crops in recent years in order to satisfy the world's biodiesel demand.

Consequently, the introduction of biodiesel as a substitute fuel for diesel fuel based on petroleum may lead to widespread harm to the environment and wildlife in these areas. Meanwhile, microalgae can produce 58,700 L/ha algal oil which can produce 121,104 L/ha biodiesel (Rodolfi et al., 2009). Microalgae do not compete with crops used on land to process grain, fodder and other food products. Oil concentrations within the dry biomass range of 20 to 50 percent by weight are given by the most common microalgae, but higher productivity can be achieved.

1.3 Objectives

i) To cultivate, extract and screen high lipid content indigenous microalgae *Nannochloropsis sp*.

ii) To maximize the lipid secretion by altering some environmental parameters as light intensity, salinity, and pH-values using 10L photobioreactor.

iii) To produce and characterize methyl esters (biodiesel) via transesterification reaction using KOH as a homogeneous base catalyst.

1.4 Scope of Study

The comprehensive scope of this research was to utilize, synthesize and optimize the harvested microalgae biomass for transesterification of biodiesel. In order to accomplish the above objectives as mentioned, the following research scopes had been identified for the first objective:

i. Microalgae sample collection in three different places for isolation purpose based on depth of seawater.

ii. Isolate high lipid productivity microalgae using serial dilution and streaking method after visual observation under fluorescent microscope.

iii. Modify the culture medium using effects of light intensity, salinity and pH-values for better enhancement of lipid accumulation

iv. Mass cultivate high lipid productivity microalgae using 10L photobioreactors which resembles batch cultivation.

v. Prepare the dry biomass of targeted microalgae which consist of high lipid content.

vi. Extract lipid from harvested dry microalgae biomass via Soxhlet extraction, Bligh and Dyer and Ultrasonic using hexane as a solvent.

vii. Transesterification of microalgae lipid using base catalyst with optimum reaction condition of methanol to oil volume ratio, catalyst amount and reaction temperature had been focused.

viii. The conversion of methyl esters are determined through thin layer chromatography (TLC).

ix. The composition of fatty acid methyl ester are determined using Gas Chromatography Mass Spectrometry (GC-MS).

1.5 Thesis outline

This research able to identify and cultivate a novel microalgae *Nannocholopsis* sp. The application of stress factor: light conditions, effect of salinity and pH values in microalgae cultivation another crucial factor in this experiment. These three elements can be crucial in cultivation to produce high lipid microalgae for biodiesel production. The optimum condition of methanol to oil volume ratio, catalyst amount and reaction temperature of biodiesel transesterification are achievable in 3 hours.

CHAPTER 2

LITERATURE REVIEW

2.1 World Energy Issue

2.1.1 World Energy Consumption Scenario

Energy is a key aspect of living and non-living things. Consumption for all fossil fuel types grows faster than production. Based on data assessed in British Petroleum's World Energy Statistical Review reports, carbon dioxide emissions have risen from 29,714.2 million tonnes in 2009 to 33,444.0 million tonnes in 2017 (British Petroleum, 2018). Once again, China has the largest increase in production, followed by the United States of America. The British Petroleum study reveals that between 2006 and 2017, global carbon dioxide emissions accelerated by 1.6%. Therefore, output of energy continues to be influenced by geopolitical events. Oil production in Libya has experienced the greatest decline in the world in the face of renewed civil unrest and oil and gas production has been interrupted in a number of other countries (British Petroleum, 2018). In the face of these disruptions and according to the International Energy Agency (IEA) report and (Bekun et al., 2019), by 2030, the planet will demand more than 50% of energy, of which China and India will account for 45%. From 2005 to 2035, global transport energy consumption is expected to rise by an average of 1.8 percent per year. Nevertheless, the possible loss of fossil fuels and the risks of the atmosphere associated with burning them has inspired many researchers to investigate the possibility of using alternative fuels.

2.1.2 Biodiesel History

Duffy and Patrick conducted the first transesterification of vegetable oil as early as 1853, several years before the first diesel engine was fully operational. By the 1890s, inventor Rudolph Diesel had made the diesel engine the favoured engine in the world for power, reliability and high fuel economy (Zahan & Kano, 2018). The French government and Dr. Diesel himself were among the early experimenters on vegetable oil fuels,

claiming that pure vegetable oils could drive early diesel engines for agriculture in remote regions where petroleum was not available at the time. Later on August 10, 1893, he demonstrated his peanut oil-powered engine and was awarded the 'Grand Prix' at the 1900 World Fair in Paris, France. The Rudolph Diesel, a single 10-foot iron cylinder with a flywheel at its base, which ran for the first time with this fuel in Augsburg, Germany, was the prime model of this first biodiesel powered vehicle. Dr. Rudolph Diesel, after whom the engine is named, held the first patent for the compression ignition engine that year (Katz & Bollella, 2020).

In the 1920s, however, diesel engine manufacturers chose to modify their engines by using the lower viscosity of fossil fuel, better known as petro diesel, rather than the biomass fuel of vegetable oil (Quah et al., 2019). Since their fuel was much cheaper to manufacture than the alternatives to biomass, both petroleum industries were able to make inroads on fuel markets, ignoring that it would cost high pollution years ahead. The near elimination of the infrastructure for the development of biomass fuel has been the outcome of the commercialization of petro diesel for many years. Before the Second World War, vegetable oil powered heavy duty vehicles were introduced in South Africa. The United States in 1978, the Aquatic Species Program, the National Renewable Energy Laboratory experimented with the use of algae as a source of biodiesel. France launched local biodiesel fuel production in the 1990s, known locally as diester, acquired through transesterification of rapeseed oil. Today, impact on the environment issues and a decreasing cost gap have made renewable fuels such as biodiesel an alternative, and International Biodiesel Day was proclaimed on August 10 in memory of Rudolf Diesel's first run in Germany. Nevertheless, their use for biodiesel production needs to be controlled so as not to cause a major problem of hunger and deforestation while providing alternative sources of energy (Balasubramanian and Steward,2019).

2.2 Biodiesel in Malaysia

In 1979, Malaysia's National Energy Policy was implemented with the purpose of providing a future energy supply that is affordable, safe and environmentally sustainable, as well as an efficient and clean use of energy. In terms of technology and expertise, Malaysia had come out with biodiesel challenges with other export markets for biofuels. For Malaysia's palm oil industry, over-supply and declining demand from China and

Europe are several other obstacles (Lam et al., 2019). The Malaysian government has therefore implemented the B10 program to reduce the problem. For the B10 programme, it is estimated that at least one million tons of crude palm oil will be used. It was announced in 2015 that 366 petrol kiosks were already selling B7 biodiesel in Sabah, Sarawak and Labuan, which meets international standards since the end of January 2015. Approximately 138,000 tons of biodiesel palm oil per year will be used by combining the 7% biodiesel palm and 93% diesel oil. In Sabah, Sarawak and Labuan, the use of B7 biodiesel is set to increase the total use of palm oil biodiesel to 576,000 tons nationwide a year (Jayakumar et al., 2017).

In Peninsular Malaysia, the B7 biodiesel project has already been fully implemented. Meanwhile, B10 programme switched from B7 on February 2019. The B10 plan aims to increase palm biofuel blending ratio in diesel from 7% to 10%. The Malaysian Cabinet announced the B10 mandate for the transport sector as part of the crude palm oil supply management mechanism to stabilize palm oil prices by rising the high level of domestic stocks and increasing energy sources sustainability. Biodiesel mixtures from B10 to B20 were also expected to implement by 2020. Malaysia faces the task of further expanding and improving the initiative to generate biodiesel through collaborations with government agencies and private sectors (The Star Online, 2019).

This work is a vision to make Malaysia the world's leading alternative fuel producers. In Malaysia is expected to see a significant increase in biodiesel production in the following years due to the various availability of mass biodiesel feedstock. Despite palm oil industry, microalgae have been most promising feedstock for biodiesel production as they have higher lipid productivity. Since the 1940s, along with a large research group on microalgae, the Solar Energy Research Institute, USA, has proposed microalgae as biomass for biodiesel production to replace the world's main fuel energy. Microalgae have become a superlative option for fuel production compared to other energy crops due to advanced photosynthetic competence, higher biomass concentration and rapid growth capacity with high lipid content (Jayakumar et al., 2017).

2.3 Microalgae

2.3.1 Diversity and characteristics of microalgae

Algae, referred to as microalgae or phytoplankton where, phyto recognized as plant whereas planktos define as made to wander, can be grouped at a microscopic level on the basis of their morphological characteristics and size. (Suparmaniam et al., 2019). Microalgae described as simple microscopic autotrophic/heterotrophic that able to grown through photosynthesis. They tend to grow in either freshwater or marine aquatic habitats in unicellular or multi-cellular form (Yin et al., 2020). Among 200,000-800,000 species range, there were approximately 50,000 species isolated and evaluated. Microalgae use CO_2 as a source of carbon, light in the form energy source and water to synthesise nucleic acids, proteins and carbohydrates and carbon-rich lipids that can later be turned into biodiesel by transesterification. This photosynthetic organism with a well-equipped nucleus, rigid/flexible cell wall and pigments is known as eukaryotic cells, where they have abundant chlorophyll within the cells (Enamala et al., 2018). The cell size range for microalgae cells is from 5 to 50 μm (Zhu et al., 2018). The composition of the microalgae cells can be explained by ionisation of surface functional groups and organic ion adsorption as charged negatively (-7.5 to -40 mV). Microalgae cells are therefore rapidly dispersed in the medium, as aggregation is prevented by their negative charge.

The diverse population of species of microalgae provides a wide variety of starting strains for their full usage and commercialisation. However, researchers and scientists have not overcome the difficulties of defining and characterizing them (Suparmaniam et al., 2019). Microalgae classification can be carried out in various ways, taking into account their differentiating characteristics, such as structural features, composition of the membrane, colour of the pigment and energy-saving molecules (Debiagi et al., 2017). *Cyanophyceae* (blue-green algae), *Chlorophyceae* (green algae), *Bacillariophyceae* (including diatoms) and *Chrysophyceae* (gold algae) can be classified into four major classes of frequently used microalgae to date. Among them, the most promising candidate for biodiesel production is the green algae (*Chlorophyceae*) taxonomic community (Sajjadi et al., 2018).

2.3.2 Crops versus microalgae as biodiesel feedstock

A few possible renewable energy sources that are mainly derived from plant residues are biochar, biodiesel, bioethanol, and biohydrogen. A wide range of raw materials are produced by biofuel processing. Biofuel and manufacturing are divided into four distinct generations based on their biomass feedstock. The first generation of biodiesel is made from plant and animal fats based on oil. Genetically modified (GM) crop production has been steadily growing since its introduction in 1996 (Sitepu et al., 2020). Genetically modified biomass of soybeans, maize and rapeseeds occupies 73.3 x 10^{13}, 46.8 x 10^{13} and 7 x 10^{13} m^2 of the global area (Callegari et al., 2019). As countries around the world started to cut down forests for plantation purposes, this created severe ecological imbalances (Abdulkhani et al., 2017). Tropical nations such as Malaysia and Indonesia might also cause deforestation, which accounts for 80% of the world's palm oil supply. This trend has also shown how large-scale deforestation has been caused by the expansion of oil crop production in the last few years to meet global biodiesel demand. Consequently, the introduction of biodiesel as a substitute fuel for petroleum-based diesel fuel in those regions could lead to substantial environmental and wildlife disruption. (Chowdhury & Loganathan, 2019).

In order to address these concerns, second generation biodiesel feedstocks were introduced. Biodiesel of the second generation is mainly made from non-edible oilseeds, waste cooking oil and lignocellulosic feedstock materials. Although feedstocks of second generation do not usually affect human food supply chain and can be cultivated in wastelands, but consequently not able to replace the amount of fuel being used in transportation sector (Mathimani & Pugazhendhi, 2019). In addition, most animal fats contain a higher volume of saturated fatty acids, making it difficult to transesterify (Singh et al., 2020). For instance, in beef tallow the saturated fatty acids constituent accounts for nearly 50% of the total fatty acids, giving it the unique properties of high melting point and high viscosity (Pal et al., 2019). In contrast, the third generations of biodiesel are mainly derived from microalgae. Because of their high yield, carbon dioxide (CO_2) absorption, and relatively easy processing, they attracted enormous interest among researchers. Based on the Table 2.1, compared to edible and non-edible feedstocks, microalgae are the least used with the highest oil yield per year. Because of their efficient photosynthetic enabling them to produce higher biomass and better growth rates

compared to other crops, thus, microalgae have known as one of the most potential alternative lipid sources in biodiesel production (Adeniyi et al., 2018).

2.3.3 Oleaginous microalgae for lipid production

Nannochloropsis sp. known as potential oleaginous ideal microalgae due to their high photosynthetic performance, high lipid productivity and relatively capable to grow well in both indoor and outdoor cultivation. *Nannochloropsis* sp. recognized to be unicellular and nonmobile microalgae which belong to Phylum Heterokontophyta along with family of Eustigmataceae (Ma et al., 2016). *Nannochloropsis* sp. is not capable to accumulate starch instead they able to produce large amount of polyunsaturated fatty acids (PUFA) and currently utilize by researchers in the production of eicosapentaenoic acid (EPA) for coronary heart disease and biodiesel production. Moreover, this particular species has also been recognised in the human diet due to its high nutritional value, which includes a great source of protein, carbohydrates and vitamins. As a source of useful pigments produced in relatively large amounts, such as chlorophyll, zeaxanthin, canthaxanthin and astazanthin, this algae genus is also well recognized (Liu et al., 2017).

Nannochloropsis sp. has been identified as one of the most promising species for biodiesel production. This species able to synthesize high lipid productivity, better growth rate, easily adaptable to environment, has low contamination risk, potential fatty acid profile, able to genetically modify and optimize (Okoro et al., 2019). Under standard laboratory growth conditions, microalgae synthesize fatty acids primarily for esterification into membrane lipids dependent on glycerol that make up about 5%-20% of the weight of the dry cell mass. Meanwhile, under controlled conditions microalgae lipid biosynthetic pathway is primarily in the form of TAGs (20 %-50%) tend to channelinto neutral lipids (Dong et al., 2016).

As depicted in Table 2.2, Turkkul et al., (2019) conducted a study on *Nannochloropsis oculata* and *Spirulina* sp., using various catalyst for biodiesel production. Meanwhile, *Nannochloropsis* sp. reported as highest lipid content compared to *Spirulina* sp.

Table 2.1 The comparison of microalgae with other feedstocks of biodiesel

Plant source	(% oil by dry cell mass)	Average oil yield (L oil/ha year)	Land usage (m^2 year/kg biodiesel)	Reference
Edible oil				
Rapeseed	41	974	12	Hajjari et al., 2017
Soyabean	18	636	18	Baskar and Aiswarya, 2016
Palm	36	5366	2	Mahlia et al., 2020
Sunflower	40	1070	11	Rezania et al., 2019
Camelina	42	915	12	Hajjari et al., 2017
Non-edible oil				
Castor	48	1307	9	Hajjari et al., 2017
Jatropha	28	741	15	Rezania et al., 2019
Jojoba	45	1413	9	Sitepu et al., 2019
Other source				
Microalgae	70	136,900	0.1	Singh et al., 2020

Table 2.2 Total lipid content of specific microalgal species

Species	Total Lipid Content (% of DW)	Reference
***Nannochloropsis* sp.**	**31-68**	**Akubude et al., 2019**
Isochrysis sp.	25-33	Ma et al., 2016
Dunaliella salina	6	Goh et al., 2019
Chlorella sp.	28-32	Desmukh et al., 2019
Neochloris oleoabundans	35-54	Yin et al., 2020
Phaeodactylum tricornutum	20-30	Akubude et al., 2019
Spirulina platensis	4-9	Yin et al., 2020

2.4 Stress factors involved in microalgae growth

2.4.1 Cultivation parameters

There are several factors required to measure algal biomass cultivation. Some of those crucial factors that closely associate with growth rate and lipid content include salinity, pH, autotrophic and heterotrophic conditions whereas the other parameters involved are temperature, nutrients, and carbon dioxide concentration. Therefore, to be successful in producing desired set of results, these parameters should be maintained and controlled.

2.4.2 Salinity

Salinity is one of the most crucial factor which affects growth and productivity of microalgae by limiting the contaminants. High salinity induces high osmotic extracellular pressure, contributing to the generation of stress within the algae cell, which responds to physiological and biochemical mechanisms (Sajjadi et al., 2018). When microalgae cells are exposed to salinity, turgor pressure restoration, ion uptake/export through the cell membrane and accumulation of osmo-protective solutes relatively become active. Membrane fluidity and permeability also will be affected in high salinity stress (Aziz et al., 2020). At high concentration, salinity tend to affect the microalgae cell, however, microalgae cultivation at optimal salinity will enhance the lipid production (Yin et al., 2020). Sodium salts such as NaCl, $Na_2S_2O_3$, $NaHCO_3$ and $C_2H_3NaO_2$ have been used in

order to enhance the lipid production of microalgae. However, NaCl reported to significantly increase the lipid content of microalgae.

As a physiological resistance response to salt tension, most microalgae control lipid biosynthesis. But the capacity for salinity tolerance of each strain is different. Meanwhile, some species known as halophilic (can tolerate high salt concentrations) and halotolerant (able to grow in saline medium for survival response) (Ashour et al., 2019). For instance, *Dunaliella salina* was recognized as halophilic species as they are relatively resistant to high salt concentration and able produce maximum biomass and lipid (Abomohra et al., 2020). Moreover, Ashour et al., (2019) reported that *Nannocholoropsis oceanica* as halophilic since they produce biomass ($0.722gL^{-1}$) per day. Halophilicspecies plays a dual role in both biodiesel and aquaculture.

2.4.3 pH

The pH value has been the fundamental aspect that directly affects the growth rate of microalgae and species composition is the acidity or alkaline base of the culture medium. The abundance and synthesis of nutrients such as iron and carbon are influenced by the pH value, which indirectly influences the growth rate (Peng et al., 2020). Due to photosynthesis and CO_2 use, the pH in microalgae cultures increases gradually during the day. Meanwhile, respiration of the cell reverses this process at night (dark) and lowers the pH level again. The concentration of carbon dioxide has a major impact on the pH of the media, and is not the only parameter that control it. The theory described by Jayaraman and Rhinehart (2015), indicates that lower pH results in a higher growth rate which is appropriate to a certain value but will cause a further decrease in the growth rate beyond that point. Though various species may have different growth impact of changes in pH values, the appropriate pH range for most species of microalgae is 7-9. The optimal pH range for the growth of microalgae, however, is strain-specific and limited. For most of them, it is between 8.2 and 8.7 (Sajjadi et al., 2018). For example, *Chlorella vulgaris* was cultivated in various pH ranges between 5.5 to 9.5 in order to find the optimal growth rate for biodiesel production, but, 8.5 supported the best specific growth rate and the lipid content was 48% of dry cell weight (Thirugnanasambantham et al., 2020). However, some species are sensitised to conditions with high alkali or high acidity. As reported, *Chlorella sorokiniana* (DOE1412) were evaluated in five different pH range (6.5, 7, 7.5,

8 and 8.5). The biomass and lipid productivity showed the highest at pH range of 6.5 (Qiu et al., 2017).

2.5 Light condition

2.5.1 Photoautotrophic

Photoautotrophic cultivation utilize inorganic carbon source such as carbon dioxide for growth; they depend on light as the major energy source which will be converted to chemical energy through photosynthesis. It is regarded as the oldest and most widely used cultivation condition for the growth of microalgae (Ma et al., 2016). It is demonstrated that through photoautotrophic cultivation the lipid content of microalgae ranges from 5% to 68% depending on the species. It has been reported in literature the highest lipid productivity of microalgae under photoautotrophic cultivation was 75.6 mgL^{-1} by using *Nannochloropsis* sp. (Ashour et al., 2019). The researcher had also demonstrated that the obtained result was favourable for biodiesel production. Since carbon dioxide is the only carbon source for the cell growth, photoautotrophic cultivation plays a major role in carbon dioxide emission as the algae consume CO_2 from the atmosphere. Moreover, Yen et al., (2016) demonstrated that integrated system of autotrophic microalgae using CO_2-based *Chlorella vulgaris* will effectively reduce carbon emissions. In photoautotrophic cultivation, the contamination risk tend to reduce compared to heterotrophic and mixotrophic cultivation (Chew et al., 2018).

2.5.2 Heterotrophic

Heterotrophic cultivation uses both carbon and energy source for microalgae growth in the absence of light (Pavithra et al., 2020). The basic medium composition will be used in this cultivation which is similar as photoautotrophic with the only exception of addition of organic carbon such as glucose, glycerol and acetate (Yin et al., 2020). Heterotrophic growth is an aerobic mechanism in which the integration of organic substrates creates energy as the final electron acceptor by oxidative phosphorylation, followed by oxygen consumption. There are also other metabolic pathways being used aerobic glycolysis (glucose breakdown) by microalgae, such as the Embden-Meyerhof pathway and the Pentose Phosphate pathway. Glucose is mainly metabolised through the Pentose Phosphate pathway, in the dark heterotrophic condition (Perez-Garcia and Bashan, 2015). As reported, a study demonstrated by Verma et al., (2020) that *Spirulina*

sp. were cultivated under heterotrophic condition where it produces highest dry cell weight $7.69 g^{-1}d^{-1}$ compared to the culture in photoautotrophic and mixotrophic. However, there are few key drawbacks of heterotrophic cultivation: (1) Limited types of microalgae species that can be grown heterotrophically, (2) costly due to the organic substrate being used (e.g., glucose, nitrogen, phosphorus and trace elements), (3) high contamination risk by other microorganisms, (4) incapable to produce light-induced metabolite.

2.6 Temperature

Owing to any cause, seasonal temperature fluctuations, daily temperature variations, and abrupt temperature variations may change the growth conditions of microalgae and therefore its efficiency of production or vice versa (Sajjadi et al., 2018).Various kinds of species of microalgae have their own optimum growth temperature where the activity and development of microalgae can be hindered or eventually stopped by higher or lower temperatures below the optimum growth temperature stage, thereby reducing the output of biomass (Huang et al., 2019). This is because photosynthetic proteins are inhibited at high temperatures, which can affect the balance of cell energy and decrease the rate of photosynthesis. Singh et al., (2015) studied on nine various types of microalgae species (*Chlorella, Spirogyra, Chlamydomonas, Botryococcus, Scenedesmus, Neochloris, Haematococcus, Nannochloropsis, Ulva*). The objective of this study is to determine the effect of temperature on their growth for biodiesel production, as observed the optimum temperature of those species were range between 20°C to 30°C. Based on their optimum growth rate at different temperatures, microalgae are divided into consequent categories which are 20 and 25°C for mesophilic species; 17°C for psychrophilic strains or 40°C for thermophilic strains. MarKose et al., (2020) found that *Nannochloropsis gaditana* doubled the lipid content from (12.4 to 29.4%) when the temperature was increased from 20 to 25°C.

2.7 Microalgae cultivation systems

2.7.1 Raceway ponds

Microalgae can be cultivated in both raceway ponds and photobioreactors. There are two types in the raceway pond system, which is open pond and covered pond for mass cultivation purpose.

2.7.2 Open ponds

Open pond system recognized as the most affordable for microalgae growth site that simply consist of natural open ponds which depending on the microalgae species which usually taken for biofuel production and the pond can be both consist of fresh water or salt water ponds. These ponds can be certainly scaled up to hectares, however, algae grazers, other algae invasions, fungal growth and high contamination of certain species of microalgae are the major key drawbacks of such open pond system (Anto et al., 2020). Nevertheless, approximately 98% of the overall production of biomass is accomplished using open pond systems, due to their high growth rate, they are certainly capable to produce 15-20 of cell weight per hectares yearly. Meanwhile, the oil content that further economizes the process is about 50-60% of the high yielding varieties of the dry mass of the algae. There are a variety of experiments on microalgal species cultivation in the open raceway pond. For instance, in an outdoor open raceway pond, growth of *Chlorella pyrenoidosa* using secondary wastewater effluent has effectively removed excess nutrients and produced the highest biomass concentration of 1.71 g/L (Dahmani et al., 2016). Similarly, Ghorbani et al., (2018) cultivated *Dunaliella salina* and *Nannochloroposis* sp. in open pond, where the obtained biomass was 0.096 g $L^{-1}day^{-1}$ and 0.208 g $L^{-1}day^{-1}$ respectively. The open pond system can therefore be considered for the processing of large amount of biomass for non-edible, non-therapeutic and renewable product like biofuel.

2.7.3 Covered ponds

In covered ponds, where invasions of other algae and fungal growth are to some point controlled, the deficiencies of open ponds are met. In these closed ponds, the large evaporation losses from open ponds, which is the major disadvantage of this system. The downside of these covered ponds, however, is that when the pond is covered, there is a substantial increase in temperature and agitation is given to manage the situation (Anto et al., 2020). Several changes have been made to the layout of the open pond and the closed pond to greatly increase the biomass efficiency of the selected strains. A thin layercascade system was installed on the roof top in a study implemented by (Thomas et al., 2015), and the microalgal cultures were held in motion through gravity on the inclined surface. At the end of the incline surface, the microalgae cultures were collected in the tank below the roof and pumped back to the roof. In the tank, which serves as a buffering

agent, the high evaporation rate on sunny days and volumetric differences on rainy days are properly controlled. Several microalgae species such as *Chlorella* sp. *and Scenedesmus* sp. cultivated in covered pond system (Raeisossadati et al., 2019).

2.7.4 Photobioreactors (PBRs)

Closed systems such as photobioreactors are designed to solve problems associated with open pond system. Compared to open pond systems, the photobioreactor provides better control of most parameters such as light, salinity, pH, temperature, etc. Generally, photobioreactor comes in a different configurations namely tubular, flat plate or vertical column type structures etc (Anto et al., 2020). In the system of photobioreactors, algal cultures are continuously pumped through and recirculated. They are transparent since these tubes are made of acrylic or glass materials, which enables the algae to perform photosynthesis and grow by allowing natural or artificial light to enter (Qin et al., 2019). By applying light emitting diodes that imitate the natural sunlight source for the indoor photobioreactor, photobioreactors can achieve yields of up to 100 gms/m^2/hr and altering the light intensity can generate very high dark photosynthesis reactions (Ma et al., 2016). Such photobioreactors are much more expensive than open pond systems, but they have many benefits as shown in Table 3.

Cultivating desired microalgae species in closed system able to eliminate contamination from foreign microalgae species, amoeba or fungi. Photobioreactor has better systems for heat dissipation and nutrient dispersion that ensure uniform and controlled algae biomass growth. There are also minimal evaporation losses in closed system, which saves a lot of necessary make-up water for open and covered ponds. In a recent study, Ajayan et al., (2019) carried out an experiment under various LED light qualities in order to determine the growth performances and lipid production of *Chlamydomonas reinhardtii*. He concluded that LEDs produced more penetration in a column photobioreactor which aid in high specific growth rate (0.514 d^{-1}) and lipid content (39.4%).

2.7.5 Tubular photobioreactor

Tubular photobioreactors are common type of photobioreactor, in which the reactors are constructed as tubes that are linked to the flow volumes in series as a parallel

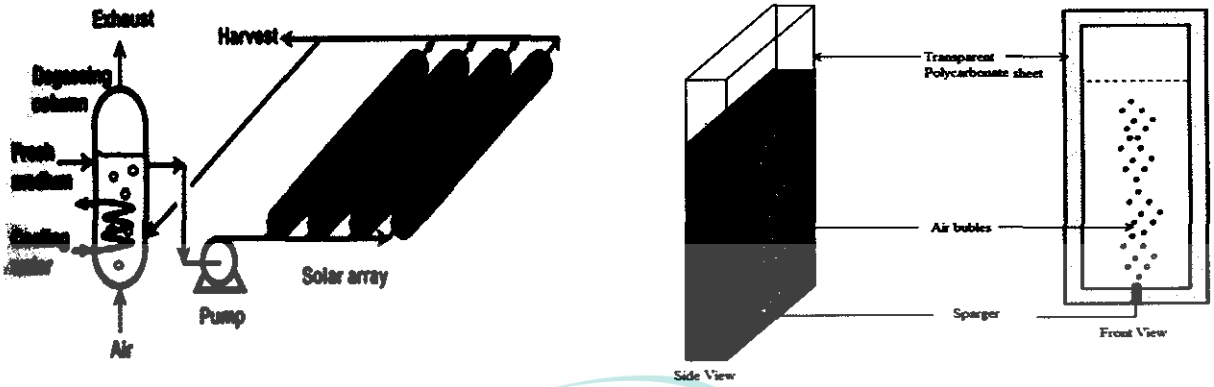
arrangement as depicted in Figure 2.2. In general, these tubes are made from glass or acrylic tubes that are targeted vertically or horizontally, and their transparency enables the sunlight penetration within the bioreactor for effective algal growth (Qin et al., 2019).

2.7.6 Plate photobioreactor

Plate photobioreactor made by plastic or glass plates, where those bioreactors are distinguished by its rectangular compartment made of transparent material approximately with the depth between 1 and 5cm as shown in Figure 2.2. Airlift recirculation is used for culture mixing inside the bioreactor. The largest total area for lighting and low oxygen build-up is supported by this design, therefore delivering the required photosynthetic performance of all photobioreactor designs. Fortunately, this photobioreactor's aeration configuration induces stress damage to the microalgae cells (Tan et al., 2020).

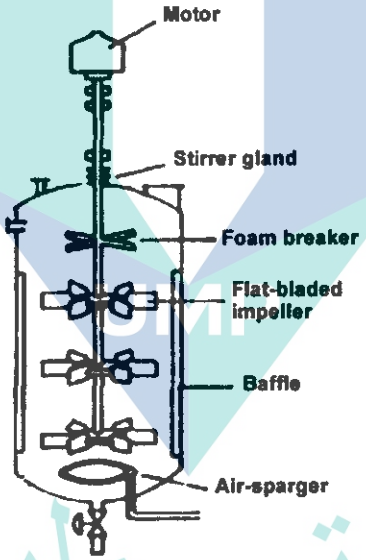
2.7.7 Vertical column photobioreactor

Vertical column photobioreactor is configured by a transparent vertical cylindrical tubing and sparger as depicted in Figure 2.1. The purpose of the sparger in this bioreactor to pumps in the air bubble where it allow homogenization take place between the transfer of oxygen and carbon dioxide (Mohan et al., 2019). Compared to other methods, this culture system provides the best gas-liquid mass transfer efficiency due to the capacity of the sparger used in this system to produce smaller bubbles that provide greater total surface area for more efficient material transfer. Moreover, the efficiency of the architecture makes it possible to provide a lower demand for energy and a simplified operating procedure (Huang et al., 2017). However, in order to conduct photosynthesis effectively, the cylindrical-shaped container does not support in illumination which is crucial for microalgae's growth. In addition, high construction cost and problems associated with cleaning the reactor are the main cause of numerous commercialisation failures in industry. There is still no commercial use of this photobioreactor design, but numerous large-scale experimental reactors have been made, comprising cultivation of *Chlorella Zofingiensis* in 40-liter vertical column outdoor photobioreactor at Guangdong, China (Huo et al., 2018).



Tubular photobioreactor

Plate photobioreactor



Vertical column photobioreactor

Figure 2.1 Types of photobioreactors in closed system

Source: Sajjadi et al., (2018)

Table 2.3 Comparative cost analysis, revenue produced, productivity of biomass in systems for large-scale algal cultivation.

Cultivation systems	Conditions	Microalgae biomass productivity (kg m^{-2} d^{-1})	Biomass cost (kg^{-1})	Biofuel type	Cost of product converted/ Product revenue generated	References
Photobioreactors	Region occupied: 5681m^2; 6 units with 132 parallel tubes per unit length (80m) and diameter (0.06 m).	0.048	$2.95	oil	$1.40/L	Anto et al., 2020
Raceway ponds (open)	Occupied area: 7828m^2; Wide(12m;82m)	0.035	$3.80	oil	$1.81/L	Anto et al., 2020
Raceway ponds (close)	Surface area: 52m^2; Length: (14m;4m):wide (0.75m): Capacity (40,000L)	0.013	-	Biodiesel	-	Bagchi et al., 2019
Raceway pond (open)	100 ha scale	Between 0.008 - 0.014	$3.83	Biofuel	$0.34/kg	Ruiz et al., 2016

2.8 Harvesting of microalgae culture

Microalgae harvesting is one of the key components of the production of desired end products from microalgae. Several studies have indicated that due to high energy demand and capital expense, it makes up 20-30% of the overall cost of production (Tan et al., 2020). In addition, all harvesting methods seek to eliminate as much culture media from the biomass of the microalgae to enable more downstream processing, such as bioactive compound extraction (Bagchi et al., 2019). In order to collect biomass, various harvesting techniques have been used, including filtration, flocculation, centrifugation, and flotation.

2.8.1 Centrifugation

In microalgae biomass recovery, centrifugation is a widely used process in which centrifugal force is used to isolate the broth. This method is therefore quick, often preferable over gravitational sedimentation and provides a high rate of recovery of biomass of up to 95% under control conditions. In addition, centrifugation is possible for all strains of microalgae, leading to the fact that the device is easy to clean and has a low chance of biomass bacterial contamination (Yin et al., 2020). Centrifugation is more acceptable to be used for retrieval of high-value goods due to its hygienic activity, which will provide high turn around on those good benefit. The risk of cell damage due to high shear forces which will cause the release of intracellular microalgae materials into culture broth is another drawback of this process (Tan et al., 2018). In general, for algal biomass harvesting, centrifugation requires 1 MJ/kg energy. For example, for the harvesting of pig waste algae, 1.4 kWh/m^3 of energy is needed, while 1 kWh/m^3 of energy is needed for the harvesting of *Scenedesmus* sp. (Mathimani and Mallick, 2018).

2.8.2 Filtration

The filtration system uses a semi-permeable membrane that can hold microalgae on the membrane by enabling the liquid media to move through, leaving behind the collection of algae biomass. This method can extract high cell concentrations from the medium, and the varying pore size of the membrane filter allows the device to match the extract contained. For example, *Coelastrum* sp. and *Spirulina* sp (Suparmaniam et al., 2019). The need for various microalgae and the more fragile species that are vulnerable to damage due to shearing can be handled. This technique, however, is very susceptible

to degradation and clogging and therefore involves regular adjustments to the new filter or membrane that may be making a big contribution to the cost of processing (Tan et al., 2018). Conversely, due to its low energy consumption and expense and no addition of chemicals, membrane micro-filtration and ultra-filtration are ideal for fragile cell harvesting and the small-scale development method of microalgae. Before processing the water, micro strainers are commonly used to isolate algae from the culture medium. The mesh size range in general is 15-64 um, allowing only very fine particles to pass through the mesh. As stated, for many microalgal strains such as *Chlorella* sp. and *Cyclotella* sp., micro-filtration is mainly used for extracting delicate, smaller algal cells with a pore size of 0.1–10 mm (Yin et al., 2020). For larger cell sizes, macro-filtration is better suited and is mainly used to filter flocs (biomass collected by flocculation). A promising candidate for the recovery of fragile algal cells could be ultra-filtration. However, due to its greater operational and maintenance costs, intense power consumption and periodic membrane replacement, this technique has not been commonly used in the harvesting process of microalgae. For instance, to generate 6% dry microalgal slurry, 0.4 kWh/m^3 energy is needed (Mathimani and Mallick, 2018). In the dewatering of microalgae, filtration has been commonly used. However, maintenance costs can be raised by the fouling/clogging phenomenon.

2.8.3 Flocculation

Flocculation is a mechanism in which cells of free floating unicellular microalgae accumulate to form a larger particle known as floc by adding flocculating agent to suppress the cells' surface charge. It is possible to divide flocculating agents into two major groups, namely chemical flocculants and bio-flocculants. In industry, low-cost and highly usable chemical flocculants such as iron and aluminium salts have been widely used. The metal salts, such as aluminium, are sulfate and iron chloride will extract 95% of the biomass of microalgae in a standard state (Chen et al., 2018). However, because of their high toxicity, the chemicals are not eco-friendly and they must be eliminated through additional treatment procedures that add to the production cost. The definition bioflocculation is typically intended to illustrate flocculation due to the secretion of biopolymers, in particular extracellular polymeric agents (EPS) or gamma-glutamicacids, preventing the insertion of chemical flocculants. Bioflocculation of microalgae relies on the high concentration of bacteria producing EPS and the ability to bind

microalgae to their surface to form flocks. A research by Nguyen et al., (2019) showed 92.0 ± 6.0% flocculating operation and 88.0 ± 2.2% nutrient removal by *Chlorella vulgaris* during bioflocculation and untreated seafood wastewater cultivation due to bacterial attachment to microalgae cells to form bioflocs. The findings also showed that the presence of bacteria helped facilitate the creation of the phase of biofloculation (Ummalyma et al., 2017).

2.9 Lipid extraction from microalgae

The efficiency of methods of cell disruption depends on the strain/species of microalgae and on the cell membrane composition and morphology. In order to break down the cell membrane, there are two techniques: mechanical and non-mechanical (Menagazzo and Fonseca, 2019). The mechanical methods are ultrasonic, high-pressure homogenization, grinding, ball mill, microwave, whereas the non-mechanical methods are osmotic shock, chemical degradation, and enzymes. Mechanical methods have the benefit of being quick and monitorable for industrial scale-up. Their energy consumption, however, is high (Sati et al., 2019).

2.9.1 Ultrasonication

This technique involves sound waves that pass through a liquid medium creating compression and rarefaction zones, resulting in cavitation, of bubble formation in the liquid medium as shown in Figure 2.2. The low pressure within the bubbles causes them to vigorously burst, releasing vast quantities of energy (Tan et al., 2020). This induces pressure and temperature fluctuations in the liquid medium and induces hot spots. There is ample energy for the falling bubbles to crack the cell walls of microalgae that produce micro jets that cause the content of the cell to solubilize. As the sound wave propagates in the culture medium at a frequency above the standard human hearing range (> 20 kHz), it produces repeated patterns of compression (high pressure regions) and rarefaction (low pressure regions). Microbubbles are produced in the rarefaction regions as a result of decreased pressure and ultimately collapse, emitting a shock wave (Anto et al., 2020). This contributes to cytoplasmic destruction, which divides the open cell bodies of microalgae. Using ultrasound-assisted technology, an improvement in oil yield by 50-500% can be achieved along with a 10-fold reduction in extraction time. Drira et al.,

(2017) demonstrated that the microalgae with rigid cell wall (*Chrollera* sp.) could produce lipids more easily than species that has flexible cell wall.

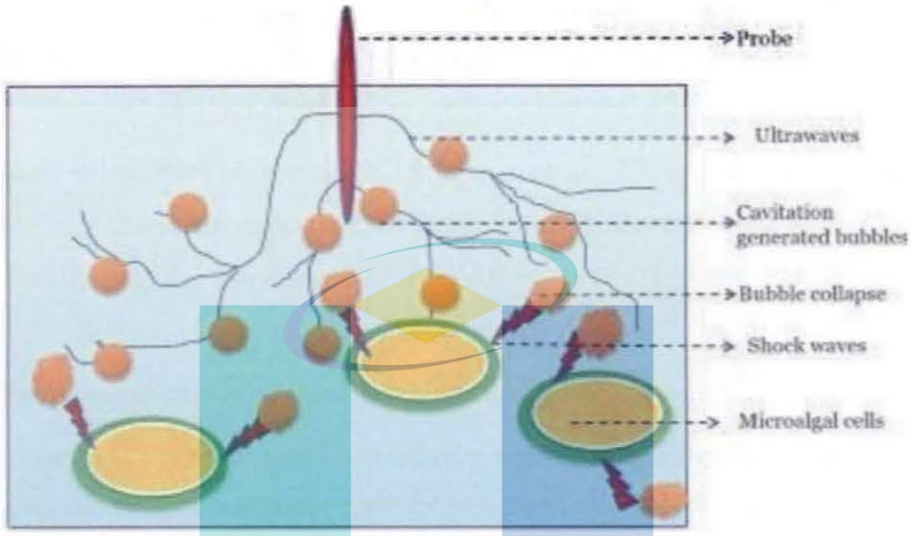


Figure 2.2 Working principle of ultrasonication for lipid extraction

2.9.2 Soxhlet extraction

The extraction of Soxhlet is a conventional method used for the extraction of lipids and carotenoids. It is carried out by using solvents at boiling temperature and atmospheric pressure, and it provides high yields and does not impact the bioactivity of the extracted molecules, even though it requires high quantities of solvents and a long extraction time (Imbimbo et al., 2019). Yusuff (2019) studied oil extraction from the green microalgae *Chlorophyta* sp. by Soxhlet. The extraction was carried out using n-hexane to ether (4:1) mixture and resulted in a production of 18.3 ± 0.4% (w/w dry biomass). In order to extract lipids, Kanda and colleagues used two separate micro-algae strains: *Chaetoceros gracilis* and *Pleurochrysis carterae*. The extractions were conducted using pure n-hexane by Soxhlet. The obtained data were *Chaetoceros gracilis* (12.3%) and *Pleurochrysis carterae* (7.5%) of dry biomass, respectively (Kanda et al., 2020).

2.9.3 Bligh and Dyer (1959) extraction

This technique requires usage of solvents such as methanol and chloroform (2:1) along with water as co-solvent for extraction and purification of lipid. A non-polar solvent dissolves neutral lipids, while a polar organic solvent dissolves cell membrane-related polar lipids (Imbimbo et al., 2020). Although n-hexane has been widely used as co-

solvent in Bligh and Dyer method but methanol has been reported had produced a higher lipid yield (Sajjadi et al., 2018). It is a quick and effective operation, thus becoming a standard method for the determination of lipid content in microalgae cells. Bonfanti et al., (2018) conducted lipid extraction using Bligh and Dyer (1959) method on *Isochrysis galbana* while the obtained data was 25.3 ± 0.2% (w/w of dry cell weight). Rasouli et al., 2018 extracted approximately 30 percent of *Chlorella sorokiniana* lipids, a value close to that recorded using the Folch method (Schipper et al., 2019), indicating that when the same strain and the same experimental protocol are followed, both methodologies are capable of extracting the same amount of lipids.

2.10 Transesterification of microalgae oil

Microalgae crude oil must be treated properly in order to turn them into suitable and commercially viable biodiesel fuel. Due to its high viscosity, free fatty acid content, low volatility, and gum formation during storage and combustion, direct use of any kind of raw oil as fuel in diesel engines is not suitable (Athar and Manaf, 2020). The high viscosity causes problems with fuel atomization and other serious problems, such as engine components and trapping of piston rings etc (Deshmukh et al., 2019). The three well-known methods for biodiesel synthesis, namely microemulsion, thermal cracking and transesterification, were studied by numerous researchers. However, transesterification method is the most suitable way of converting microalgae crude oil into biodiesel (Anto et al., 2020). Transesterification often referred as alcoholysis where the reaction done with or without catalyst to form biodiesel (FAME) and glycerol to a triglyceride present in fat or oil with alcohol as illustrated in Figure 1.

As the mechanism is reversible, it takes an excess amount of alcohol to move the equilibrium reaction in the forward direction (Yin et al., 2020). As the most reactive groups, triglycerides are composed of one glycerol [$C_3H_5(OH)_3$] and three fatty acids (R-COOH), indicating that fatty acids most influence the oil and fat characteristics (Chen et al., 2018). Triglycerides are transformed into diglycerides in the first phase of the transesterification reaction, followed by the next reaction where diglycerides converted into monoglycerides and finally to glycerol, at every step, one alkyl ester molecule from each glyceride attained (Figure 2). The low cost solvent methanol act as esterification reagent in this reaction (Kanda et al., 2020). For example, compared to those obtained from ethanol, biodiesel from methanol has slightly greater boiling and melting points and

slightly lower viscosities. Moreover, methanol also does not form an azeotrope and is convenient to recycle, but with water, ethanol forms an azeotrope.

Transesterification from crude oil microalgae can be done with or without the presence of catalyst. There are three types of catalyst that can be utilized in this reaction alkaline, acidic and enzymatic (Sajjadi et al., 2018). According to the solubility of the catalyst in the reactant, the transesterification reaction can be homogeneously or heterogeneously catalysed such as sulphuric acid, KOH or NaOH etc. Based on their solubility in the reactants, both acidic and alkaline catalysts can either be homogeneous or heterogeneous (Ramesh et al., 2019). Depending on the free fatty acids (FFA) content of the oil, reactions can take place either in a single stage with an acid or basic catalyst or in two stages with both acid and basic catalysts. Esterification of FFA with the assistance of alcohol in the presence of acidic catalysts occurs in the first stage, resulting in the formation of biodiesel and water (Pal et al., 2019). In the second step, in the presence of an alkaline catalyst, triglyceride transesterification occurs with methanol.

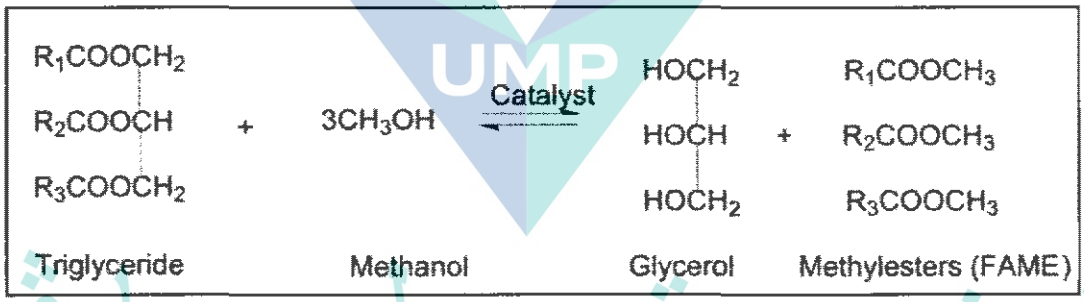


Figure 2.3 Overall equation of transesterification

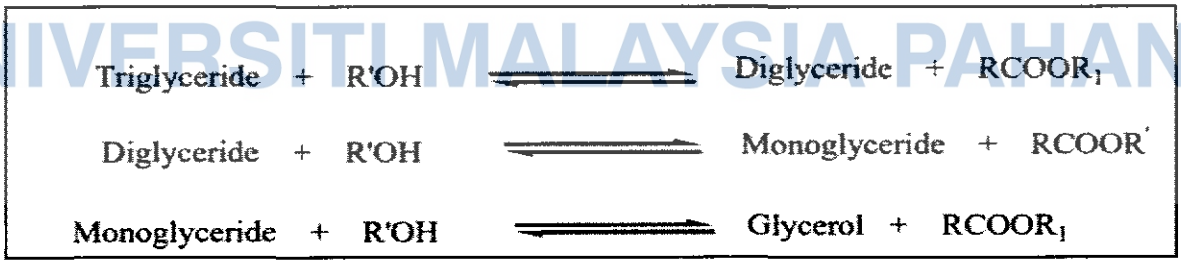


Figure 2.4 Stepwise reactions of transesterification of triglyceride

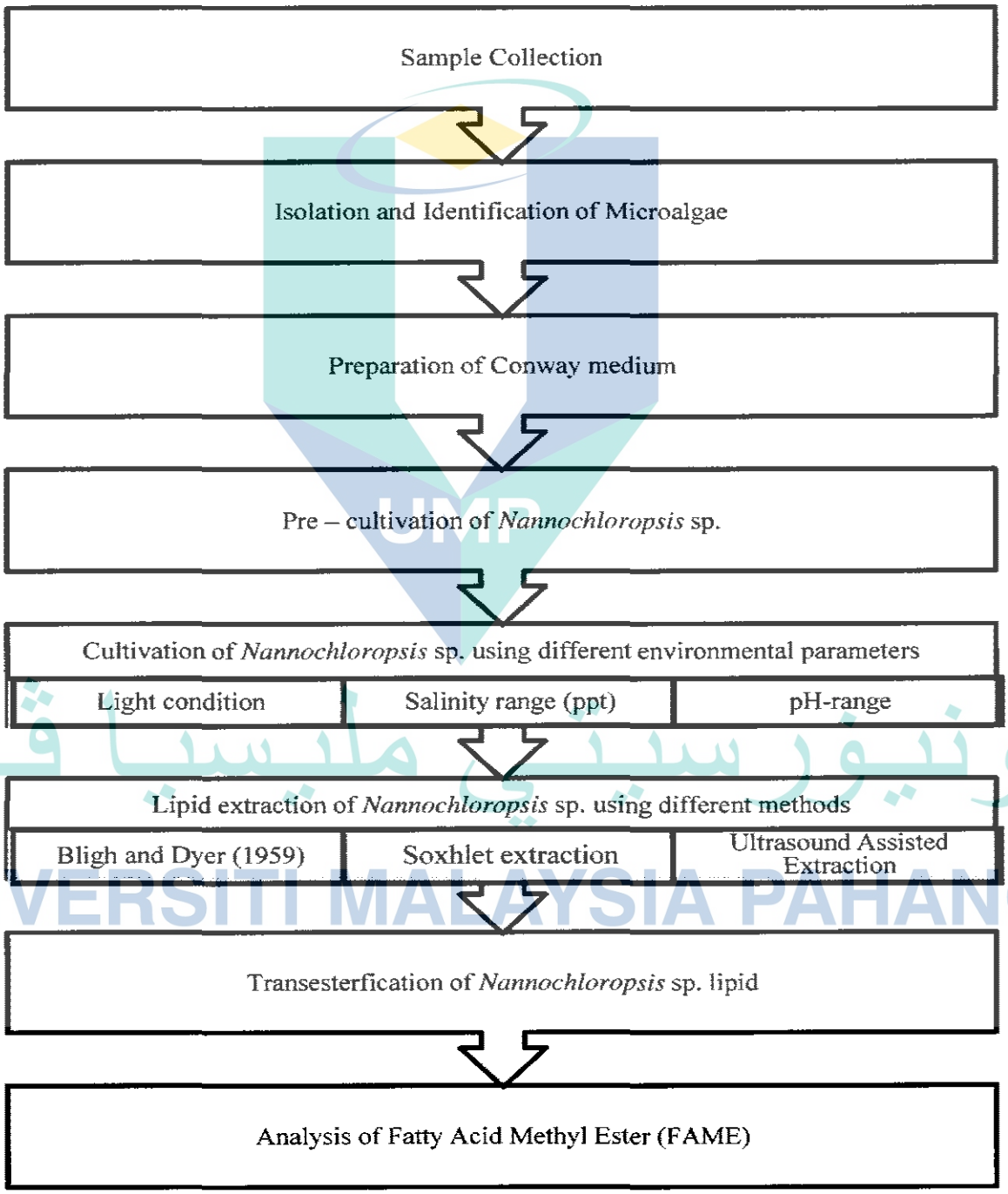
2.11 Lipid composition of microalgae

A key that determines the suitability of microalgae as a biofuel feedstock is the amount and ratio of saturated and unsaturated fatty acids. In general, unsaturated fatty acids, in particular palmitolleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and palmitic acid saturated fatty acids (16:0), are the main components of microalgae-generated oil with a limited proportion of saturated stearic acid fatty acids (18:0). In *Aurantiochytrium* sp., C22:5 + C22:6 (39.4%) in *Schizochytrium limacinum*, C20:5 (25%) in *Porphyridium cruentum*, some microalgae may also synthesise a large number of polyunsaturated fatty acids such as C22:6 (42%) (Sajjadi et al., 2018). The FAME properties derived from microalgae strongly depend on the composition of the fatty acids of the microalgae. For instance, since long-chain saturated fatty esters dramatically increase the boiling and melting point of biodiesel, thebiodiesel produced from the microalgae fat with lower saturated fatty acid content presents better cold temperature properties (Suparmaniam et al., 2019).

Biodiesel containing a large amount of unsaturated compounds, however, is oxidised more easily than standard diesel, resulting in insoluble sediments interfering with the performance of the engine (Goh et al., 2019). Therefore, when searching for effective microalgae strains for biofuel production, consideration should be given to the ability of microalgae to produce a high amount of lipid and a high quality of fatty acid composition (Tan et al., 2020). In addition, most species of microalgae are capable of surviving under extreme conditions where their growth rate, lipid content and composition of fatty acids are significantly affected.

CHAPTER 3

METHODOLOGY



3.1 Materials

Microalgae sample was collected from Pantai Teluk Cempedak, Kuantan coast, Pahang. Potassium hydroxide (KOH), 99% methanol and 99% *n*-hexane was purchased from Merck, Germany. Sodium chloride (NaCl), sodium nitrate ($NaNO_3$), zinc sulphate ($ZnSO_4$), copper sulphate ($CuSO_4$), concentrated HCL procured from Sigma Aldrich. All the chemicals mentioned above is analytical grade. The TLC aluminium sheets was purchased from Merck. MEGAWAX column was procured from MEGA, Italy. Plankton net was purchased from LabFriend Malaysia.

3.2 Sampling site description

The sample for this research project was collected from Pantai Teluk Cempedak, Kuantan Coastal Region, an estuary in Pahang state of Peninsular Malaysia between the latitude of 3°55'31"N and longitude of 103°22'23"E. It drives 13 north of Kuantan town, passing the fishing village of Beserah. Approximately 26.7km from Universiti Malaysia Pahang (UMP) to Pantai Teluk Cempedak. Figure 3.1 and Figure 3.2 had shown the Pahang state map and Pantai Teluk Cempedak for a better clarification.

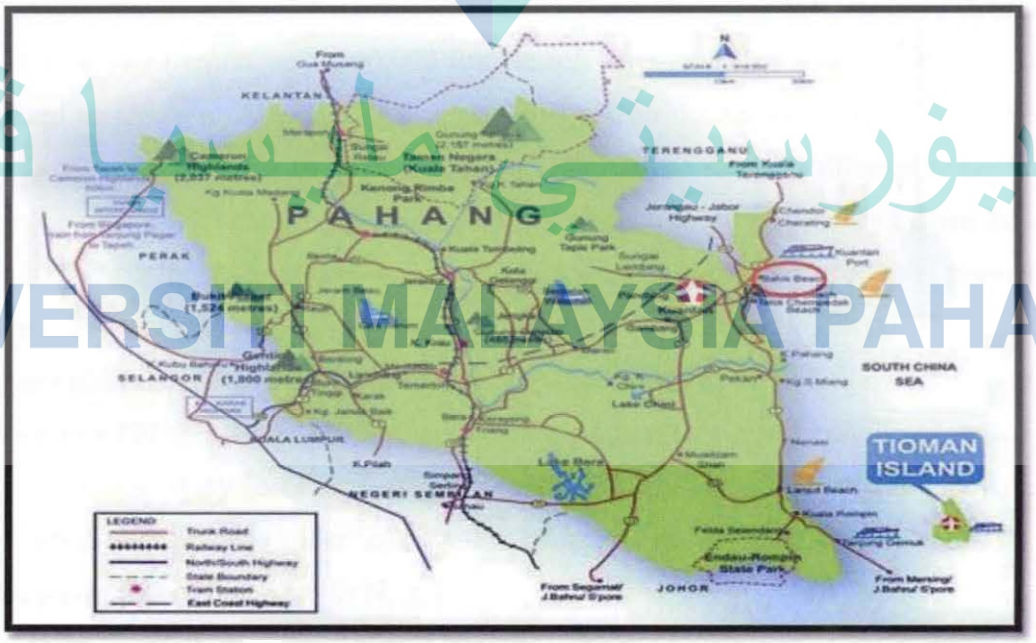


Figure 3.1 The Pahang map of Teluk Cempedak region

Source: Wikipedia, (2009)

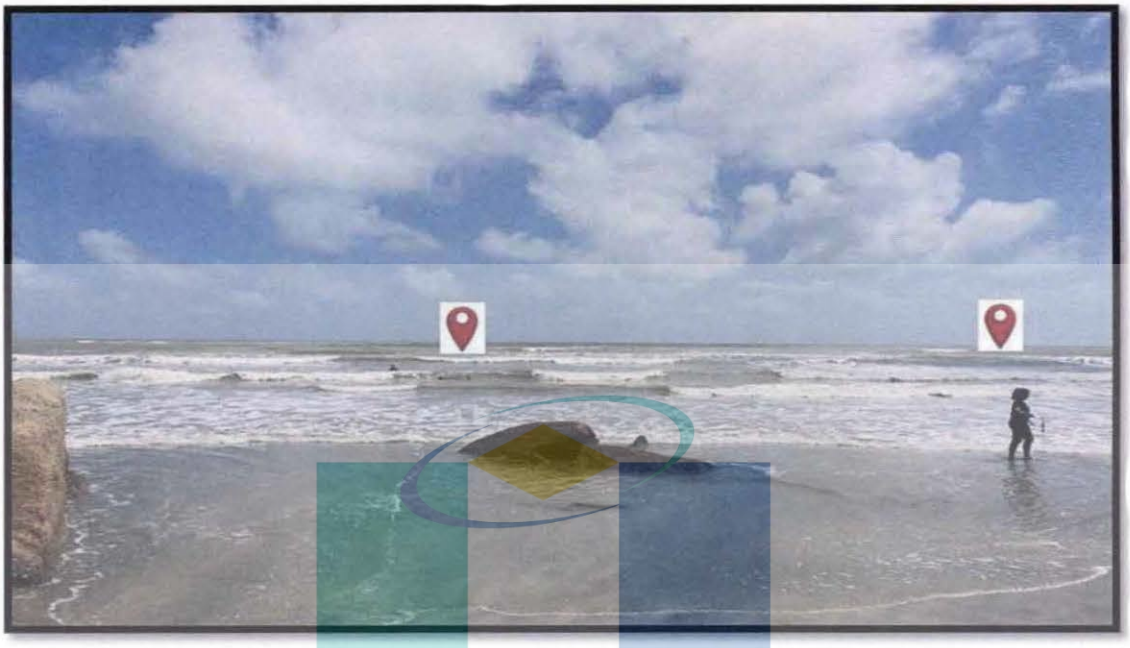


Figure 3.2 The sample collection area Pantai Teluk Cempedak, Kuantan

3.3 Sample Collection

The sample collection was carried out during midday period where the potential source areas of microalgae were exposed to sunlight at the maximum light intensity. The sample collection was made in three different location based on the depth of seawater. Meanwhile, the distance from each location from another was 5 meters. The natural light intensity able to reach up to 60,000 lux during afternoon. Microalgae which is more attracted to photoautotrophic mode appear to float on the surface region of in order to absorb energy from light for photosynthesis. Microalgae sample collected using 0.5µm plankton net which is made up by the bolting silk cloth as depicted in Figure 3.3. The collected algae samples added with few drops of 5% formalin in order to prevent the action of protozoan engulfing the pytoplanktons (Wainright, 1987). The collected samples placed in bottles and centrifuge tubes respectively, for isolation purpose at room temperature (37°C ± 2). All the bottles and centrifuge tubes were labelled and sealed with parafilm respectively in order to avoid contamination. The collected samples taken to laboratory and placed under continuous illumination of 20,000 lux by 50W cool fluorescent type of lamps at 28 °C ± 2.

Figure 3.3 Plankton trawl net

Source: Wikipedia, (2013)

3.4 Isolation and Identification of Microalgae

Standard plating methods were used to separate algal populations in order to isolate single microalgal organisms from the sampling site. To separate the microalgae colonies, several media compositions were utilized such as BG11 medium (Stanier et al., 1971), Walnes/Conway (Walne, 1970) and basal medium. The collected samples were diluted (1×10^{-5}) accordingly to aid the isolation process. To plate these diluted samples, sterilised plastic petri dishes (100 × 15 mm) containing approximately 40 mL of solid agar medium were used. 1mL of the diluted sample was transferred and distributed uniformly over the surface to a media plate. The inoculated samples were placed in a controlled temperature (25±2°C) with continuous exposure of 12,000 lux by cool fluorescent lamps for 14 days (Govindan et al., 2019). The inoculated samples were monitored daily to fix the suitable medium for isolation along with microalgae growth curve. The grown algae colonies were streaked aseptically on additional sets of nutrient media and placed back into the clean culture room respectively. This method of streaking was repeated until isolation into axenic cultures of unialgal was achieved. The number of colonies that were shifted to other nutrient media plates from each dilution plate depending on the amount of contamination and the identification of the colonies present based on the morphology of the colony and each isolate's microscopic cellular morphology (Kanda et al., 2020). The grown microalgae cultures were examined morphologically using fluorescence microscope OLYMPUS, BX 53 and Field Emission

Scanning Electron Microscope (FESEM) using a Joel, USA JSM-7008 FESEM from Central Laboratory, Univeristi Malaysia Pahang.

3.5 Preparation of Conway medium

Conway medium preparation includes the preparation of macronutrient, trace metal and vitamin solutions for stock solutions. The chemical compositions dissolved in sterile distilled water, in order to prepare stock solutions. Table 3.1 showed composition of Conway medium. 1mL of solution A (macronutrient), 0.5mL of solution B (trace element), 0.1mL of solution C (vitamins) were transferred to 1000mL of filtered and sterilised seawater (30ppt).

Table 3.1 Composition of Conway medium

Stock	No.	Substances	Amount
I	Solution A (per liter distilled water)		
		$MnCl_2.4H_2O$	0.36g
		H_3BO_3	33.6g
		EDTA	45g
		$NaH_2PO_4.2H_2O$	20g
		$NaNO_3$	10g
		$FeCl_3.6H_2O$	1.3g
II	Solution B (per 100mL distilled water)		
		$ZnCl_2$	2.1g
		$CoCl_2.6H_2O$	2g
		$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9g
		$CuSO_4.5H_2O$	2g
		Concentrated HCL	10ml
III	Solution C (per 200mL distilled water)		
		Vitamin B1	0.2g
		Vitamin B12	10mg
	Solution A		1mL/L
	Solution B		0.1 mL/L
	Solution C		100µL
	Sterile Seawater (30ppt)		1L

Source: Walnes, (1970)

3.6 Pre – cultivation of *Nannochloropsis* sp.

The identified *Nannochloropsis* sp. was pre-cultivated in 100 mL of Conway medium with adjusted pH of 7.5 in a 250 mL Erlenmeyer flask on a rotary shaker at 100 rpm constantly until it reaches stationary growth phase. They were cultivated until obvious signs of algae growth were seen, especially the full green flask. The pure culture then scaled up to 2L Erlenmeyer flask containing 90% (v/v) of *Nannochloropsis* sp. culture with 10% (v/v) of Conway medium to 1L. *Nannochloropsis* sp. cultured under the light intensity of 15 µmol m^{-2} s^{-2} along with sterile aeration in order to avoid the biomass from settling and causing accretion. The cultured medium was also swirled manually five to six times a day. The culture was examined 2-3 weeks approximately regularly for microalgae growth curves (Show et al., 2017).

3.7 Cultivation of *Nannochloropsis* sp. under growth parameters

The growth of microalgae is highly dependent on stress factors, and variables in the state of cultivation vary from one species to another. The pure *Nannochloropsis* sp. culture was optimised with three different variables include light, salinity and pH in order to determine the best growth for biodiesel production.

3.7.1 Cultivation of *Nannochloropsis* sp. under different light condition

The effect of light in two different conditions was applied, photoautotrophic and heterotrophic respectively. As for heterotrophic condition, the cultures of *Nannochloropsis* sp. was kept at 18:6 light/dark days (Wahiddin et al., 2013). Meanwhile, photoautotrophic cultures were placed under continues exposure for 24 hours of 35µmol photons m^{-2} s^{-1} under fluorescent light. The cultures were maintained and aerated continuously at 25 ± 2°C.

3.7.2 Cultivation of *Nannochloropsis* sp. under different salinity

NaCl has been used as a source for salinity effect on *Nannochloropsis* sp. 20, 30 and 40ppt was studied in this research by adding in Conway medium composition in order to determine the potential concentration in producing biomass and lipids. Each culture condition was studied with continuous illumination of 35µmol photons m^{-2} s^{-1} under a standard laboratory temperature of 25°C ± 2°C.

3.7.3 Cultivation of *Nannochloropsis* sp. under different pH

pH is one of the crucial factor as it decides the solubility and availability of CO_2 along with other nutrients. The pH ranges were adjusted using 1M of HCL and 1M NaOH. The specific growth rate and lipid content was further being studied using various pH range 5, 6, 7, 8 in order to determine the suitable range of pH for *Nannochloropsis* sp. Continuous exposure of 35 µmol photons m^{-2} s^{-1} and the temperature was constantly maintained at 25°C ± 2°C with regular aeration.

3.8 Mass Cultivation of *Nannochloropsis* sp. using 10 L photobioreactor

The photobioreactor (PBR) was disinfected using sterile deionized water that contains 0.04% NaOCl and 0.2% NaOH and the following steps with three washes of sterile deionized water leaving it for 24 hours with strong aeration. The total volume of the photobioreactor, which was made of 0.6 mm thick glass material, is 10 L. The PBR were placed in clean culture room where six series of white cool light-emitting diode (LED) lamps were attached to the outer wall of the photobioreactor as shown in Figure 3.4. *Nannochloropsis* sp. pure culture with optimised stress factor of best growth which was salinity 30ppt, adjusted pH of 8 along with photoautotrophic condition was further transferred from 2L Erlenmeyer flask to photobioreactor. The algal culture supplemented with sterile aeration which enables uniform nutrient dissolution and light penetration along with efficient gaseous exchange in the photobioreactor. A 20 µmol m^{-2} s^{-1} of light intensity exposed for 24 hours at constant temperature of 25± 2°C for two weeks. The culture were routinely monitored by withdrawing 1mL of culture to determine the specific growth curve of *Nannochloropsis* sp using Genesys 10S UV-Vis spectrophotometer, Thermo Fisher Scientific at 680nm wavelength.

Figure 3.4 Photobioreactor with 5L of *Nannochloropsis* sp. culture

3.9 Determination of Dry Cell Weight (DCW) and Growth Rate

The dry cell mass was determined gravimetrically. 100 mL of microalgae culture was collected routinely and centrifuged (9000g, 10min). The pellet was recovered and rinsed twice by resuspending in distilled water and retrieved again by centrifugation. The wet biomass was then dried at 60 °C for 3 hours in a vacuum drier and weighed. The pellet's measured mass and the culture sample's initial volume were used to determine the original sample's dry biomass concentration. For plotting the growth curves, the dry cell mass concentration was estimated by measuring the optical density of a culture sample diluted with fresh medium at 680nm optical density (Govindan et al., 2019).

3.10 Harvesting of Microalgae Biomass

Like other microorganism microalgae experience four growth phases: lag, exponential (development), stationary and phase of death or lysis. They convert photonic energy, water and CO_2 to sugars whereas sugars are converted to macromolecules such as Triacylglycerols (TAG) (Moazami et al., 2012). After the microalgae culture has moved into the stationary phase, the microalgae separated from water in order to obtain their biomass by undergoing downstream process. 50 mL of microalgae culture added

into centrifuge tube and centrifuged at 5000 rpm for 5 min. After centrifuge, the supernatant removed and the remaining pellet placed in -80°C freezer for 5 days to reduce the moisture content to 0%. The freezed microalgae pellet transferred to freeze drier in order to attain powder form (Chowdhury et al., 2019).

The lyophilized microalgae biomass was then pulverized using pestle and mortar in order to reduce the particle size as shown in Figure 3.5 which was captivated during the pulverization of biomass.

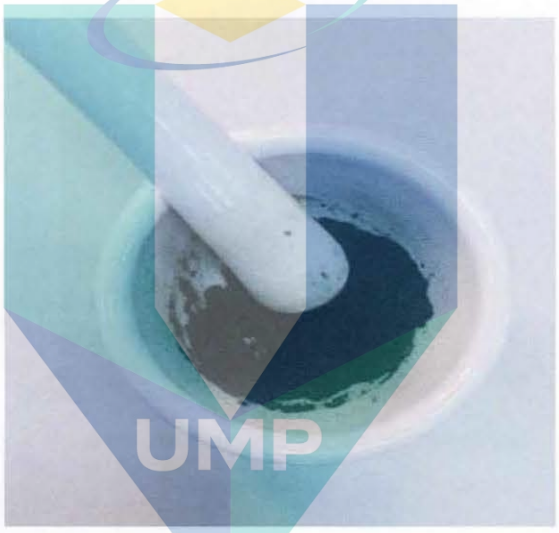


Figure 3.5 Ground biomass of *Nannochloropsis* sp.

3.11 Microalgae lipid extraction methods

3.11.1 Soxhlet Extraction Method

The obtained dried powder from freeze dried microalgal biomass was extracted using soxhlet method. Soxhlet method utilizes the non-polar solvent *n*-hexane to perform the lipid extraction of *Nannochloropsis* sp. In order to allow quantification of the lipid extract, the hexane phase from each extraction was collected in a pre-weighed flask before it was heated to oven dryness (80°C). Approximately 5g of biomass sample was weighed in a cellulose thimble with 3-5 small rocks (to improve the drainage). The mixture was covered with cotton wool (to help in solvent drainage). The packed thimble was soaked overnight in *n*-hexane for cell distruption where the cell wall tends to break and easily release the lipid from intracellular. This process aid the lipid extraction. The overnight soaked thimble with biomass was then placed in a chamber of Soxhlet unit. Meanwhile,

the whole set up was placed on a heating mantle and allowed the *n*-hexane to boil at 60°C as shown in Figure 3.6, to extract the lipid for 7.5 h at the rate of 10 refluxes per hour, pure *n*-hexane (300 mL) was used. The mixture was then subjected to rotary evaporator for solvent separation. The extracted lipid was calculated by the following formulae (3.1).

Figure 3.6 Microalgae lipid extraction using Soxhlet method

$$\text{Lipid yield} = \frac{\text{Lipid extract weight}}{\text{Total biomass weight}} \times 100\% \qquad 3.1$$

3.11.2 Bligh and Dyer extraction

The lipids were extracted using the Bligh and Dyer (1959) method from freeze-dried biomass, as previously used for lipid recovery from various microalgae (Chisti, 2017). In short, 0.2 g of freeze-dried biomass was applied to a solvent mixture of chloroform (1 mL), methanol (2 mL), and deionized water (0.8 mL). The resulting slurry was combined with the vortex for 2 min and allowed to rest at room temperature for 4 h. Then, chloroform (1 mL) was included and the vortex mixture was mixed for 30 seconds. Deionized water (1 mL) was included and the vortex was mixed for 30 s with the suspension. The resulting suspension (4150 g, 10 min) was centrifuged and allowed to break into three layers. Methanol and water from the top layer was thrown away. There was a recovery of the chloroform layer (the third layer from the top). Twice as much of the remaining biomass was removed using 100mL micropipette, Eppendorf. Total lipids

were measured gravimetrically in a combined chloroform extract by evaporating the extract (50 °C) in a pre-weighed aluminium dish in a clean fume hood (Govindan et al., 2019).

3.11.3 Ultrasonic Extraction

Approximately 5g of microalgae biomass was weighed and n-hexane: methanol (2:1, v/v) was added in 50mL falcon tube. The mixture was vortex for 20 sec. The sonication was executed using a Sonics Vibra. The vortexed mixture was then placed in ultrasonic bath for 1h at 60°C ± 5°C of 20 kHz (to make the small bubbles to collapse). During cavitation, high pressure and high-speed liquid jets shape shearing forces around the algae cells and mechanically split the cell structure and enhance the transfer of material promoting lipid extraction. After 1h, to protect against overheating, the samples were kept in an ice bath for 5min. The mixture was then centrifuged at 5000rpm for 10 min. The upper layer of the mixture was collected for transesterification as shown in Figure 3.7.

Figure 3.7 Extracted crude oil of *Nannochloropsis* sp.

3.12 Transesterfication of *Nannochloropsis* sp. lipid

Approximately 5g of microalgae oil was weighed and added with 1% potassium hydroxide (KOH) catalyst with the volume ratio of 0.5:1 of MeOH/oil. The mixture was then vortex for 15 sec and placed it in ultrasonic bath at 20 kHz, 40°C for 1.5 hours. The two layers was formed in falcon tube and the upper layer collected. The collected upper

layer was transferred to 2ml centrifuge tube. The sample was pre-weighed before placed in centrifuged. The collected upper layer sticky solution placed in fume hood for 6h to allow the excess methanol to evaporate (Bhuyar et al., 2020).

3.13 Analysis of Fatty Acid Methyl Ester (FAME)

3.13.1 Qualitative Analysis of FAME

Thin layer chromatography (TLC) analysis was conducted using TLC plates from Merck, in accordance with the protocols defined by (Chen et al., 2020). TLC analysis is done to ensure the transesterfication is complete by converting the lipid into FAME. The TLC analysis was conducted on an aluminium sheet coated with silica gel (20 cm × 20 cm, silica gel 60 F254) were purchased from MERCK (Germany). A mixture of solvent of chloroform and petroleum ether (3:2) was used to mix the solvent and iodine vapour was used for visualization of spots. The R_f value was calculated as the following formulae 3.2.

$$Rf\ value = \frac{Distance\ travelled\ by\ solute}{Distance\ travelled\ by\ solvent} \quad 3.2$$

3.13.2 Quantitative analysis of FAME

Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine the FAME. GC-MS is utilized for sample information based on the Mass Spectrometry library database. Separation of lipid components in *Nannochloropsis* sp. were carried out using capillary column Mega-Wax MS (length 30m x diameter 0.32mm x film thickness 0.50μm) and the pressure was 14.790psi. The outcome of GC-MS data is used to evaluate the methyl ester compound in the sample. 0.2mL of the transesterified oil was dissolved in 0.8mL of hexane and injected into GC-MS; Agilent7890A. The total sample injection volume was 1μL and helium used as a carrier gas. The injection temperature was set as 250 °C. The column temperature was initially 70 °C for 1 min and was raised to 270 °C at rate of 5 °C/min. The total analysis time was 54 min.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and preliminary screening of collected microalgae samples

Around twelve microalgae species had been collected from Teluk Cempedak Coast, Kuantan for further morphology identification. The green algal growth was seen in the medium of Conway within 10-15 day after inoculation. In order to isolate those collected microalgae species plating method were conducted respectively. The twelve isolated species were examined under fluorescence microscope. Among the collected and isolated marine microalgae include green algae *Nannochloropsis* sp., *Tetraselmis* sp., *Chrollera* sp., *Spirulina* sp., and diatoms *Chaetoceros* sp., *Amphora* sp., *Amphiphora* sp., *Navicula* sp., *Nitzshia* sp., *Gyrosigma* sp., *Neidium* sp., *Cyclotella* sp., from seawater while some of the microalgae struggled to adapt in standard laboratory environment and thus, those found to be dominant during cultivation are those who are sufficiently robust enough to grow further in bioreactors under controlled conditions. A similar study was conducted recently by Bhuyar et al., (2020), where the researcher successfully isolated *Chorella* sp. and cyanobacteria sp. from Teluk Cempedak Coast, Kuantan for bioremediation study.

Among the isolated strains, many strains showed poor growth upon transfer from the agar plates to liquid medium during preliminary cultivation and were therefore discarded. Only six species able survive and cultivated in the constant laboratory environment as shown in Figure 4.1. Since the cells were completely motile and homogeneously distributed across the flask, *Nannochloropsis* sp. was by far have good visibility and the highest growth observed when compared with other microalgae . In liquid medium, the *Tetraselmis* sp. isolates developed relatively slow, and this was evident by the delayed growth and very low cell numbers. Since cyanobacteria have only a low lipid content and are therefore of no potential, no cyanobacteria have been isolated into a unialgal community. During the preliminary cultivation of unicellular microalgae, the colour of cultures ranged from dark green to brown while most of the green algae demonstrated the characteristic of yellow-green coloration with exception of few brown

strains as well. Green algae (*Tetraselmis* sp., *Chorella* sp.) and diatoms were found abundantly but the growth rate was low when compared with *Nannochloropsis* sp.

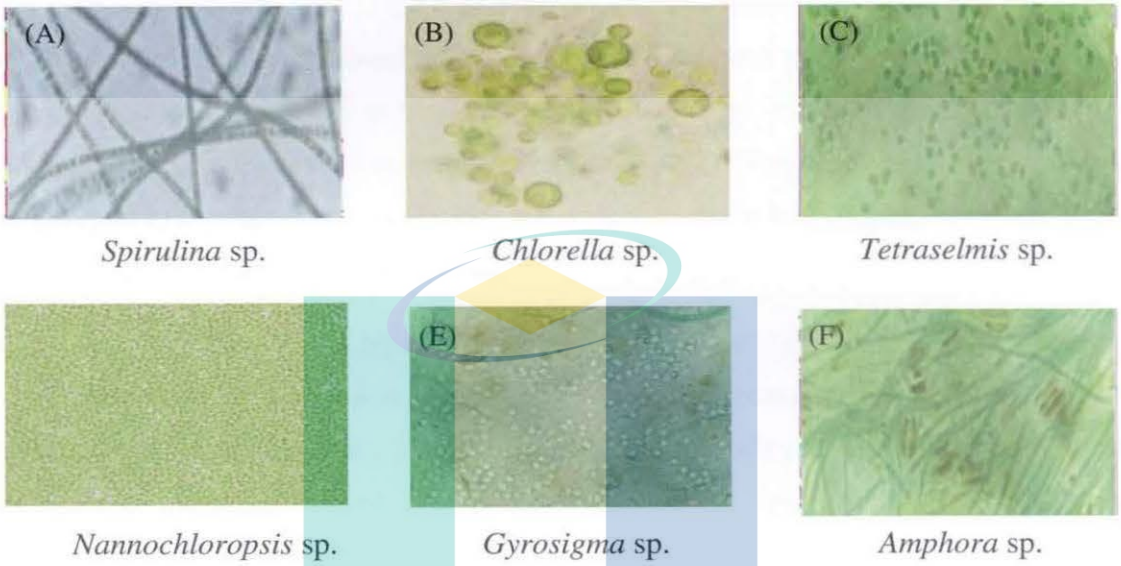


Figure 4.1 Fluoresence microscope image at 100X of six isolated species which cultivated in laboratory environment

4.2 Identification and morphological characteristic of *Nannochloropsis* sp.

The isolated species were identified by determining their morphological features. As depicted in Figure 4.2 and Figure 4.3, fluorescence microscope and FESEM species analysis revealed a single-cell structure of the isolate with spherical to oval cells along with smooth cell wall with 2-3µm diameter under stationary growth phase respectively (Ma et al., 2016). The small size of the *Nannochloropsis* sp. has been the advantage for the species itself as due to the surface ratio of smaller cells that facilitates the integration of nutrients at a significantly faster rate, they are able to grow rapidly than the larger microalgae species. The species was identified using fluorescence microscope (Lubian 1982; Hibberd 1971). *Nannochloropsis* sp. cells were yellow-green color, unicellular and free-floating. The green colour of cells indicating the chlorophyll pigments. The cell dimensions and shape of the isolate under study agree with the dimension demonstrated for other strains of *Nannochloropsis* sp. (Subasankari et al., 2020). There was a visible identification of a single parietal chloroplast in the cell. A similar study had reported that *Nannochloropsis* sp. consisted with unique structure include the lamellate vesicles present in the cytoplasm; the parietal yellow-green chloroplast; and the connection

between the chloroplast cover and the nuclear envelope (Fietz et al., 2005). Based on the observation on Figure 4.2, the morphology of *Nannochloropsis* sp. under fluorescence microscope there were small spots with different size were seen visibly. Silverberg (1976) had demonstrated that lipid present in the cytoplasm of *Nannochloropsis* sp.in the form of 30nm droplets in size. These unicellular microalgae have many pyrenoids, however, pyrenoids are not observed in this study. Researcher also indicated that pyrenoids are rarely observed in *Nannochloropsis* sp. (Yu et al., 2007). *Nannochloropsis* sp. known to be grouped in Eustigmatophyceae class are widely found in both marine environment as well as brackish water (Hu et al., 2014).

Nannochloropsis sp. reproduce through cell division and it is a valuable source of pigments with high production levels of chlorophyll a, zeaxanthin, canthaxanthin and astaxanthincan (Lubian et al., 2000). Because of their ability to develop rapidly, these algae are of industrial interest and synthesize significant quantities of TAG which are potential for biodiesel production and high-value polyunsaturated fatty acids (e.g. eicosapentaenoic acid) and withstand broad environmental and culture conditions. In earlier studies, it has been reported that *Nannochloropsis* sp. contains high amount of lipids from 31% to 68% based on the dry cell weight (Chisti, 2007). Damiani et al., (2008) and Pyle et al., (2008) have confirmed that *Nannochloropsis* sp. lipid extraction has the following composition include triglycerides: 37.74% along with non-polar hydrocarbons, isoprenoids: 8.72% and polars, glycolipids, phospholipids: 3.54%. In addition, it has been reported that *Nannochloropsis* sp. has been the best candidate for both aquaculture and biodiesel production. This is due to its unique characteristerics that conatins high nutritional value, ease of cultivation, lack of toxicity, right size of cells and digestible cell wall meet the selection criteria for both aquaculture and biodiesel production (Khatoon et al., 2014). For instance, *Nannochloropsis* sp. able to produce high amound of triglycerides, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) can be produced on their own up to 68% of the total dry cell mass (Rodolfi et al., 2009).

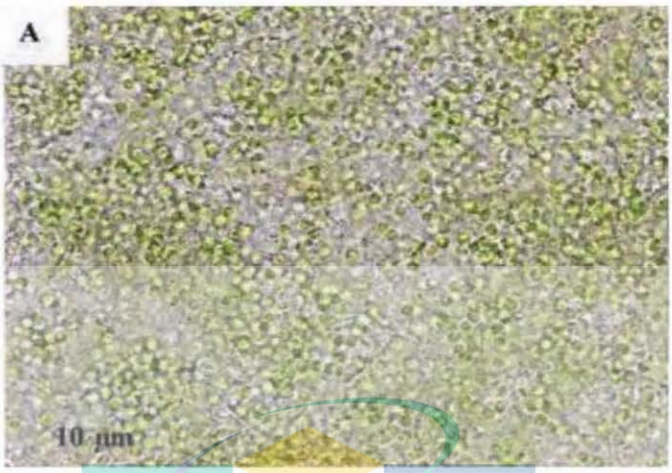


Figure 4.2 Fluorescence micrograph (A) of *Nannochloropsis* sp. under 100X

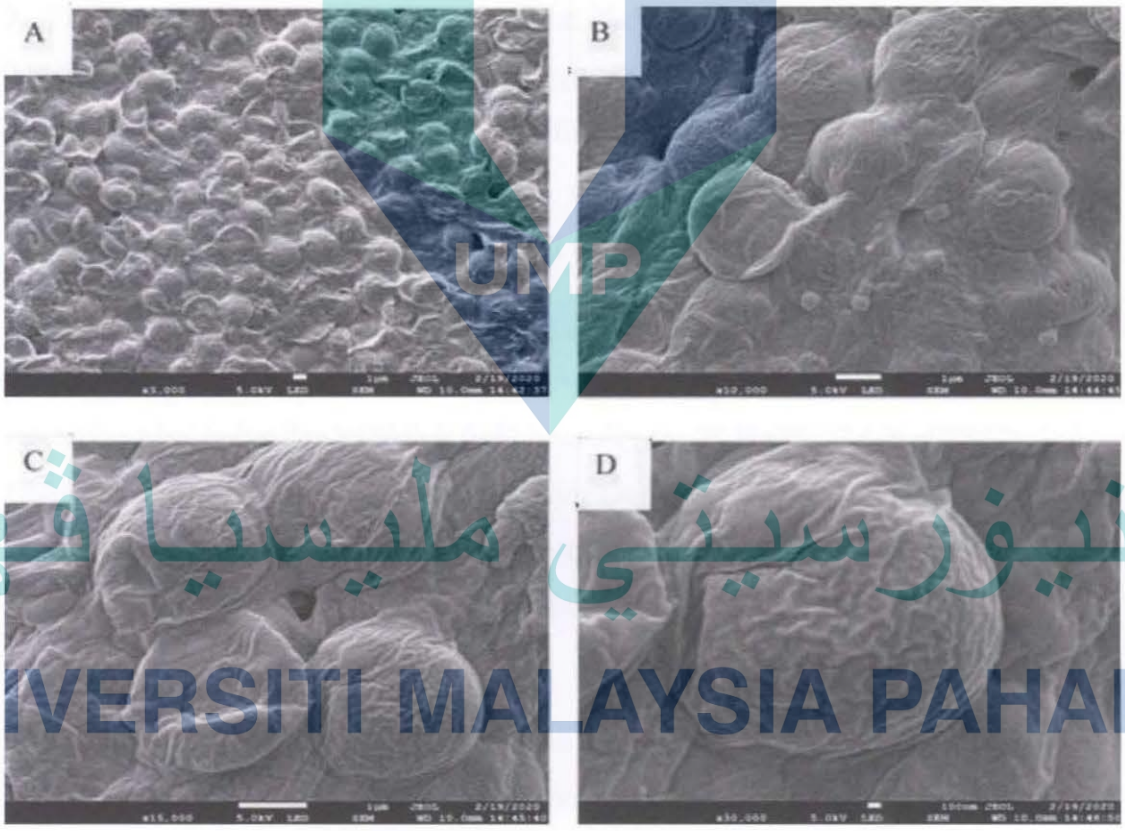


Figure 4.3 FESEM micrograph of *Nannochloropsis* sp. under 3000x (A), 10,000x (B), 15,000x (C) and 30,000x (D)

4.3 Mass cultivation of *Nannochloropsis* sp. under growth parameters

4.3.1 Mass cultivation of *Nannochloropsis* sp. under light condition

i) Effect of photoautotrophic condition

Light is the basic energy source and important factor in photosynthesis and is necessary for microalgae photoautotrophic development. Therefore, the use of sufficient light wavelength and light intensity are the main factors that can influence or even regulate the production of biomass and lipids in microalgae. After 1 day of incubation, the exponential growth process began and ended on the 15th day with a maximum dry cell weight of 0.502±0.015g L^{-1}. The total dry cell weight was harvested using freeze dried method as illustrated in section 3.10, respectively. Meanwhile, the total obtained lipid of *Nannochloropsis* sp. was 0.109±0.012g L^{-1} whereas the total lipid percentage was 21.7% for photoautotrophic condition. The *Nannochloropsis* sp. in this present study which cultivated under 100 μ mol $m^{-2}s^{-1}$ experiences better growth rate but the biomass and lipid production relatively low. These findings of this research was further supported by Wahidin et al., (2013) where the author cultivated *Nannochloropsis* sp. in photoautotrophic and the biomass and lipid production was regularly monitored.

This is possibly due to the fact that growth at a higher intensity of light has been inhibited, so it does not need an extended photoperiod cycle to be quicker. Photo inhibition may occur due to higher light intensity for 24 hours. The availability and light intensity of photosynthetic cultures are major factors influencing productivity (Sajjadi et al., 2018). Thus, *Nannochloropsis* sp. which cultivated under photoautotrophic failed to enhance the lipid production due to photo inhibition.

Light which is too strong can be stressful, leading to low biomass and lipid production as shown in Figure 4.4 when the culture incubated continuously for 24 hours in 100 μ mol $m^{-2}s^{-1}$. The rate of photosynthesis is directly proportional to the intensity of light at the illumination intensity above the light compensation limit. However, damage to the photosynthetic receptor system occurs within a few minutes at high illumination intensities, and this effect is known as photoinhibition (Wahiddin et al., 2013). This results were visibly known by the change of colour from green to brown and subsequently the microalgae growth rate was decreased from day 9. In a similar study where the

researcher noticed a severe drop in growth rate when the microalgae culture was cultivated for long photoperiods (24:0), this phenomenon occurred due to photoinhibition (Richard, 2003). As indicated in earlier studies, dry biomass production in many phytoplankton increased under high light illumination, which is the predicted high light response that usually causes an increased reproduction until the intensity of the saturation point was experienced, and photoinhibition was subsequently observed, restricting further development of biomass. This phenomenon is usually caused by photooxidation reaction within the cell as the excessive light cannot be penetrated in to the photosynthetic apparatus. Thus, different species of microalgae will react to light intensity and photoperiod respectively.

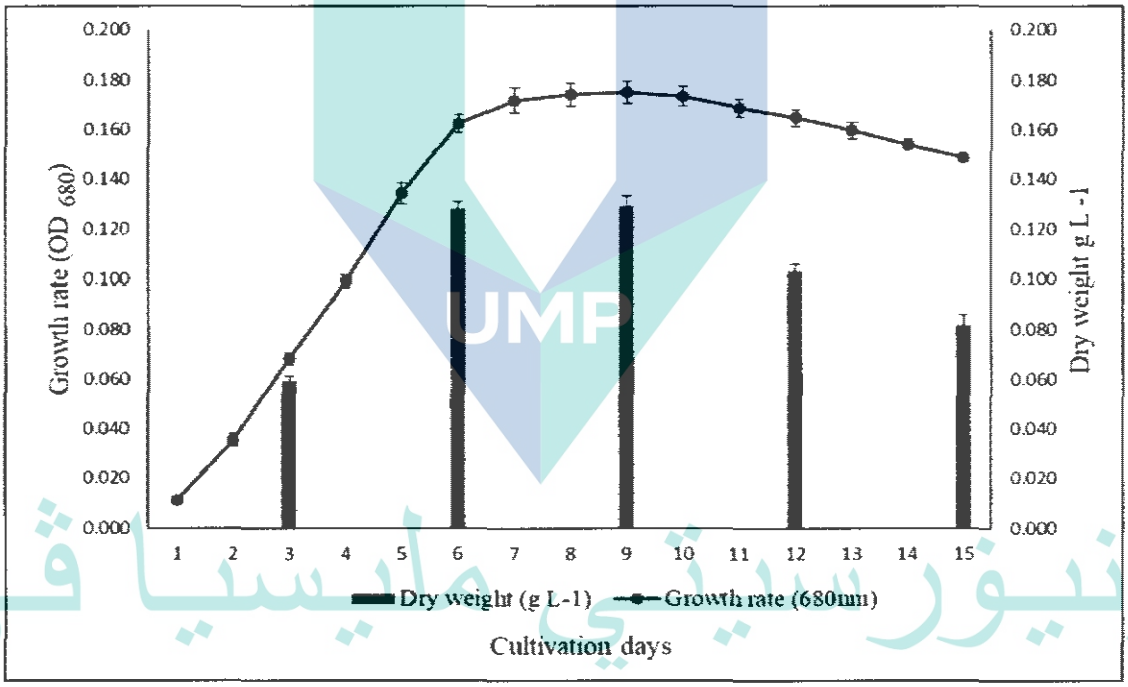


Figure 4.4 Growth rate (OD_{680}) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under photoautotrophic condition.

ii) Effect of heterotrophic condition

Several researchers revealed that microalgae growth is directly proportional to the total amount of light illuminated per day, meanwhile, in other studies researchers discovered that growth of phytoplankton can be controlled by photoperiod instead in order to obtain high biomass and lipid production. Figure 4.5 illustrates the cell growth rate and concentration of dry cell weight (g L^{-1}) respectively for photoperiod of 18:06

light: dark cycle cycles for 15 days. The cell growth rate was measured routinely at 680nm wavelength between switch of light to dark cycle. The results revealed that cell growth curve pattern of *Nannochloropsis* sp. was different from photoautotrophic. The cultures reached stationary phase on the 9th day whereby the growth pattern from Figure 4.5 reveals that the *Nannochloropsis* sp. cultures grew linearly. The results was easily visible because of the colour change of the *Nannochloropsis* sp. cell, usually it can be seen after 8 hours of dark cycle every day the culture was more darker, hence, this indicates that as the light: dark cycle length exceeds the turnover time of the photosynthetic unit, optimum photosynthetic efficiencies was achieved. This results further supported by Kumar et al., (2010), whereby the researcher reported that when the photoperiod exceeded the microalgal photosynthetic unit turnover time, the full photosynthetic efficiencies could be reached. Moreover, compared to photoautotrophic 24:0 photoperiod of 18:06 light to dark cycle significantly increased the cell growth rate.

In photosynthetic activity and growth rates of microalgae, photoperiod is also a decisive factor (Jacob-Lopes et al., 2008). Meanwhile, the total average dry cell weight was (0.714±0.003gL^{-1}) whereas the lipid production was (0.212±0.004gL^{-1}). The results have demonstrated that high growth rates of biomass appear to lead to high lipid content. Microalgae cultivated at light and dark cycles, reported by Richardson et al. (1983), showed remarkable changes in their total chemical components, pigment intensity and photosynthetic capacity. In this research, the total lipid content obtained under photoperiod was 29.6% which is relatively higher compared to lipid content in photoautotrophic. In another study, Wahiddin et al., (2013) have reported that the growth rate, biomass concentration and lipid content was noticeably higher when *Nannochloropsis* sp. was shift from 12:12h light: dark cultivation to 18:6 light to dark cycle. The total obtained lipid was 27.95% when the authors cultivated *Nannochloropsis* sp. in 18:6 light: dark cycle. As shown in a comprehensive study on different light regimes on diatom lipids by Brown et al., 1996, photoperiods or light: dark cycles at different growth phases also have a major impact on algal lipid composition.

In this present study the optimum temperature of *Nannochloropsis* sp. was fixed accordingly, following with salinity at 20ppt and pH 7.5. Qin (2005) recorded that the maximum biomass and lipid content for *B.braunii* was provided by a photoperiod of 12 h light: 12 h dark, 23°C temperature, and 0.8755% NaCl, and 30-60 W/m^2 light intensity irradiance. Therefore, *Nannochloropsis* sp. which is cultivated in photoperiod of 18:6

light: dark cycles had the ability to produce high lipid content compared to photoautotrophic condition.

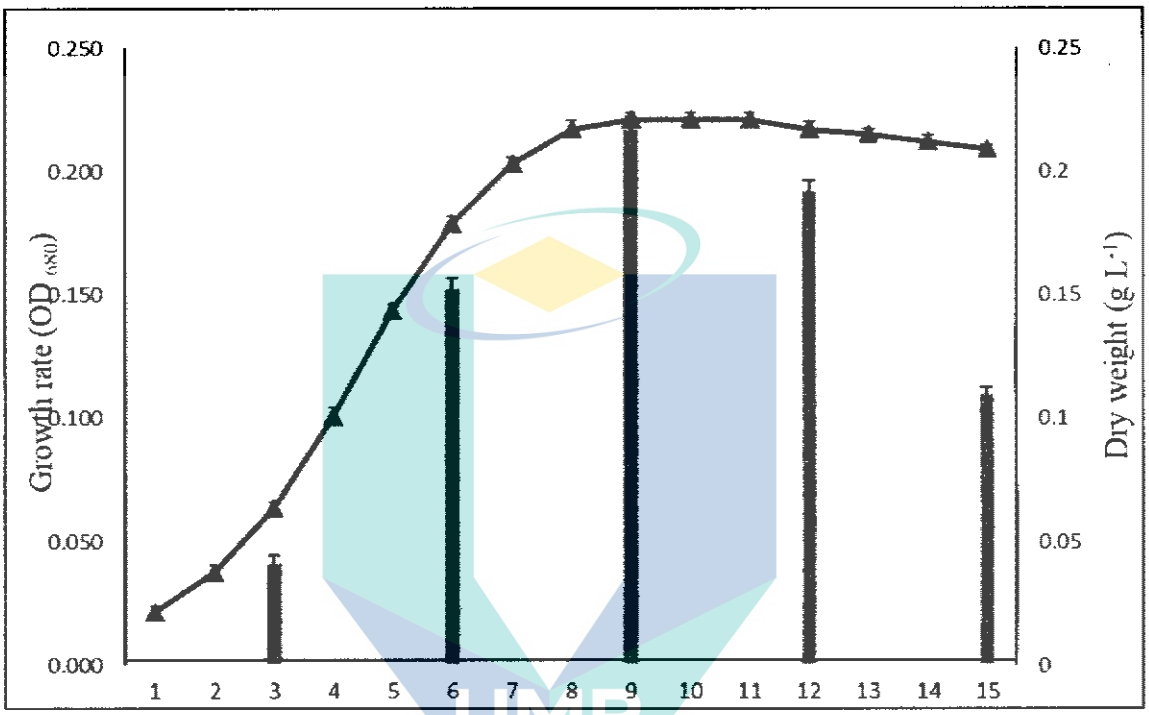


Figure 4.5 Growth rate (OD $_{680}$) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under heterotrophic condition

4.3.2 Mass cultivation of *Nannochloropsis* sp. under different salinity

i) Effect of 20 ppt of salinity

Variations of salinity plays an important role in microalgae growth as well as lipid production. Salinity generates ions of sodium that are necessary to enhance microalgae photosynthesis by intracellular regulation of pH, inorganic nutrient uptake and alkalinity tolerance. As vividly seen in Figure 4.6, the growth rate of *Nannochloropsis* sp. grew well during the exponential phase, however, the stationary phase was only last for two days. The growth and the dry cell weight of *Nannochloropsis* sp. dropped gradually up to 15th day of cultivation. Each experiment was done in triplicate respectively. As observed, during the cultivation the culture's colour range changed from dark green to colorless which it is a sign for death phase where the chlorophyll decreased and the retardation of cell division take place. Thus, the average dry cell weight was (0.921±0.002g L-1) whereas the total lipid production was (0.25±0.02g L-1). Meanwhile, the total lipid yield

of *Nannochloropsis* sp. grown under 20 ppt was 27%. The increase in dry cell weight on 6th day this could be due to the accumulation of intracellular lipid in *Nannochloropsis* sp. cell (Su et al., 2010).

This study was in agreement to an earlier report, Rai et al., (2015) indicated that cultures at 0.2M salinity showed a better growth compared to 0.5M, however, the lipid production was significantly low. The researcher also indicated that photosynthesis studies suggest that the production of adenosine triphosphate and flavin mononucleotide by the Hill reaction requires a chloride ion. Moreover, *Nannochloropsis gaditana* was cultivated in different salinity range (20, 25, 30, 35 and 40ppt) at standard laboratory temperature and light intensity. Among them 25ppt showed a positive trend of growth and lipid production up to 35.8% but at 20ppt showed a negative trend of growth and lipid production, 18.4% (MarKose et al., 2020). Based on the literature findings, the researchers confirmed that the optimum range of salinity between 25PSU to 40PSU for better growth and lipid accumulation (Bartley et al., 2013).

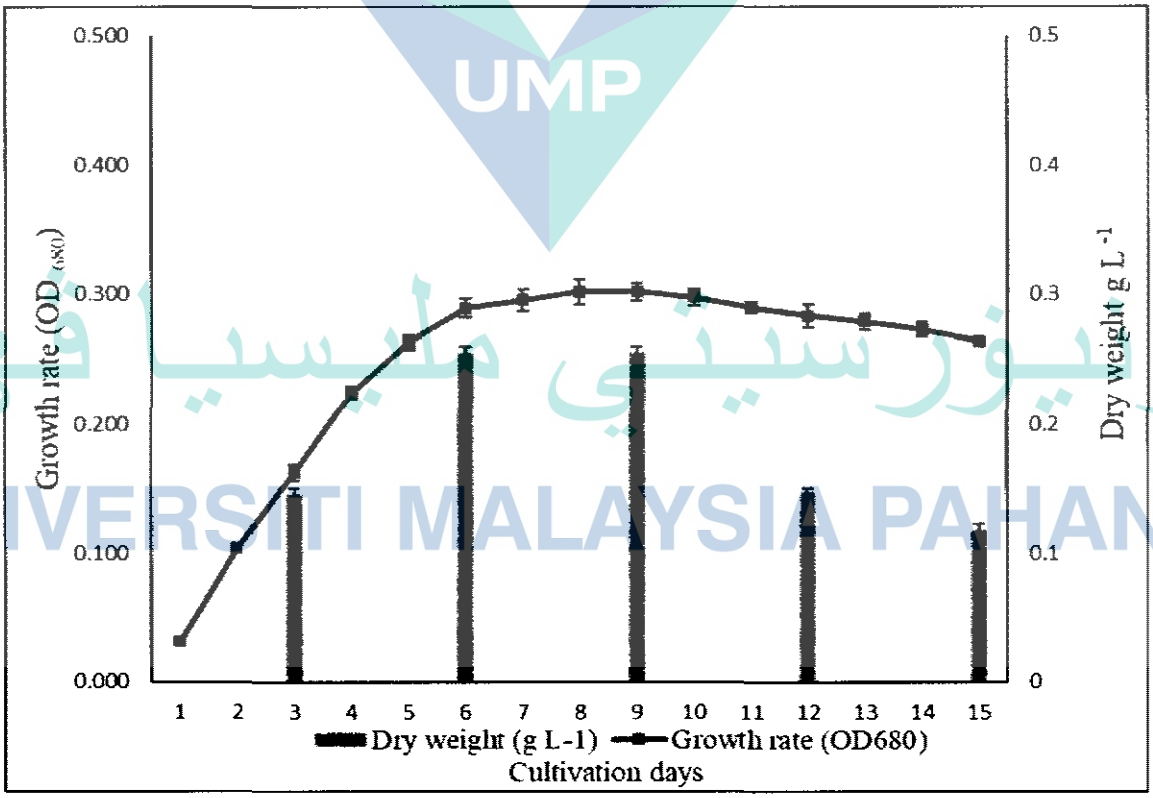


Figure 4.6 Growth rate (OD $_{680}$) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under 20ppt of salinity

ii) Effect of 30ppt of salinity

As vividly seen in Figure 4.7, dry cell weight of *Nannochloropsis* sp. was weighed on every three alternate days. The culture achieved the maximum dry cell weight (1.191±0.007g L^{-1}) and lipid production was (0.362±0.003g L^{-1}) at 30ppt. As observed, during the cultivation the colour of the culture was dark green throughout 15 days. This indicates that the culture was growing healthy without any contamination. Meanwhile, the average lipid yield was 30.3%. As observed from growth curve of Figure 4.7, the stationary phase sustained up to 10^{th} day whereas in 20ppt the culture started to drop drastically after day 8. This indicates that the *Nannochloropsis* sp. culture was fed with enough nutrient specifically the range of salinity. The maximum oil yield was obtained under 30ppt of salinity this could be due to the stress developed during the salinity increase. Therefore, the stress eventually prompts the lipid accumulation in the microalgae cell.

Generally, the salinity stress above 30ppt will tend to inhibit the growth of the cell, resulting in significantly lower biomass concentrations. Upon high salinity condition, the cell growth inhibition will be occurred due to the high external ion concentrations and excessive ion flow into the cells. In high salinity (>30ppt), cellular water potential can be diminished by osmotic stress, inducing excessive salt ion absorption into the cells, and thereby causing cellular ion imbalance (Bartley et al., 2013).

Meanwhile, less than 30ppt of salinity, the *Nannochloropsis* sp. cell grew linearly but the lipid production was significantly low. Below 30ppt of salinity, the external water potential increased, contributing to the absorption of water into the cell, significantly resulting in the contraction of cell volume and inner osmotic pressure against the cell membrane to modify the osmotic balance (Sajjadi et al., 2018). Nevertheless, the current *Nannochloropsis* sp. cell seemed could not tolerate the low salinity condition when cultivated in 20ppt. The osmotic modification was failed to achieve under lower salinity condition, inducing deadly damage to cell membranes and organelles. Since *Nannochloropsis* sp. is a marine microalga, a certain optimal concentration of salts is considered necessary to maintain the osmotic balance of the cell (Bartley et al., 2013). The current *Nannochloropsis* sp. which was utilized in this study was significantly tolerable to 30ppt which is almost equivalent to the standard sea water salinity range (35ppt).

Research by Bartley et al., (2013) confirmed that the salinity increase from 22 to 34ppt can cause a substantial rise in lipid content of 35% by weight. The researcher also had indicated that for growth and lipid development, each algal species has an optimal salinity level and this level can vary for different species depending on the physiological condition. In this present study, *Nannochloropsis* sp. produced 30.3% lipid content when cultivated in 30ppt. The stress of salinity influences different physiological and biochemical processes associated with the growth and production of *Nannochloropsis* sp. Due to its important role in causing changes in fatty acid metabolism, it can contribute to an increase in the lipid content of microalgae (Kalita et al., 2011). Meanwhile, another similar study demonstrated by Hu (2004), who reported that when the salinity increases from 10% to 35%, there will be a significant increase in lipid content in regard to osmotic pressure. *Nannochloropsis* sp. in this study grew linearly and reached the stationary phase on day seven whereas *Nannochloropsis* sp. which cultivated in 20ppt the stationary phase was only lasted for two days and tend indulge into death phase on the consequent days. Specific processes such as control of ion uptake and distribution through the cell membrane, turgor pressure regeneration, and accumulation of osmo-protective solutes and stress proteins were enabled when cells were exposed to salinity, contributing to a stable growth state of the *Nannochloropsis* sp. cell in 30ppt of salinity in this study.

In earlier study reported by Khatoon et al., (2014), where they cultivated *Nannochloropsis* sp. and *Tetraselmis* sp. in different ranges of salinity (20,30 and 40ppt) among them cultures in 30ppt tend to produce higher lipid content compared to other salinity ranges. In the present study, the *Nannochloropsis* sp. grew steadily up to stationary phase at 30ppt. This phenomenon agreed with Mohammady et al., (2014) where the researcher greatly demonstrated that in order to create cell division within *Nannochloropsis oculata*, 30g L^{-1} is the best salinity range for better growth. A similar study was made by Rao et al., (2017), in which the researcher specified that several halotolerants have a mechanism of responding that enables their presence in saline medium. These species generate metabolites that actually protect them and maintain an osmotic balance from salt injury between the cell and its surrounding environment. Increases the salinity level might trigger a slight rise in total lipids in the cytoplasm of microalgae due to the accumulation of tiny molecules such as glycerol as a response to osmotic pressure. In this current study, the biomass concentration of *Nannochloropsis* sp. tended to increase gradually from day 3 up to day 9 in 30ppt of salinity boosted the high

lipid content in the intracellular of the cell. This phenomenon occurred due to the lipid synthesis induction which usually triggered by photosynthesis relief under a variety of stress conditions, where excess electrons through the conversion of glyceraldehyde-3-phosphate to TAG, NADPH or ATP is scavenged. In contrast, there are studies reported that *Nannochloropsis* sp. to show higher growth rate and lipid secretion at 22ppt. The author illustrated that the lipid content of the *Nannochloropsis* sp. raised from 20.2% to 26.2%, when the salinity concentration shifted from 35 to 22ppt (Kim et al., 2015). Hence, species-to-species rely on the optimal salinity for microalgal cell growth, and their response to salinity variation is also species-specific. In this present study of *Nannochloropsis* sp. which cultivated in 30ppt of salinity demonstrated the better growth and highest lipid production of 30.3% respectively. Therefore, 30ppt of salinity found to be the optimal for *Nannochloropsis* sp. in order to obtain high lipid content for biodiesel production.

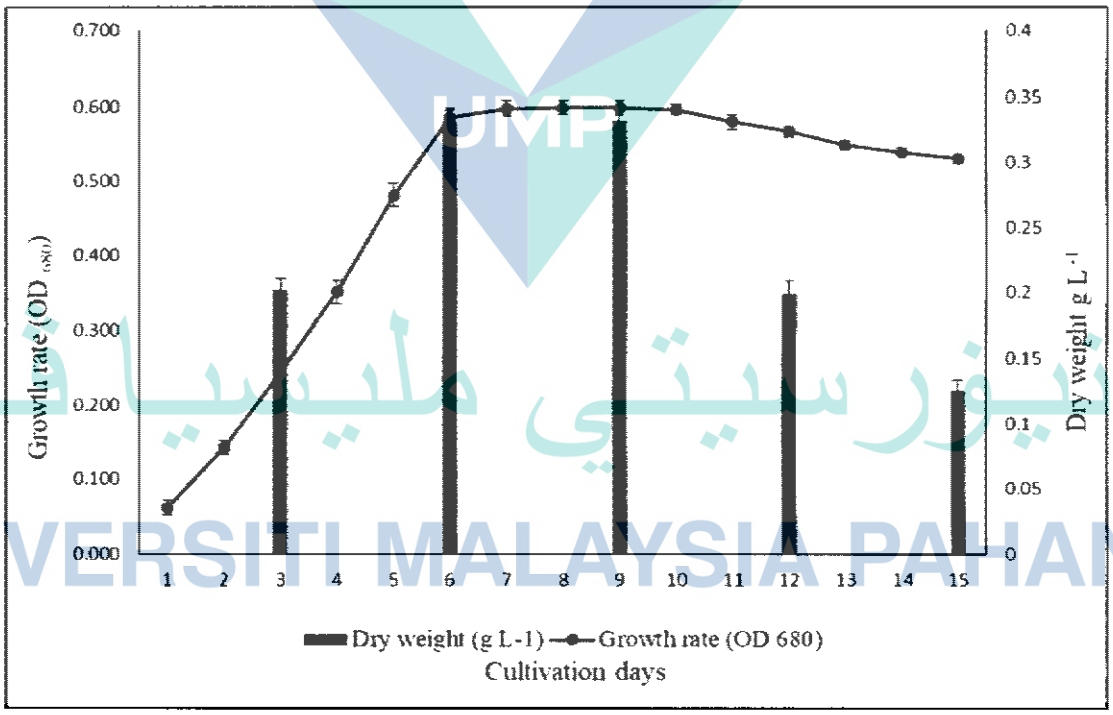


Figure 4.7 Growth rate (OD_{680}) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under 30ppt of salinity

iii) Effect of 40ppt of salinity

Based on the observation from Figure 4.8, *Nannochloropsis* sp. which cultivated in 40ppt of salinity grew inconsistently and indulged themselves into lysis phase. The total average dry cell weight 0.302±0.01g L^{-1} and lipid production was 0.05±0.002g L^{-1}. The total lipid content was relatively low 16.5%. Bartley et al., (2013) indicated the higher salinity contributed to a lesser abundance of microalgae cells which consistent with the present study where the *Nannochloropsis* sp. failed to survive in 40ppt. *Nannochloropsis* sp. showed a higher lipid production during the cultivation at 30ppt compared to 20 and 40ppt. This is because at high concentration, salinity be able to damage the cell, however, an optimal amount of salt capable to increase the lipid production of microalgae cell (Sajjadi et al., 2018). A culture mode that allows an optimal growth rate and allows lipid enhancement was recommended by researchers to help with the inconsistency between lipid production and biomass production yield.

Moreover, Pal et al., (2011) noticed a massive decrease in chlorophyll content during the late stationary phase at 40g L^{-1}. This is consistent to present study where the colour range of the culture was started to fade from dark green this is due to the decrease in chlorophyll content and photosynthetic material when cultured in 40ppt of salinity. The growth of *Nannochloropsis* sp. has been suspended on the day 10 where the growth rate dropped massively. Fatma et al., (2007) explained that the proliferation of microalgae has been suspended at high salinity due to the accumulation of compatible solutes acting as osmoprotectants to regulate metabolic enzymes. Meanwhile, in this present study *Nannochloropis* sp. could only able to produce 26.3% when the cell harvested in late stationary phase where this experiment found to be consistent with Bartley et al., (2013) whereby the researcher cultivated *Nannochloropsis salina* in 46PSU salinity range, the resulting outcome of lipid production was 21.8% which is lower than the present study. On the other hand, as a mechanism of physiological resistance to salt stress, most microalgae control lipid biosynthesis. However, the capacity of each strain to tolerate salinity is different.

Furthermore, Bartley et al., (2016) observed salinity range (14.5-41.5ppt) for *Nannochloropsis salina*. *Nannochloropsis salina* grew well in 28ppt and produced maximum biomass concentration of 7.417$g^{-1}L^{-1}$. The researcher noticed a drastic dropped of growth rate when the microalgae cultivated at 41.5ppt. This is totally accordance to the present study where the cultivated *Nannochloropsis* sp. failed to survive at extreme

condition of saline. In earlier studies by (Renaud and Parry 1994) had indicated that the most suitable salinity range for *Nannochloropsis* sp. is 25-30ppt. In contrast, other researcher had found that salinity tolerance can be different among marine microalgae whereby few diatoms and green microalgae has the tendency to grow at extreme saline condition above 100ppt. However, the capacity of each strain to tolerate salinity is different (Hu and Goh, 2006).

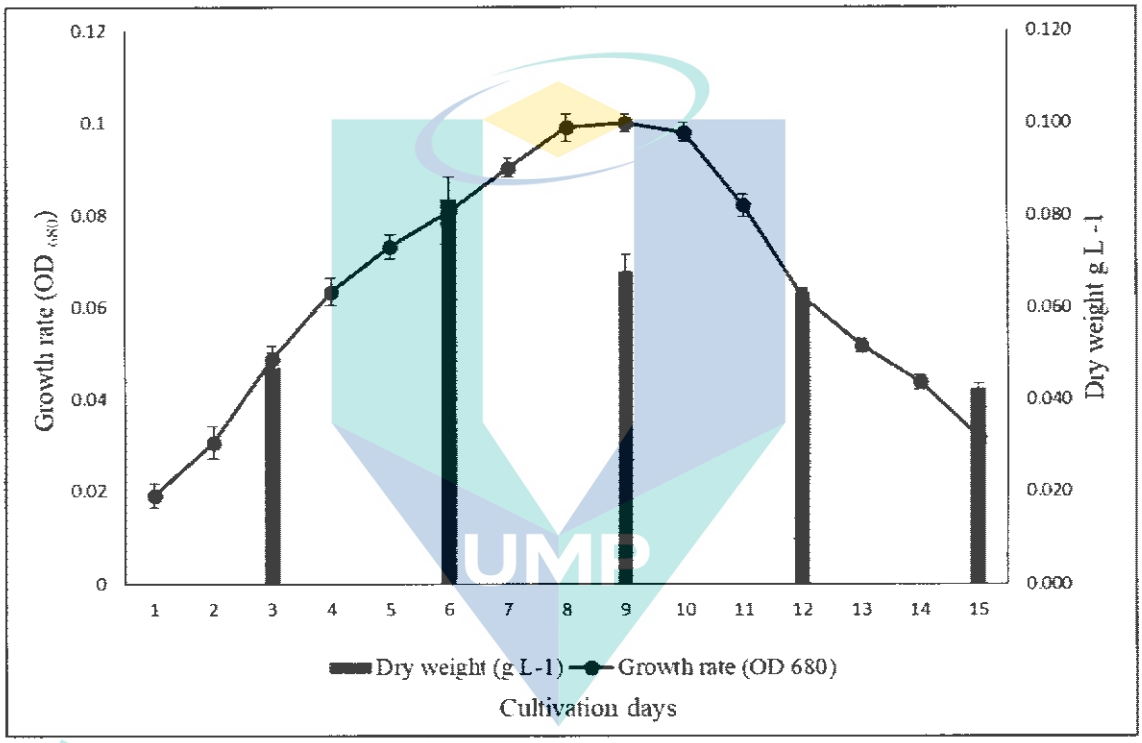


Figure 4.8 Growth rate (OD $_{680}$) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under 40ppt

4.3.3 Mass cultivation of *Nannochloropsis* sp. under different pH variations

i) Effect of pH range of 5

Based on the growth curve as shown in Figure 4.9, the growth rate of pH 5 grew relatively slow and started to drop after day 7. Meanwhile, the total average dry cell weight of *Nannochloropsis* sp. was (0.357±0.005g L^{-1}) and the lipid production was (0.071±0.004g L^{-1}). The total lipid content in this present study was 19.8%. Similarly, MarKose et al., (2020) cultivated *Nannochloropsis gaditana* under pH 5 and observed a massive decrease in growth and lipid content. This findings was further supported by Difusa et al., (2015), cultivated *Scenedesmus* sp. in different pH conditions where it was

that the biomass productivity was only 0.08±0.02g $L^{-1}d^{-1}$ whereas in alkaline state the species showed the trendemous increase of biomass and lipid production. However, species such as *Chlorella* tend to grow rapidly at the pH value of 5.0, (compared to pH of 4.0, 4.5, 5.5, 6.0, 6.5 and 7.0) and its growth inhibited under alkaline pH variations. Therefore, most of the algae species are prone to acidic pH than alkaline pH range.

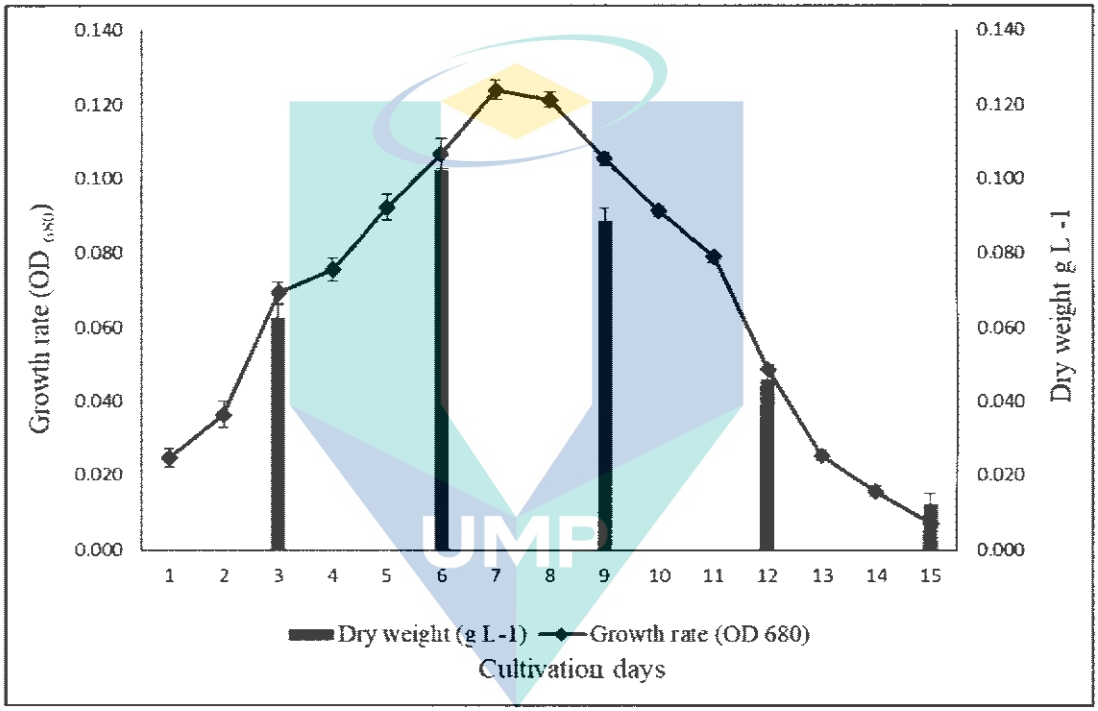


Figure 4.9 Growth rate (OD 680) and dry cell weight (g L-1) of Nannochloropsis sp. under pH 5

ii) Effect of pH range of 6

Based on growth curve as shown in Figure 4.10, the growth rate of pH 6 grew slowly and started to drop after 6th day. Meanwhile, the average of total dry cell weight of *Nannochloropsis* sp. which harvested using a freeze dried method was only at (0.559±0.02g L^{-1}) and the lipid production was (0.131±0.005g L^{-1}) whereas the total lipid content was 23.4%. The amount of dry cell weight and cell growth rate shows that *Nannochloropsis* sp. failed to survive in pH 6. This present study was similar to Bartley et al., (2014) findings where the researcher cultivated *Nannochloropsis salina* in various pH range from 5-10. The researcher observed that the microalgae grew poorly in pH5 and pH6 compared to other ranges.

Meanwhile, a similar study made recently by MarKose et al., (2020), whereby at pH 5 the attained lipid of *Nannochloropsis gaditana* was 19.4% during log phase whereas 15.5% was obtained during stationary phase it was consider the lowest compared to other pH ranges in his study. Low growth rates at high pH may be associated with the fact that by producing CO_2, high pH would limit carbon accessibility, which could have led to reduction of cell growth. Furthermore, in the present study the harvested dry cell weight of *Nannochloropsis* sp. using freeze dried method showed relatively lower lipid production 33.3%. This present study was significant to (Kumar and Saramma, 2018) studied different pH range in *Nannochloropsis salina* whereby the lipid production at pH 6 was the lowest (80µg/mg) compared to other pH ranges in their study. The researchers had indicated that the algal cell membrane is entirely permeable to H+ and OH- ions, and cellular functions can be impaired by the intensity of such ions, causing death at high H+ concentrations (very low pH). The below mentioned results in Figure 4.10 are in agreement with other studies. Khatoon et al., (2014) had cultivated *Nannochloropsis* sp. and *Tetraselmis* sp. in different pH ranges where the researcher noticed that both the species failed to survive in acidity condition. He also indicated that the difference in pH range caused changes in the total lipid content in both *Tetraselmis* sp. and *Nannochloropsis* sp. when cultivated in pH 5.5. On the other hand, Khalil et al., (2010) noticed a sharp decline of dry biomass weight 47% of *Dunaliella bardawil* in pH 4 meanwhile he shifted the pH value to alkaline condition the biomass weight attained up to 72.23%. However, according to Pronina et al., (1981), in the presence of intrathylakoid membrane-bound carbonic anhydrase, a large amount of bicarbonate (HCO_3^-) will move through illuminated thylakoids where the pH is near 5 and dehydrate to CO_2. Then the resulting CO_2 can escape into the pyrenoid from the thylakoids. A second version is also possible in which the membrane channels can be formed by the membrane bound carbonic anhydrase of mammalian cells (Wistrand, 1984).

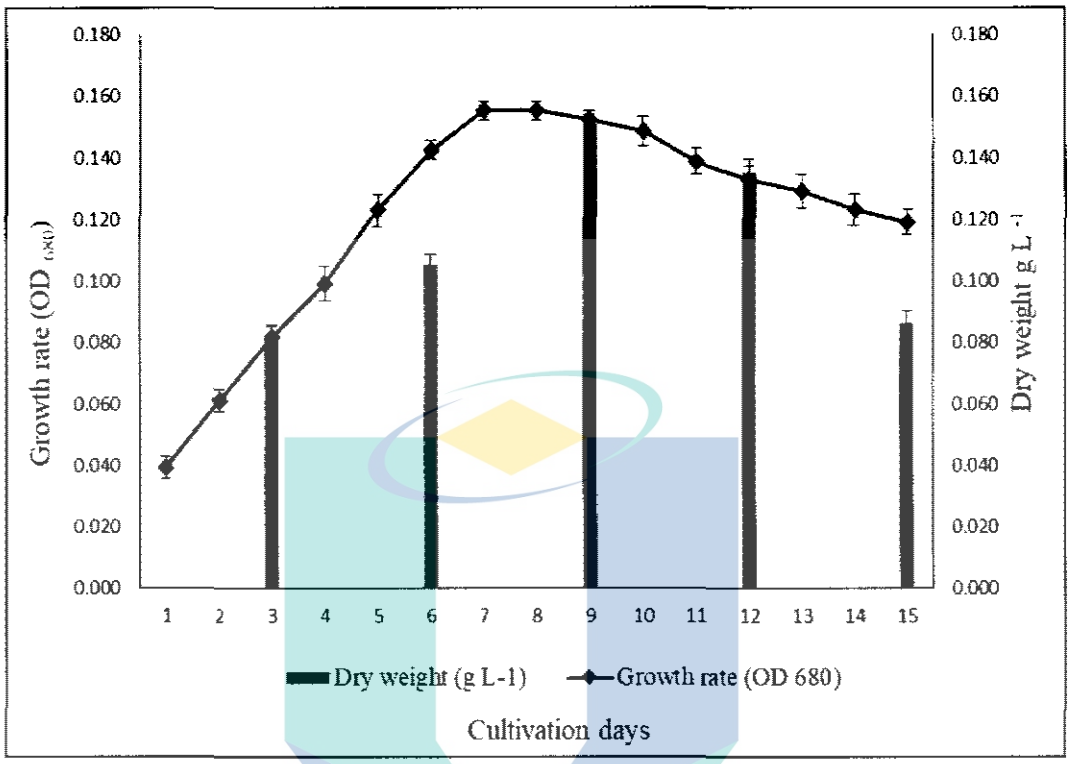


Figure 4.10 Growth rate (OD_{680}) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under pH 6

iii) Effect of pH range of 7

Figure 4.11 represents the growth curve of *Nannochloropsis* sp. by dry biomass weight (g L^{-1}) and optical density under pH 7. The experiment was repeated three times in order to ensure the accuracy and the average values are reported. After day 1 of incubation, *Nannochloropsis* sp. which cultured under pH 7 grew well and reached the stationary phase at day 7 whereas the death phase started on day 11. Meanwhile, the dry cell weight (DCW) of *Nannochloropsis* sp. was (0.620±0.006g L^{-1}) whereas the lipid production was (0.182±0.02g L^{-1}). The total lipid content of this present study was 29.3%. *Nannochloropsis* sp. survived well in pH 7 but it produces low amount of lipid inthis study. This experiment was agreed with MarKose et al., (2020), where the *Nannochloropsis gaditana* survived well when cultivated in pH 7 but relatively produces low lipid content, DCW% (29.4±1.87) compared to other pH ranges in the study. Meanwhile, in previous study Moheimani et al., (2013) explored the effects of different pH ranges of lipid production in *Tetraselmis suecica* and *Chlorella* sp. where he concluded that pH 7 and pH 7.5 is the ideal range for lipid accumulation of the species

respectively. However, in this present study it is known that *Nannochloropsis* sp. which cultured under pH 7 not able to produce high lipid content since that was the prime objective of this study.

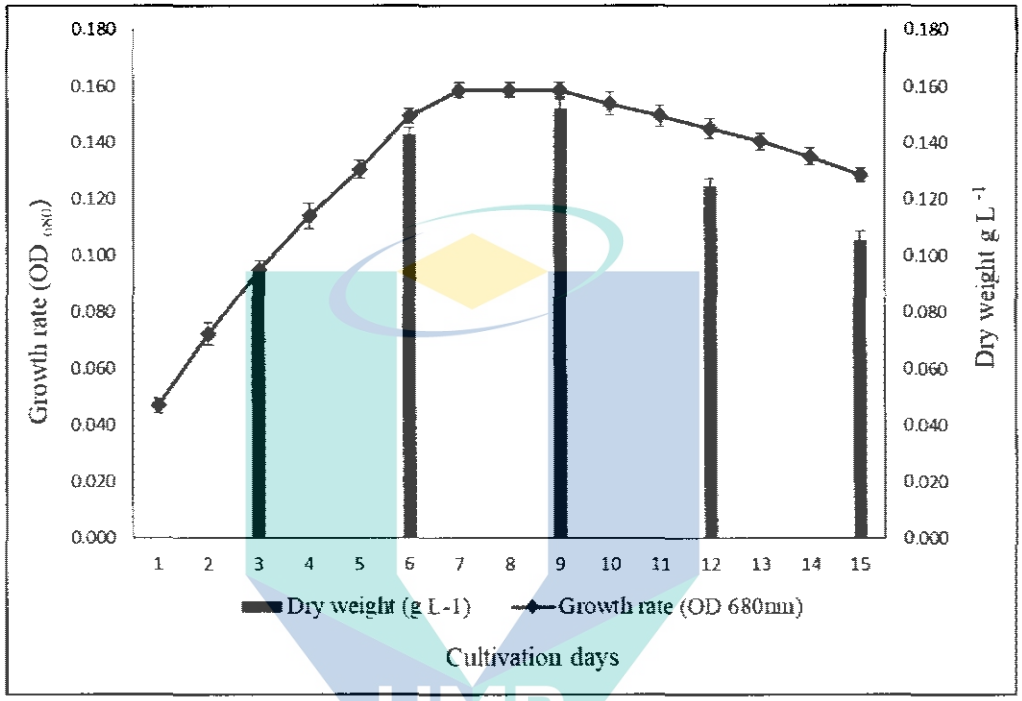


Figure 4.11 Growth rate (OD_{680}) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under pH 7

i) Effect of pH range of 8

Based on Figure 4.12, the growth curve represents the optical density and dry cell weight (DCW) of *Nannochloropsis* sp. which cultivated for 15 days respectively. After day 1 of incubation, the *Nannochloropsis* sp. cell grew well and reached the stationary phase. Therefore, the activation of ACCase (the main enzyme in lipid biosynthesis) is pH dependent, so the pH of the culture medium plays an important role in influencing the aggregation of lipids in the algae. In this present study, the dry biomass weight was (0.839±0.02g L^{-1}) meanwhile, the lipid production was (0.309±0.01g L^{-1}). The dry biomass weight was higher compared to pH 7 and the total lipid content of *Nannochloropsis* sp. which cultivated in pH 8 was (36.8%) which was relatively higher than lipid content in pH 7. From the data obtained, it was observed that the pH of Conway medium has a significant effect on cell growth and lipid accumulation of *Nannochloropsis* sp. Compared to acidic conditions (pH 5 and pH 6) did not enhance the cell growth rate and lipid content of *Nannochloropsis* sp. Therefore, a linear decline was observed in both

acidic condition. However, *Nannochloropsis* sp. showed a steady growth rate whereby maximum biomass concentration was observed in pH 8 but in pH 7 showed a slight slow growth rate. Based on the data obtained in this current study, *Nannochloropsis* sp. the ideal pH range was 8-9. *Nannochloropsis* sp. which cultivated in pH 8 grew linearly and reached the stationary phase on day 7 and lasted up to day 10.

This present study was accordance to Bartley et al., (2013) where the researcher explored different pH ranges in *Nannochloropsis salina* in order to find the optimum pH range to maximize the lipid accumulation. The researcher indicated that pH 8-9 might be ideal to maximum the growth, dry cell weight and lipid content. At slight alkaline pH stress (above neutral = 8), *Nannochloropsis* sp. comparatively decreases the content of glycolipid and polar lipids while increasing the accumulation of TAG, which is not dependent on levels of limitation of nitrogen or carbon. Meanwhile, when acidic pH range (pH 5 and 6) is introduced in *Nannochloropsis* sp. cultivation the growth rate was inhibited. PH conditions that are too acidic are known to cause the function of enzymes involved in the microalgae metabolism process to be inactivated and leading to lysis of the cell.

In another study, Spolaore et al., (2006) found that *Nannochloropsis oculata* showed the best growth rate at pH of 8.4. Similarly, MarKose et al., (2020) demonstrated that *Nannochloropsis gaditana* reached a highest growth rate at pH 8 whereby the maximum oil content was 34.6% which was recorded in the same pH. In the present study, the *Nannochloropsis* sp. showed the maximum growth and oil content was recorded in alkanine pH. The reason might be due to as the alkaline pH reduces the cell release, therefore prompting lipid accumulation. In earlier research, Guckert and Cooksey reported in 1990 that the alkaline pH tends to develop autospore on *chlorella* CHLOR-1 and therefore reduces the release of cells, hence, stimulating lipid accumulation. In the present study, *Nannochloropsis* sp. which cultivated under pH 7 showed a higher growth rate and dry cell weight compared to pH 8. However, the lipid content was relatively lower than *Nannochloropsis* sp. which cultivated in pH 8. The same pattern was seen in the lipid content of *Chlorella* sp. in earlier studies. The researcher had indicated that the lipid content increased to 23% at pH 8, although the highest production of biomass at lower pH was observed (= 7) (Rao et al., 2015). Evident from morphological observations, research conducted by Guckert and Cooksey (1990) shows that conditions of alkaline pH stress inhibit microalgae growth; as a result, the energy is redirected for TAG biosynthesis.

In another study by Liu et al., (2007) also found that *Chattonella marina* growth remained unchanged in the standard seawater pH range (pH7.5&8.5), although major growth decreases were observed as the pH increased beyond pH 9. According to Sajjadi et al., (2018), the acceptable pH for *Nannochloropsis* sp. is 7.5 to 8.5. Maintaining culture pH in the optimal range is crucial due to the disruption of cellular processes by extreme pH, full culture collapse can occur (Razzak et al., 2015). PH conditions that are too acidic are known to cause the function of enzymes involved in the microalgae metabolism process to be inactivated. Such metabolic disorders hinder development and can result in the death of cells (Yustinadiar et al., 2020). In contrast, invading organisms such as ciliates and diatoms reported to grow maximum at pH 6 and lower at elevated pH values (Bartley et al., 2013). Other research has demonstrated that the *Urotricha castalia* freshwater ciliate shows positive growth above pH 6.5 but they did not show any significant difference in lipid content (Weisse and Stadler, 2006). In this present study, the *Nannochloropsis* sp. which cultivated under pH 8 shows significant differences in lipid yield compared to other pH ranges. Thus, pH 8 is the most ideal condition in order to produce high lipid content in *Nannochloropsis* sp. for biodiesel production (Khalil et al., 2010).

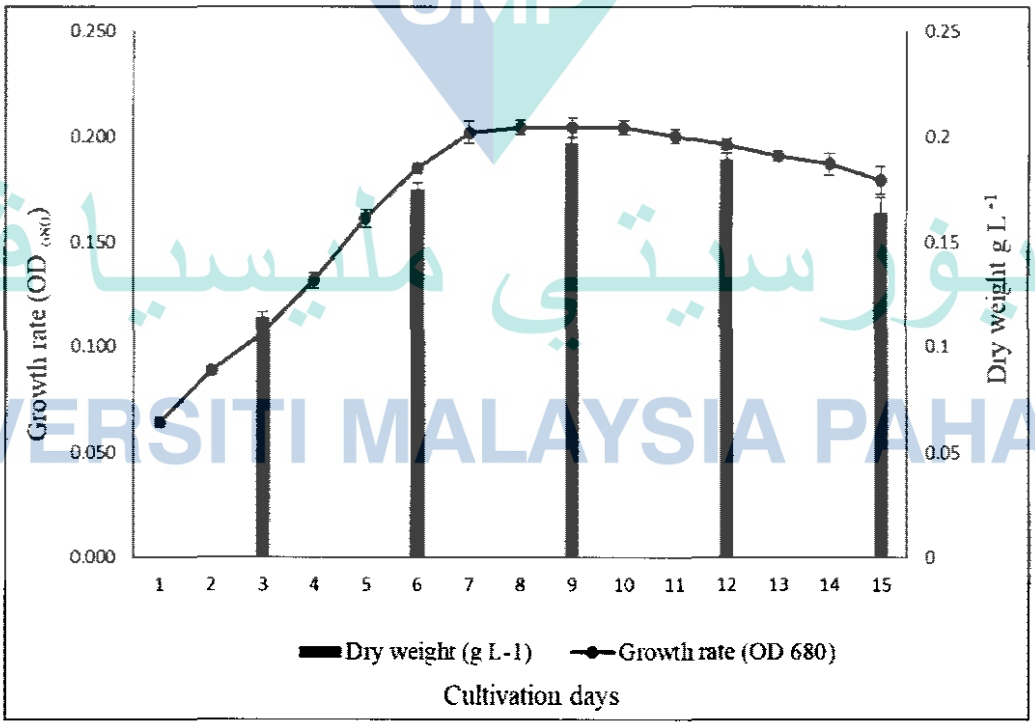


Figure 4.12 Growth rate (OD_{680}) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under pH 8

iv) Effect of pH range of 9

Figure 4.13 illustrates the growth rate and dry cell weight of *Nannochloropsis* sp. under pH 9. After 1 day of incubation, the growth has begun and a sharp decline can be seen after day 6 where it can be said that *Nannochloropsis* sp. started to venture into death phase. Meanwhile, the dry cell weight of *Nannochloropsis* sp. which harvested through freeze dried method was gradually reduced as observed in Figure 4.13. The total harvested biomass was (0.289±0.03g L^{-1}) whereas the total lipid production was (0.04±0.01g L^{-1}). The total lipid content was 13.8%. The total average dry cell weight and lipid production was relatively low compared to pH 7 and 8. A similar study reported by MarKose et al., (2020), whereby *Nannochloropsis gaditana* was cultivated under different pH ranges. The researcher had indicated that *Nannochloropsis gaditana* grew slowly in pH9 and pH 10. In the present study the total lipid yield was 13.8% whereby the total obtained lipid yield was much lesser compared to other pH ranges. Similarly, MarKose et al., (2020) reported that he have noticed drastic drop in lipid yield (14.7%) when *Nannochloropsis gaditana* was cultivated under pH 9.

The present study results are in accordance to Mandotra et al., (2014) where the *Scenedesmus abundans* survived well in a wide range of pH (5-9). With an increase in the pH range from pH 5 to 8, the quantity of biomass increased, further changes in pH (pH 9) had a negative impact on the concentration of biomass. The researcher also indicated that the pH of the culture medium plays a significant role in order to control the lipid accumulation in the algae. Meanwhile, in the present study the growth of *Nannochloropsis* sp. significantly dropped after day 6. Hansen et al., (2007) said that the increased pH is the cause of growth constraints in the in log phase and stationary phase.

In contrast, *Scenedesmus* sp., grows stronger under alkaline conditions (pH > 9) and, under these conditions, has higher lipid productivity (Gardner et al., 2011). High alkali conditions usually seriously inhibit the normal growth of most microalgae, while acidic conditions favour the growth of microalgae and the accumulation of oil (Sajjadi et al., 2018). It is also understood that high pH levels are often related to toxic blooms of microalgae or red tides (Berge et al., 2012). However, in this present study it is known that *Nannochloropsis* sp. which cultivated under pH 9 produces significantly low biomass concentration and lipid content respectively. Therefore, pH 9 condition is not conducive to cultivate *Nannochloropsis* sp. for biodiesel production.

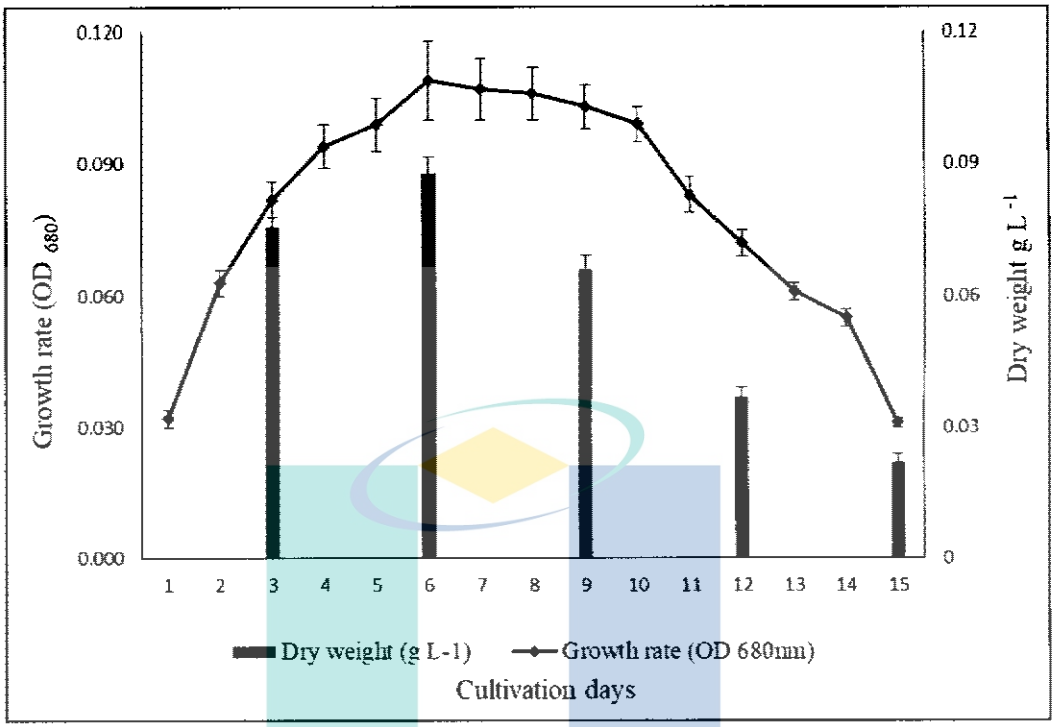


Figure 4.13 Growth rate (OD $_{680}$) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under pH 9

4.4 Optimization of environmental parameters for *Nannochloropsis* sp.

Figure 4.14 illustrates the cell growth rate of *Nannochloropsis* sp. under photoperiod of 18:6 light/dark cycle, salinity of 30ppt along with 8 pH variation for 20 days in 10L photobioreactor respectively. As depicted in Table 4.1 the reading of the culture was taken up to 20 days. Meanwhile, the experiment was done for three times in order to ensure the taken reading are consistent. Initially, those parameters were examined separately in order to determine the optimum parameter for *Nannochloropsis* sp. All experiments were conducted in triplicates. The cell growth curve pattern of *Nannochloropsis* sp. was seen visibly as they grew linearly and the stationary phase was achieved on day 13. The cells experience the exponential growth from day 3 and remained in linear phase until day 12. During this period the biomass gradually increased and the total average biomass was 0.874±0.02g L^{-1} while the total lipid content was 0.567±0.01g L^{-1}. The total lipid production of *Nannochloropsis* sp. when cultivated under combined stress factor was 64.8%. Meanwhile, there is no any study has been reported on *Nannochloropsis* sp. on highest lipid yield. This finding was similar to where the author cultivated *Nannochloropsis oceanica* with different environmental factors whereby the total lipid content was 64% (Wan et al., 2013). This findings was further supported by

Che et al., (2019) where he noticed a massive difference when he shifted 12:12 light to dark cycle to 18:6 in 14L photobioreactor.

The lipid content of *Isochrysis galbana* increased from 65.2% to 71.1% (w/w) respectively. Initially, when *Nannochloropsis* sp. was cultured under photoautotrophic and heterotrophic condition with fixed salinity at 20ppt and pH range (7.5), the biomass and lipid production was relatively lower compared to heterotrophic condition. The light-dark cycle was one of the crucial factor in this experiment which significantly affects the growth, biomass and lipid production by approaching the photosynthetic unit turnover time (Krzeminska et al., 2013). On the contrary to the present study, Khoeyi et al., (2011) demonstrated that increase in light duration from 8:16 to 16:8 during *Chlorella vulgaris* cultivation had showed a promising change in biomass concentration. Indeed, a light/dark regime enables an increase in the final biomass and lipid production. The need for a dark phase was clarified by two reactions governing photosynthesis, a light-dependent photochemical phase and another, a light-independent biochemical dark phase (Khoeyi et al., (2011). The compounds which produced during the light dependent phase NADPH (dinucleotide phosphate nicotinamide adenine) and ATP (tri-phosphate adenine) are synthesize to produce the metabolic molecules necessary for growth are used in the dark phase. Dark reactions, or enzymatic reactions, occur within the chloroplast of stroma (Wahidin et al., (2012). Meanwhile, in this present research after a 8 hour of dark cycle the colour of culture noticeably became more into darker green this is probably due to vigorous photosynthetic efficiency. During the light cycle, water is hydrolyzed in order to form oxygen while during the dark cycle carbon dioxide is absorbed by cell component through the Calvin cycle (Gatamaneni et al., 2018). It has been reported that *Nannochloropsis* sp. make up the lipid in the dark cycle as well (Rastogi et al., 2017).In this present study, the obtained lipid content might not only come from the light condition but also other stress factors such as salinity. Initially, *Nannochloropsis* sp. was cultivated in three different salinity concentration (20, 30 and 40ppt) in order to determine the optimum salt concentration for biomass and lipid content of *Nannochloropsis* sp. Among them 30ppt was the most optimum concentration for *Nannochloropsis* sp. and produces high lipid content of 35% whereas 20ppt and 40ppt produces significantly lower lipid content.

Similarly, Bartley et al., (2013) exposed *Nannochloropsis salina* was subjected to a functional salinity unit of 10, 22, 34, 46, and 58 ppt at different salinity levels. As a

marine microalgae, it had the highest growth rate at 22 ppt and the highest concentration of biomass at 22 and 34 ppt salinity levels. At salinity levels of 58 ppt and < 10 ppt, *Nannochloropsis salina* showed no growth (Bartley et al., 2013). Therefore, it can be said that 30ppt of salinity level is the most ideal for lipid production in *Nannochloropsis* sp. In this present study, pH was fixed at 8 where it was one of the crucial factor in boosting the TAG accumulation (Bartley et al., 2013). Thus, the combined stress factor include 18:6 L-D heterotrophic condition, salinity at 30ppt and pH at 8 was the most ideal factors in order to enhance the lipid production in *Nannochloropsis* sp. which can be able to produce biodiesel. As depicted in Table 4.2, the statistical analysis of using Anova has concluded that the *F* value of is higher than *F critical* value this shows that there is a significant differences between the data within the three replicated experiment.

Table 4.1 Reading of Growth rate (OD 680nm) and Dry cell weight g L^{-1} under optimized environmental parameters

Days	Growth rate (OD 680)	Growth rate (OD 680)	Growth rate (OD 680)	Dry Cell weight (g L^{-1})	Dry Cell weight (g L^{-1})	Dry Cell weight (g L^{-1})
1	0.075	0.071	0.079	0.023	0.028	0.031
2	0.125	0.119	0.121	0.123	0.234	0.456
3	0.261	0.257	0.251	0.145	0.121	0.122
4	0.355	0.338	0.348	0.134	0.137	0.141
5	0.462	0.422	0.443	0.122	0.119	0.125
6	0.575	0.521	0.542	0.132	0.231	0.234
7	0.643	0.633	0.651	0.132	0.135	0.138
8	0.772	0.748	0.761	0.142	0.147	0.149
9	0.856	0.828	0.839	0.123	0.122	0.118
10	0.899	0.881	0.887	0.302	0.306	0.31
11	0.922	0.901	0.939	0.234	0.231	0.239
12	0.949	0.956	0.953	0.234	0.344	0.421
13	0.989	0.996	0.982	0.213	0.231	0.342
14	0.989	0.996	0.982	0.862	0.231	0.311
15	0.989	0.996	0.982	0.375	0.369	0.378
16	0.976	0.984	0.969	0.102	0.123	0.234
17	0.967	0.978	0.953	0.192	0.128	0.145
18	0.958	0.967	0.942	0.132	0.176	0.149
19	0.951	0.959	0.936	0.123	0.126	0.177
20	0.942	0.948	0.929	0.123	0.119	0.126

Table 4.2 Statistical analysis of optimized parameters using Anova

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0.768108	1	0.768108	363.0357	5.01E-19	4.149097
Columns	0.709711	7	0.101387	47.91931	3.53E-15	2.312741
Interaction	0.645607	7	0.09223	43.59105	1.37E-14	2.312741
Within	0.067705	32	0.002116			
Total	2.191131	47				

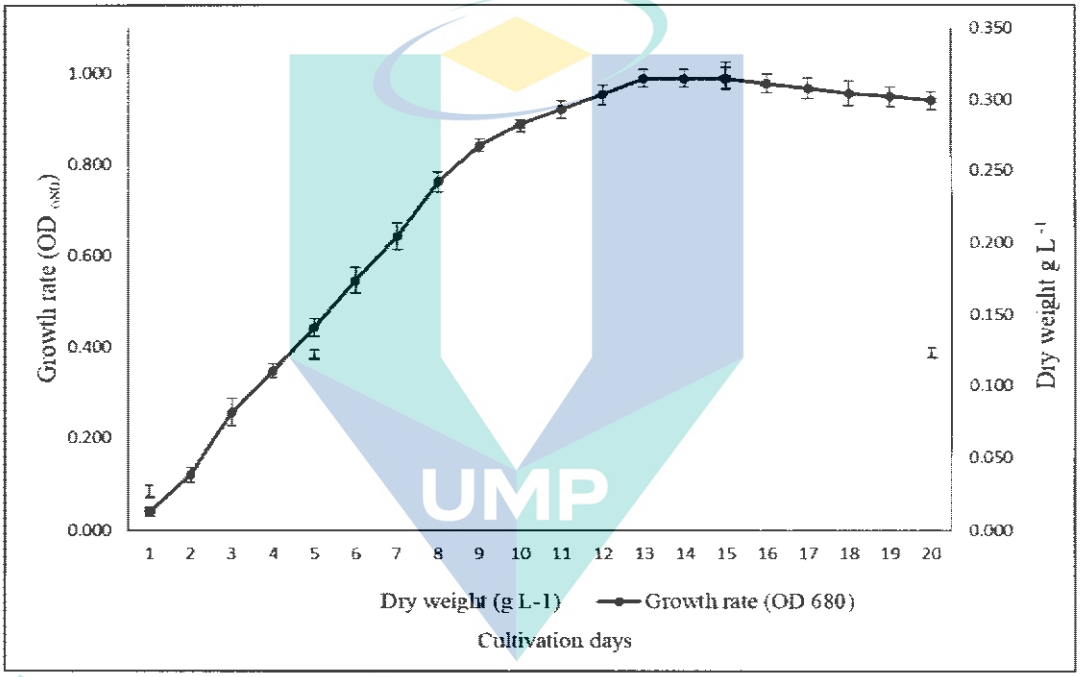


Figure 4.14 Growth rate (OD $_{680}$) and dry cell weight (g L^{-1}) of *Nannochloropsis* sp. under optimized condition of heterotrophic condition, salinity (30ppt) and pH 8.

4.5 Comparison of lipid extraction methods for *Nannochloropsis* sp.

4.5.1 Bligh and Dyer extraction

Three different methods of lipid extraction were used in this study to determine the most suitable extraction method to extract the highest lipid yield and to increase the efficiency of the result on lipid content variations. The influence of Bligh and Dyer (1959), Ultrasound-assisted and Soxhlet extraction methods on the yield of lipid recovered from *Nannochloropsis* sp. by using hexane: methanol (1:1 v/v). Soxhlet extraction produced 64.8±0.88% whereas Bligh and Dyer (1959) and ultrasound-assisted extraction (UAE) extraction produces 25.4±1.30% and 41.03±1.20%, respectively. Among those lipid extraction methods that has been used in this present study, soxhlet

technique had relatively better efficiency as demonstrated in Figure 4.15. Meanwhile, Ultrasound assisted and Bligh and Dyer (1959) showed lower efficiency. Similar study was reported by Natarajan et al., (2014) where he analyzed the UAE with *Nannochloropsis* sp. and *Chlorella* sp. wet biomass and discovered that microalgae with rigid cell wall (*Chlorella* sp.) able to release intracellular lipids more easily than microalgae that has flexible cell wall microalgae (*Nannochloropsis* sp.). The cause of this phenomena as *Nannochloropsis* sp. cell wall tend to curled up during cell disruption and ended up forming small pockets by storing the lipids of the membrane.

4.5.2 Soxhlet extraction

In this present study, lipid extraction from Bligh and Dyer method demonstrated the lowest lipid efficiency of 25.4%. This present findings was further supported by Escorsim et al., (2018), by using chloroform: methanol (1:2 v/v) as extraction solvents the obtained lipid was only 7.4%, where the researcher indicated that did not obtain substantial variations in lipid yields from *Acutodesmus obliquus*. Moreover, the use of harmful solvents such as chloroform and methanol causes adverse health and environmental risks and is the key downside of the algae oil process of Bligh and Dyer (1959) extraction. Instead hexane has been reported as a low toxicity solvent which can substitute chloroform/methanol (Halim et al., 2011). Organic solvents such as hexane have shown the most efficiency in this study as it has the tendency to degrade the microalgae cell walls easily as the microalgae oil highly soluble to inorganic solvents. The low boiling point of hexane was the crucial physical property in this study as it facilitates the removal after lipid extraction. Hexane was used in experiment as it is reusable and inexpensive.

4.5.3 Ultrasound-sonication extraction

In this present study, it is known that Soxhlet extraction has showed a better efficiency than other lipid extraction methods were used in this study. As shown in Figure 4.15, the lipid yield of Soxhlet extraction showed at 64.8%. At each cycle of solvent evaporation and condensation, soxhlet continuously replenishes *Nannochloropsis* sp. biomass with fresh solvents and minimizes the usage of solvent. Hence, it increases the efficiency of extraction to reach highest lipid yield from the biomass sample. Lipid yield of ultrasound – extraction was much lower compared to soxhlet extraction. Ultrasound

uses cavitation and acoustic streaming to break the cell wall of *Nannochloropsis* sp. and can induce the mixture of solvent with biomass. Although the energy input and extraction time is lower but ultrasound will tend to damage the cells. Hence, the lipid efficiency will be disrupted due to strong cavitation. The requirement relatively low temperature (65 ℃) makes the Soxhlet method of extraction cell penetration better compared to Bligh and Dyer and ultrasound. Kanda et al., (2012) obtained highest lipid yield content by using Soxhlet extraction of dried biomass using hexane as solvent compared to microwave method.

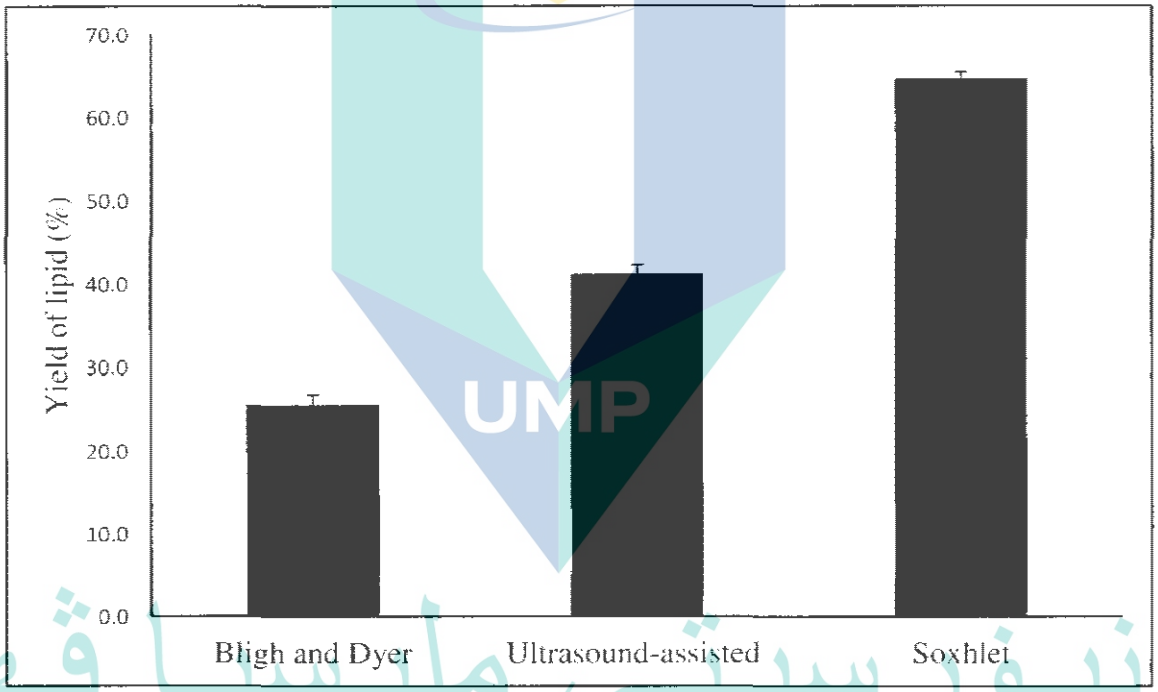


Figure 4.15 Lipid yield under different extraction methods

4.6 Transesterification of *Nannochloropsis* sp. oil

The transesterification process of triglycerides has been applied in synthesis of biodiesel. In this study, transesterification of the extracted lipid oil has been used with the ratio of 1.5:1 (MeOH:oil) with 1% of KOH as catalyst at low temperature and pressure. The reaction time is 1.5 hours. This present study was supported by Ashokkumar et al., (2014) where he evaluated the effect of five different alkaline catalyst in order to determine the efficieny in transesterification process. Transesterification catalysts including [$Ca(OH)_2$, NaOH, KOH, $NaOCH_3$, and $KOCH_3$] and the authors found that

largest ester yield was obtained using potassium methoxide whereas sodium methoxide produces significantly lower yield. $Ca(OH)_2$ has shown the worst catalytic efficiency. This study was further supported by Chen et al., (2012), whereby the researcher utilized three different alkaline catalysts (NaOH, KOH and $KOCH_3$) during transesterification of *Nannochloropsis* sp. oil. Among them, 2% of KOH tend to work better by completing the transesterification reaction within 2 hours at 65°C. However, a few studies reported that high baseline catalyst levels (above 2%) resulted in reaction mixture saponification and TAG loss by interfering with the phase of downstream separation. Thus, utilization of within 2% catalyst is reasonable in transesterifcation process (Veljkovic et al., 2006).

In contrast, Velasquez-Orta et al., (2013) observed that the dried biomass of *Nannochloropsis* sp. was able convert to biodiesel using suphuric acid as catalyst in transesterification process. The researcher indicated that the methyl esters and lipid composition was potentially influenced by the composition of the microalgae cells and not by the biomass salinity. However, acid catalysis has some disadvantages compared to alkaline catalysis: although an acid catalyst is less costly than an alkaline catalyst, the reaction needs more acid catalysts than the alkaline reaction. Moreover, Miao and Wu (2006) have established the fact that acid catalysis requires a high molar ratio but disproven the requirement of high temperature.

The potential variables in the quality of methyl ester are the volume ratio of alcohol to oil (Saravanan et al., 2017). Methanol has been selected as a possible variable in this study as it has a short chain that is sufficient to be applied as fuel in diesel engines. In this reaction, the longer chain group can also be used, but the issue is that the reaction will proceed until all the oil has been converted into glycerides and soap instead of esters. Methanol has shorter reaction time compared to ethanol and is commonly recognised for low cost and physical and chemical benefits (Huang et al., 2010). Reaction time is another critical indicator in the transesterification process, as the reaction time is increased, the quality of fatty acid ester increases to a maximum, if the length of the reaction is further extended after reaching this point in time, it does not have a direct effect on the yield of the commodity. In the present study, 1.5 hours was the optimum time for trigylcerides of *Nannochloropsis* sp. to completely convert to methyl ester (biodiesel). This study was further supported by Ashokkumar et al., (2014), whereby he extracted oil from *B.braunii* TN101 and during the transesterification he visualized using TLC plates in order to determine the formation of methyl ester, at 150th minute achieving the maximum

conversion of methyl ester and it remained practically unchanged as the reaction continued further.

One of the most significant indicators influencing biodiesel production the temperature of the reaction mixture is the phase. As the oil temperature increases, its viscosity decreases, which results in a higher speed of reaction. In this research, the sample was placed in ultrasonication for cavitation and according to literature findings the optimum temperature of *Nannochloropsis* sp. oil when completely converted to biodiesel was at 60°C. Rahman et al., (2017) indicated that a maximum conversion was took place at 65°C for 25 minutes when *S.maxima* oil was converted to biodiesel through transesterification. The researcher also demonstrated that the effect of temperature on the efficiency of product generation can be explained by the fact that a rise in temperature results in a decrease in the viscosity of microalgae oil, which increases both the mixing of oil and methanol and the formation of esters at the same time. Thus, reaction time and temperature plays a crucial role in conversion of methyl ester from *Nannochloropsis* sp. oil.

4.6.1 Qualitative analysis of FAME using TLC technique

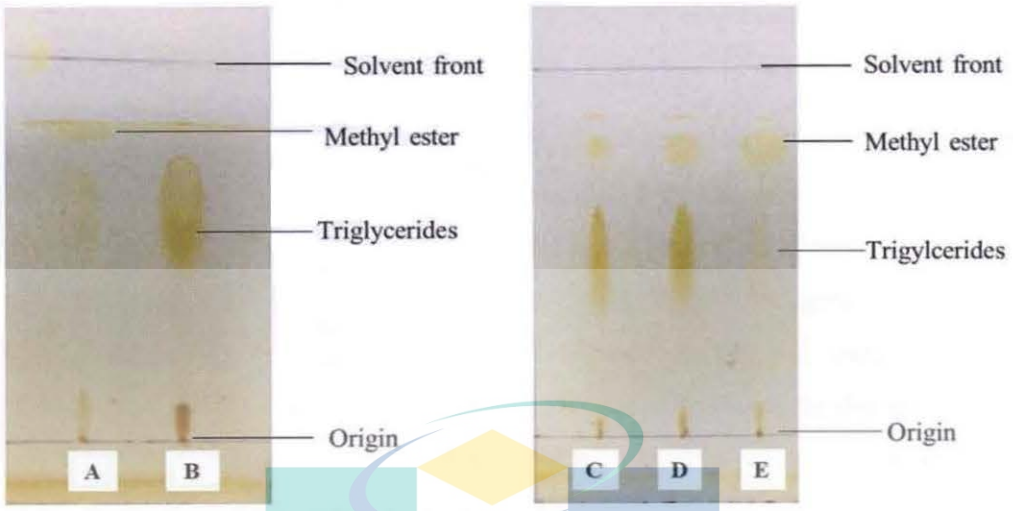
Thin layer chromatography is an analytical technique which known to be one of the simplest and most widely used method for preliminary evaluations of separations from microalgae oil to biodiesel by transesterification. Figure 4.16 demonstrates the presence of FAME and TAGs. Fraction A has been mixed with methyl ester and microalgae oil in order to differentiate both of the component. Meanwhile, fraction B was microalgae oil in order to show the presence of triyglycerides. The TAG oil was mixture of monoglycerides, diglycerides and triglycerides whereas the transesterified methyl ester was equivalent to industrial biodiesel. Each material used in this study was eluted with *n*-hexane. Meanwhile, the transesterification conducted using 0.1% of KOH as catalyst in an extracted oil from *Nannochloropsis* sp. During the transesterification process, 2µL of sample was spotted on aluminium sheet coated with silica gel using a capillary tube every 0.5 consequent hours up to 1.5 hours. This is to ensure the microalgae oil was completely converted to methyl ester through transesterification.

As for methyl ester and triglycerides, the retention factors were determined as R_f by using the formulae 3.2, the R_f value of the distance travelled of *Nannochloropsis* sp.

was 0.93cm. The compound with the higher R_f value is less polar since it interacts with the polar adsorbent on the TLC plate less strongly. The features of high non-polarity were exhibited by the methyl esters and triglycerides here. Meanwhile, the colour of the fraction C on Figure 4.16 the triglyceride separation was dark yellow whereas there was a small spot of methyl ester began to form. On the next consequent hours huge methyl ester spot can be seen visibly on fraction E whereby this indicates that the transesterification process was successfully converted to methyl ester from microalgae oil.

In the present study, aluminium sheet coated with silica gel TLC plates were utilized to spot the lipid samples. Aluminium is mainly used to distinguish lipids based on the degree of unsaturation with various fatty acyl compositions, since Ag+ forms a complex of double bonds of unsaturated fatty acids with the π electrons, resulting in a decreased mobility of these fatty acids (Fuchs et al., 2011). In contrast, Boric acid (H_3BO_3) is utilize for the identification of the various diacylglycerols isomers as well as the separation of isomeric phospholipids. H_3BO_3 forms complexes with compounds containing neighbouring hydroxyl groups and allows these compounds to migrate slower (Allan and Cockroft, 1982). However, researchers had indicated that aluminium coated TLC plates known to improve the separation of lipids and can be easily done (Wang and Gustafson, 1992).

In this study, iodine has been used to visualise the lipid on droplets of triglycerides and methyl esters. This is because iodine vapours able to form non covalent and brown complex lipids. However, iodine is not capable to stain saturated lipids, whereas iodine cannot be removed completely from highly unsaturated lipids (containing, for example, arachidonoyl residues) because iodine is chemically bound to the double bonds. Similarly, spraying of 50% sulphuric acid on TLC plates is also one of the common method being used in studies but sulphuric acid is corrosive and it takes minimum 1 hour for the lipids to be seen visible. Therefore, iodine is the most efficient method as it reduces experiment time and easily forms single bond of lipids (Fuchs et al., 2011).



A= *Nannochloropsis* sp. oil and methyl ester, **B**= *Nannochloropsis* sp. oil, **C, D and E** are after 0.5 h,1h and1.5 h of reaction, respectively

Figure 4.16 TLC plate showing the separation and presence of oil and methyl ester based on different reaction time.

4.6.2 Quantitative analysis of FAME using GC-MS

The composition of fatty acids determines the properties of biodiesel due to the chemical characteristics of fatty acids, such as the length of the carbon chain and the level of unsaturation. Therefore, fatty acid composition of *Nannochloropsis* sp. was demonstrated in Table 4.1. As depicted in Figure 4.17, the most crucial fatty acid that present in *Nannochloropsis* sp. was oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:3) and stearic acid (C18:0). These findings are consistent with Knothe, 2008, that the most common fatty acids found in microalgal lipid acid were recognized as oleicacid, linoleic acid and palmitic acid. Meanwhile, as seen in Table 4.1 MUFA is the most dominant composition in *Nannochloropsis* sp. which can provide oxidation stability. However, PUFA and SFA was significantly low in *Nannochloropsis* sp. In order to be considered an appropriate candidate for the production of biodiesel, it is not only desirable to produce high lipids, but also an appropriate fatty acid composition is crucial. In general, physico-chemical properties are highly influenced by the amount of carbon and the degree of saturation of fatty acid methyl esters. Regulatory agencies are also describing a set of biodiesel requirements. In Brazil, for example, there is concern about oxidative stability. For mixtures blended of petrodiesel with more than 10 percent biodiesel. In order to comply with this specification, microalgal oil must contain relatively low concentrations

of PUFA that are more prone to oxidation due to high levels of insaturation (Sajjadi et al., 2018).

Subsequently, microalgal oils containing high levels of C16 and C18, in particular monounsaturated fatty acids such as palmitic acid (16:0) and oleic acid (18:1), has been reported that to have a fair balance of biodiesel quality because of their ability. The best balance between the desired characteristics (cold filters plugging point, viscosity, lubricity, amount of cetane, heat of combustion, density and oxidative stability). According to Table 4.1 *Nannochloropsis* sp. can be considered as the most promising candidate for biodiesel production. This is because among other fatty acids oleic acid found to be the highest (72.60%) among the rest of the MUFA composition, with 8.86% PUFA. Nevertheless, it has high content of TAG 64.3% (according to demonstration in section 4.5). These findings were further supported by Mahmoud et al., (2015) where he cultivated and extracted oil from three different microalgae species (*Chlorella vulgaris, Scenedesmus quadricauda* and *Trachelomonas oblonga*). Conversely, among the microalgal species studied, *Scenedesmus quadricauda* exhibited the highest content of oleic acid, making it the most suitable for producing biodiesel of good quality 25.97%.

Moreover, it has been reported that stress factors that has been used in this study is one of the most crucial factor that could affect the fatty profile of the intracellular lipid of microalgae (Sajjadi et al., 2018). The fatty acid profile of intracellular lipids confirms that, compared to the regulation, the relative content of monounsaturated fatty acids [palmitic acid (C16:1) and oleic acid (C18:1)] increases significantly with high salinity and high alkalinity without any addition of salt. These compounds are primarily made up of neutral lipids, which in turn facilitate the production of biodiesel. On the other hand, the proportion of polyunsaturated fatty acids (PUFA), especially linolenic acid (C18:3), has significantly decreased in this study. It has been reported that such modifications are in the process of preserving the membrane fluid and avoiding its destruction (Xia et al., 2014).

A similar trend was reported by Xu et al., (1997) in which by varying the salinity in the growth medium of *Dunaliella* sp., the fatty acid composition was changed. The proportion of total saturated and monounsaturated fatty acids increased in cultures as salinity increased from 0.4 M to 4 M NaCl, while total polyunsaturated fatty acids decreased. The researcher concluded that rising salinity in the growth medium increases

the degree of saturation of fatty acids. The same pattern was also seen for the polar lipid fatty acid composition in *Dunaliella salina Teodoresco* due to a change in NaCl concentration. In contrast, the saturated compounds have not dramatically decreased in the case of *Botryococcus braunii*, but they are changing remarkably. C18:0 decreases considerably in the intercellular of this microalga, although C16:0 increases. There is also a remarkable C22:0 and C24:0 content that is not produced under control conditions. Therefore, in the present study *Nannochloropsis* sp. the optimum concentration of salinity was 35ppt where the species tend to produce high lipid content by stressing the cell by producing high TAG, hence, boosting the lipid composition of *Nannochloropsis* sp. particularly by producing high oleic acid.

Nevertheless, pH stress in the culture medium also affects the lipid composition accumulated in microalgae (Sajjadi et al., 2018). In this present research, the best optimal pH variation was at 8. The accumulation of TAG was much higher compared to other pH variations. A similar study was reported by Guckert and Cooksey, 1990 alkaline pH stress substantially decreases Chlorella's glycolipid and polar lipid content while growing the accumulation of TAG, which is not based on levels of nitrogen or carbon limitation. However, the resulting outcome is lower total lipids with higher saturated (C16:0) and monounsaturated (C18:1) compositions. Hence, at optimum pH stress *Nannochloropsis* sp. produces high monounsaturated fatty acids by adapting to the stress of the cell. Moreover, temperature is one of the factor that affects the lipid composition of *Nannochloropsis* sp. In this research, the culture medium of *Nannochloropsis* sp. had a temperature range of 25 °C±2. The lower palmitic acid (13.36%) was prolly due to the low temperature in the medium. A study had demonstrated that at high temperature (29 °C±2) will enhance the percentage of palmitic acid (Hu and Gao, 2006).

Apart from temperature, contain of nutrient such as sodium nitrate and sodium silicate in Conway medium able to increase Acetil CoA carboxylase enzyme, which is a dominant precursor for making lipid in microalgae. The high percentage of MUFA content in *Nannochloropsis* sp. is due to the harvesting them at stationary phase. The results of total dry cell weight are a good indication for the biodiesel criteria but the content of the fatty acid composition are also a factor. Based on a study by Sajjadi et al., 2018 the percentage of lipid composition specifically MUFA was higher at stationary phase than in exponential phase. This is because during the stationary phase the nutrient

content was lower, cell division slowly decreased, and *Nannochloropsis* cells began to store lipids.

In general, high quantity of oleic acid and palmitic acid is most desirable in reducing the cardiovascular events. It also makes Nannochloropsis sp. a promising biomass for biodiesel and food industry, as it has Table 4.3 illustrated oleic acid in majority and the second highest content was palmitic acid. In earlier studies, Borowitzka, 1997 demonstrated oleic acid and palmitic acid as the main key components of the fatty acid fraction. Palmitic and oleic fatty acids, provided by aerobic desaturation and chain elongation, act as substrates for major membrane glycerolipid constituents (Erwin, 1973). In addition, physicochemical property of biodiesel was done in this study to ensure the converted Nannochloropsis sp. oil was reflected the international ASTM and EN standards. One of the physicochemical property was done in this research was refractive index. The physical property of FAME was evaluated for refractive index (nD 20 °C) as 1.4472. As unsaturation and the number of carbon atoms increase, the biodiesel refractive index increases. Again, that would clarify the disparity in the experimental results of the refractive index. Based on the obtained results, Nannochloropsis sp. was the most suitable candidate for biodiesel production.

Table 4.3 Lipid composition of *Nannochloropsis* sp.

	Structure	**Composition (%)**	
		Present work	**Previous work* (FAME range)**
Oleic acid	C18:1	72.60	14.10 – 45.40
Palmitic acid	C16:0	13.35	4.63 – 18.20
Linoleic acid	C18:3	8.86	1.19 – 12.20
Stearic acid	C18:0	3.07	1.10 – 7.10
Palmitoleic acid	C16:1	1.20	0.11 – 17.80
Eicosanoic acid	C20:0	0.44	0.63 – 1.52
Gadoleic acid	C20:1	0.28	
ΣSFAs		16.86%	0.87 - 1.50
ΣMUFAs		74.08%	
ΣPUFAs		8.86%	

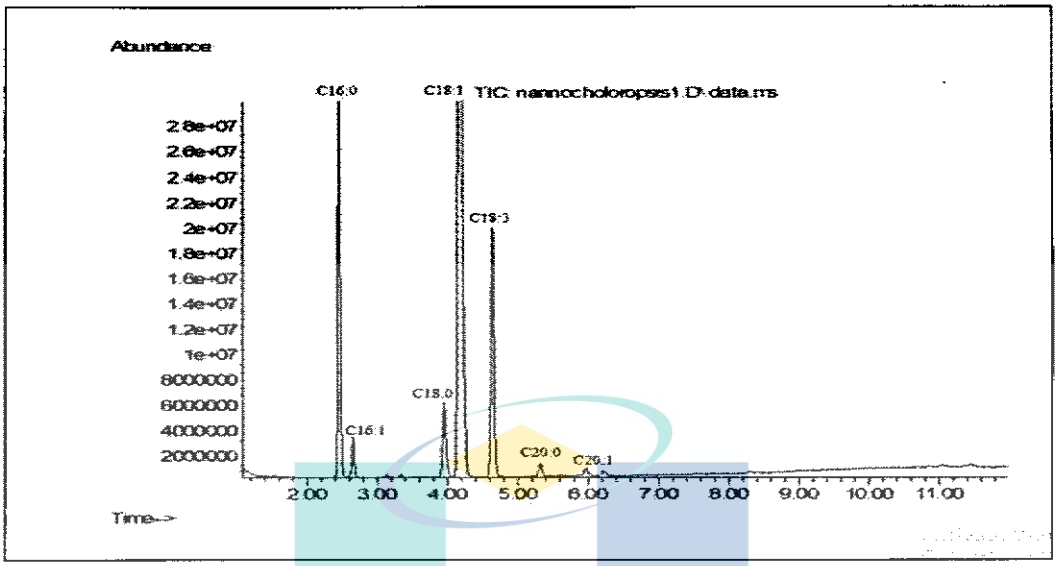


Figure 4.17 Chromatogram of methyl ester from *Nannochloropsis* sp. using GC-MS

Source: Kawk et al., (2020); Rahman et al., (2019) ; Moazami et al., (2012); Chini et al., (1999); Sukenik et al., (1993)

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Based on the results of the study, potential microalgae species were isolated and identified from the three different location which vary in depth of seawater from east coastal region, Kuantan, Pahang. Among seven isolated marine microalgae, *Nannochloropsis* sp. is an ideal candidate which successfully survived and produced high lipid content. The effect of 18:6 light/dark cycle of heterotrophic condition, salinity at 30ppt along with pH variation of 8 significantly increased the biomass and lipid production of *Nannochloropsis* sp. Under optimized condition, the combined stress factors of *Nannochloropsis* sp. which grown in 10L photobioreactor obtained highest biomass concentration of 0.874±0.02gL^{-1} and lipid production was 0.567±0.01gL^{-1} after cultivated for 20 days. *Nannochloropsis* sp. was separated by centrifugation at 6000 g to obtain biomass. Among lipid extraction methods been used in this study, Soxhlet extraction was found to be the highest (64.8±0.88%) compared to ultrasound assisted extraction (41.03±1.20%) and Bligh and Dyer (1959) (25.4±1.30%). Transesterification of *Nannochloropsis* sp. oil was done using 1% of KOH as a catalyst to convert into fatty acid methyl ester. Transesterification of the extracted oil was done using 1.5:1 volume ratio of methanol:oil in 1.5 hours reaction time at 65 °C. Thin layer chromatography was done to ensure the completion of tranesterification from *Nannochloropsis* sp. oil to biodiesel. GC-MS chromatogram depicts potential fatty acid methyl ester include oleic acid ($C_{18:1}$) 72.6%, palmitic acid ($C_{16:0}$) 13.35%, linoleic acid ($C_{18:3}$) 8.86%, stearic acid ($C_{18:0}$) 3.07%, palmitoleic acid ($C_{16:1}$) 1.20%, eicosanoic acid ($C_{20:0}$) 0.44% and gadoleic acid ($C_{20:1}$) 0.28%. Among lipid compositions, MUFA was the highest which indicates that *Nannochloropsis* sp. is the promising candidate for biodiesel production. The physical property of FAME was evaluated for refractive index (nD 20 °C) as 1.4472.

5.2 Recommendations

Overall, the research was successfully evaluated a series of experiments on enhancement of lipid in microalgae species, and a several recommendations are proposed to boost the whole research as listed below:

i. For further research, different light colours should be used in heterotrophic condition to determine the growth and lipid production.

ii. Molecular study with genetically modified *Nannochloropsis* sp. for higher lipid secretion.

iii. Different catalyst should be used in transesterification to identify the best catalyst.

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1. Paramasivam,P., Palanisamy, K.M., Maniam, G.P., Rahim, M.H.A., Govindan, N. Lipid production from *Nannochloropsis* sp. grown in palm oil mill effluent. *Proceedings of International Conference of Sustainable Earth Resources Engineering 2020 (SERIES 2020),* 641, 012-021.

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3. 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 Environmental Communication*, 2-2, 1-7.

4. 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),* 641, 012-022.

CONFERENCE

1. International Conference of Sustainable Earth Resources Engineering 2020. (SERIES 2020) 'Exploring Future Sustainable Engineering Technology' on 3rd May 2020.