PHYCOREMEDIATION USING *BOTRYOCOCCUS* SP. AS NUTRIENTS REMOVAL IN ORGANIC WASTEWATERS COUPLED WITH HYDROCARBON PRODUCTION

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"I hereby declare that the work in this Doctoral Thesis is my own except for quotations and summaries which have been duly acknowledged."

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DEDICATION

Especially to my beloved family, my supervisors and friends.

For giving me infinite care and blessing.

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ABSTRACT

Rapid population growth and industrial development are expected to contribute extremely to the world environmental crisis due to the excessive wastewater generation, global warming, climate change and increased use of petroleum fuels. In response to the problems, new technology via phycoremediation to reduce the wastewater contamination coupled with production of sustainable hydrocarbon has received much interest worldwide. Thus, the aim of the study is to produce the hydrocarbon from microalgae, *Botryococcus* sp. combined with phycoremediation of domestic wastewater (DW) and food processing wastewater (FW). The *Botryococcus* sp. locally isolated from the tropical rainforest. The optimisation study proved that the *Botryococcus* sp. grew well in the temperature of 23-33°C, the light intensity of 243 μmol $m^{-2}s^{-1}$ and 24 hours of light exposure. In fact, this *Botryococcus* sp. much more tolerated with the outdoor condition when integrated with wastewater phycoremediation in term of biomass productivity and wastewaters bioremediation. The best microalgae concentration was performed at 10^6 cells/mL for both wastewaters. The highest removal of nutrients (TP, TN and TOC) in DW and FW up to 100% and 92.8%, respectively under outdoor condition; while 95.4% and 76.4%, respectively under indoor condition. Selected heavy metal (Zn, Fe, Cd, Mn) study showed a very significant reduction (p<0.05) for both wastewaters as influenced by culture conditions. In flocculation harvesting, alum indicated the best coagulant to recover microalgae biomass from DW with efficiency up to 99.3% while chitosan showed a good candidate to harvest *Botryococcus* sp. from FW with efficiency about 94.9%. This study notably found that different culture media used in cultivation produced difference kinds of hydrocarbon compounds. As known, the biggest contribution of this algae oil as biofuel feedstock that potentially contributes to the development of renewable energy technology. Moreover, the hydrocarbon compounds obtained also have bright perspective to be used as a chemical value added in any related industry.

ABSTRAK

Pertambahan penduduk yang pesat dan pembangunan industri menyumbang kepada krisis alam sekitar akibat daripada penjanaan airsisa berlebihan, pemanasan global, perubahan iklim serta peningkatan penggunaan bahan api. Dengan itu, teknologi baru melalui *phycoremediation* untuk pemulihan pencemaran airsisa disamping berpotensi mengeluarkan biojisim untuk hidrokarbon amatlah diperlukan. Oleh itu, tujuan kajian ini adalah untuk menghasilkan hidrokarbon dari mikroalga, *Botryococcus* sp. yang di intergrasikan dengan *phycoremediation* airsisa domestic (DW) dan airsisa pemprosessan makanan (FW). *Botryococcus* sp. diperolehi daripada hutan tempatan. Kajian membuktikan bahawa *Botryococcus* sp. membiak dengan baik pada suhu 23-33°C, keamatan cahaya 243 μmol $m^{-2}s^{-1}$ dan 24 jam pendedahan cahaya. *Botryococcus* sp. lebih mengemari keadaan luar apabila digabungkan dengan air sisa dari segi produktiviti biojisim dan rawatan airsisa. Kepekatan mikroalga yang terbaik adalah pada 10^6 sel/mL. Penyingkiran tertinggi nutrien (TP, TN dan TOC) dalam DW dan FW sehingga 100% dan 92.8% dalam keadaan luar; manakala 95.4% dan 76.4% dalam keadaan tertutup. Penyingkiran logam berat (Zn, Fe, Cd, Mn) menunjukkan pengurangan yang ketara (p<0.05) untuk kedua-dua airsisa tersebut. Darisegi penuaian, alum menunjukkan agen terbaik untuk menuai mikroalga daripada DW dengan kecekapan sehingga 99.3% manakala chitosan bagus untuk menuai daripada FW dengan kecekapan pada 94.9%. Kajian ini mendapati bahawa terutamanya perbezaan media yang digunakan dalam penanaman menghasilkan sebatian hidrokarbon yang berlainan. Seperti yang diketahui, sumbangan terbesar minyak alga ini adalah sebagai bahan mentah untuk bahan api bio yang berpotensi menyumbang kepada pembangunan teknologi tenaga boleh diperbaharui. Selain itu, sebatian hidrokarbon yang diperolehi juga mempunyai potensi yang besar untuk digunakan sebagai bahan kimia tambahan dalam mana-mana industri yang berkaitan.

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LIST OF SYMBOLS AND ABBREVIATIONS

μ_{max}	-	Maximum growth rate
D_d	-	Division per day
t_d	-	Doubling time
sp.	-	Species
H	-	Hydrogen
C	-	Carbon
GHG	-	Greenhouse gases
P	-	Phycoremediation
X_0	-	Initial cell concentration
X_m	-	Maximum cell concentration
Mn	-	Manganese
Zn	-	Zinc
Cd	-	Cadmium
Fe	-	Iron
°C	-	Degree Celsius
M	-	Mole
H	-	Hour
N	-	North
E	-	East
k	-	Coefficient rate constant
t	-	Time
BOD	-	Biochemical oxygen demand
COD	-	Chemical oxygen demand
TP	-	Total phosphorus
TN	-	Total nitrogen
DW	-	Domestic wastewater
FW	-	Food processing wastewater

TOC	-	Total organic carbon
TSS	-	Total Suspended Solid
IC	-	Inorganic carbon
TC	-	Total carbon
ICP-MS	-	Inductively coupled plasma mass spectrometry
BBM	-	Bold's Basal Medium
GC-MS	-	Gas chromatography-mass spectrometry
FT-IR	-	Fourier transform infrared spectroscopy
RSM	-	Response surface methodology
DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase chain reaction
OD	-	Optical density
APHA	-	American Public Health Association
ASTM	-	American Society for Testing and Materials
UTHM	-	Universiti Tun Hussein Onn Malaysia

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Wastewater is well known to contain contaminants that can negatively impact the environment if not controlled in terms of pollutant load removal. This is because wastewater containing nutrients such as nitrogen and phosphorus can affect the natural ecosystem particularly the aquatic lives. Large amounts of water used for domestic and industrial purposes result in the generation of large volume of wastewater loaded with nutrients (Danilović *et al.,* 2013). In addition, wastewater may also contain heavy metal pollutants, especially in industrial wastewater eg. Iron, cadmium, zinc, ammonia etc. (Danilović *et al.,* 2013). Furthermore, the presence of heavy metal and organic compound in wastewater can cause long-term problems (Chan, Salsali & McBean, 2014; Onalo, Matias-Peralta & Sunar, 2014; Travieso *et al.*, 1999). Considering all these facts on wastewater, issues as such need to be solved correctly without contributing other problems.

Meanwhile, Sriram & Seenivasan (2012) stated that the wastewater is a word to represent the water with poor or low quality that contains a high amount of pollutants and microbes. Thus, discharging wastewater directly into the water body may lead to the serious environment and human health problems. Without any compromises, pollutant loads in wastewater should be removed to ensure compliance with the local effluent standard before discharging into the environment. Nowadays, selection of treatment method is one interesting topic among the researchers either conventional, bioremediation or advanced method. Phycoremediation is a branch of bioremediation technique in wastewater treatment. According to Phang, Chu &

Rabiei (2015), phycoremediation is the use of either macro or microalgae for the removal or biotransformation of pollutants including nutrients and toxic chemical from wastewater. In the past, microalgae have attracted much attention as an alternative to the conventional treatment method. Microalgae wastewater treatment is an eco-friendly approach that offers the advantages of a cost effective way of removing pollutant loads (Sriram & Seenivasan, 2012). The previous study has reported that the use of algae to treat wastewater has been in practice for over 40 years (Ahmad, Khan & Yasar, 2013) and the first description of this application was reported by Oswald in 1957. Wastewater bioremediation, (phycoremediation) technology can also be combined with hydrocarbon production. Since the hydrocarbon was obtained from biological plant or algae, then it's called as bio-hydrocarbon. In the terminology of its chemical, the hydrocarbon is an organic compound consisting entirely of hydrogen (H) and carbon (C). Hydrocarbon production from microalgae refers to the lipid or oil content that can be obtained from algae biomass. The most popular hydrocarbon product that has been investigated from microalgae recently is biofuel (Rawat *et al*., 2011). These algae do not only produce biofuel but also great potential to create other bio-based product such as fertiliser, animal foods and bioactive chemicals (Sivakumar & Rajendran, 2013). According to Amaro, Guedes, & Malcata (2011), microalgae such as *Botryococcus* sp. produced highest up to 75% of oil in dry weight basis, which has high potential as a new bio-based product for renewable energy development. Therefore, this species was chosen as the microalgae that conducted in this study.

As the demand for energy continues to increase globally, fossil fuel usage will likewise continue to rise. There is still a plentiful supply of fossil fuels at reasonably low cost, although this is likely to change in the future. More critically, though, rising use of fossil fuel is unlikely to be sustainable in the longer term, principally due to the attributed increase in greenhouse gases emission and the environmental impact of this emission on global warming (Hill *et al.*, 2006).

Compared with other forms of renewable energy (e.g. the wind, tidal and solar), biofuels allow energy to be chemically stored and conveniently be used in existing engines and transportation infrastructures after blending to various degrees with petroleum diesel (Singh & Gu, 2010). This biodiesel is, in essence, a set of monoalkyl esters of long- chain fatty acids – and at present is derived chiefly from the acylglycerols of plant oils. Besides being renewable, biofuel is also non-toxic and

biodegradable (Meher, Vidyasagar, & Naik, 2006).These are just a few of the reasons why a renewed interest has arisen in recent years towards producing hydrocarbon from microalgae. Microalgae clearly present a few advantages: they have much higher biomass production compared to terrestrial plants (Singh & Gu, 2010). In addition, microalgae need a lower rate of water renewal compared to the terrestrial crop. Moreover, microalgae only require sunlight and few inexpensive nutrients (phosphorus and nitrogen) to grow (Aslan & Kapdan, 2006). Therefore, hydrocarbon production from microalgae is expected to offer new opportunities to diversify income and fuel supply sources and to promote employment in rural areas. Furthermore, such venture aims to develop long-term replacement of fossil fuels, and to reduce GHG emissions, boosting the decarbonisation of transportation fuels and increasing the security of energy supply (Mata, Martins, & Caetano 2010).

1.2 Problem statement

The escalating population each year lead to the development of various industries is producing huge amounts of wastewater. To date, population clock of Department of Statistic Malaysia (2016) has estimated the number of world population to about 7 billion while in Malaysia itself, population clock shows 31 million people currently. Since the number of people is increasing steadily each year, the probability of causing an accretion in wastewater is high.

By that, production of wastewater coming from residential well known as domestic wastewater increase dramatically (Uwidia & Ademoroti, 2011). In Malaysia, it has been estimated that the citizens generate six million tonnes of domestic wastewater every year. Locally, the volume of domestic wastewater from residential areas can significantly impact the quality of life if released into the waterways freely. In fact, Asadi *et al.* (2013) reported that, in some rural areas of Malaysia, domestic wastewater was discharged into the river directly. So, this kind of scenario needs to handled and mitigated wisely since domestic wastewater considered a complex mixture containing water together with common constituents such as organic and inorganic matter and microorganism (Huang *et al.*, 2010). Moreover, Sperling (2007) stated that the consumption of water by community size, for example in an average town inhabited by 50,000 – 250,000 population be able to use the water up to 120 – 220 L/inhab.d. Indirectly, this amount is able to generate

the production of domestic wastewater that expected harmful to the public health and environment. Even worse, 90% of wastewater in developing countries is discharged into the river, lake, sea and environmental without any treatment (WWAP, 2012; Zhu, 2014). Thus, it can threaten public health and food security and affect access to safe and clean water for drinking and bathing. Domestic wastewater problem needs to be solved professionally to give awareness about the dangers of the wastewater on health and well-being of nature. This was the motivation behind the present study.

Generation of wastewater is not only caused by domestic consumption but also due to industries which are rapidly growing, especially in developing countries. An example is the food industry. Food processing in Malaysia is a vital industry that plays an important role in economic development especially for small-scale production (Ahmed, 2012; Shamsudin *et al.,* 2011). Interest in the food processing industry is related to the production, consumption and export-import activities to boost country revenues and income. According to the Malaysian Industrial Plan 2006-2020 period, the food processing industry's investments are expected to increase each year up to RM24.6 billion in 2020 (Ayupp & Tudin, 2013). In food processing factories, there is a huge amount of water used, directly generating a volume of wastewater from washing and processing activities (Shin *et al.,* 2015; Tenca *et al.*, 2013; Vanerkar, Satyanarayan, & Satyanarayan, 2013). Consequently, on a global scale, the food industry generates wastewater which has a significant effect on the environment which is similar to that of municipal wastewater if uncontrolled discharged into the water bodies (Gentili, 2014). In addition, 85% of small-scale industries did not provide the facility of primary treatment to their wastewater and about 87% of these industries dispose their contaminated effluent directly into the municipal drainage (Pattanshetti & Gawande, 2015). This may due to the lacking of effective policy and poor enforcement from the local authority. Moreover, most of the small-scale industries do not have enough investment and solely rely on the government grant to run their daily business and lead to the poor management of their waste especially wastewater (Pattanshetti & Gawande, 2015). Therefore, the creation of innovative and sustainable idea to treat the food wastewater is highly required so that any food industry wastewater must meet the lowest requirement quality of the effluent standard before released to the environment. The food processing wastewater such as food and milk processing industries consumed large volume of water then characterized by high BOD (442 –

523.5 mg/L) and COD (8960 – 11900 mg/L) with fats, oil and grease and other nutrient such as nitrogen, phosphorus and potassium (Qasim & Mane, 2013). All these nutrients are suitable for the growth of microorganism that can cooperate to absorb the pollutant loads like algae. (Ji *et al*., 2015) also reported that food processing wastewater was rich in nutrient including nitrogen (1385 mg/L), phosphorus (108 mg/L), calcium (ND), iron (24.7 mg/L), aluminium (316.4 mg/L) and total organic carbon (14898 mg/L). Recently, increasing concern on environmental and health risks evaluations are demanded with a more rigorous control of wastewater specifically, promoting the development of new treatment technologies capable of dealing with toxic organic pollutants (Syafiie *et al.,* 2011). In addition, it has become a priority to develop wastewater treatment technology employing environmentally friendly approaches and economical systems with the minimal use of chemicals.

Continuous increases in populations not only produce unconceivable wastes but also fossil fuel consumption which may be possibly exhausted in the near future. Hydrocarbons from fossil fuel are considered unsustainable because they are non-renewable. Apart from that, it also contributes to the release of CO_2 gas that has negative effects on climate change and the environment. Renewable carbon neutral and transport fuels are necessary for environmental and economic sustainability. Hydrocarbon-derived from oil crops is a potential renewable and carbon neutral alternative to petroleum fuels. Unfortunately, oil from crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the existing demand for hydrocarbon in the future (Chisti, 2007). Therefore, there is a need to find the alternative solution to replace the existing use of fossil fuel. The bio-hydrocarbon form phycoremeditioan in this study is one of the innovative steps to explore the potential for sustainable of alternative biofuel.

Furthermore, conventional wastewater treatments are expensive and require high energy input. In fact, conventional process generates large amount of sludge, and handling and disposal this sludge is one of the challenges works to the present technology. Therefore, high operational and maintenance of treatment plant make it less economical approach. These challenges are able to be overcome by using phycoremediation process (Rawat *et al.,* 2016). This has been proven by the cultivation of microalgae such as *Scenedesmus* sp., *Chlorella* sp., *Demodesmus* sp. and *Spirulina platensis* using wastewaters simultaneously reduce the pollutants in

wastewater. Similarly, green microalgae *Botryococcus braunii* also had been successfully employed in phycoremediation process of different wastewater. For instance, *Botryococcus braunii* was able to remove 93.3% of TP in unsterilized swine lagoon wastewater (Liu *et al.*, 2013). In the treatment of domestic wastewater, *Botryococcus braunii* was able to eliminate phosphate and ammonium up to 99% and 99.6%, respectively when 100% concentration of municipal wastewater was used (Can *et al.*, 2013). This has led to the selection of *Botryococcus* sp. as the best microalgae candidate in phycoremediation of wastewaters. Recently, Atiku *et al.*, (2016) revealed that this microalgae genus (*Botryococcus* sp.) was able to remove ammonium ion (NH_4^+) from greywater about 92%-98%. Moreover, *Botryococcus* sp. used in this study locally collected and known as indigenous species where having high metabolic pathway and highly tolerant with local climate. Biomasses resulted from phycoremediation process can be used as a biofuel feedstock and value-added chemical in biotechnology industry. These factors were the motivation behind the application of *Botryococcus* sp. in the present study.

1.3 Objectives of study

The objectives of this study are:

i. To optimise the specific growth rate and biomass productivity of *Botryococcus* sp. at the different situation of environmental factors.

ii. To develop a new technique employing microalgae *Botryococcus sp.* in treating wastewaters (domestic and food processing wastewater).
    - To examine the efficiency of the integration of *Botryococcus* sp. cultivation with domestic and food processing wastewater.
    - To identify the suitable growth rate and biomass productivity of *Botryococcus* sp. in domestic and food processing wastewater.

iii. To evaluate the potential of biomass production from microalgae biomass cultivated using closed photobioreactor.
    - To optimise the harvesting efficiency via flocculation process using inorganic and organic coagulants.
    - To measure the biomass production from microalgae cultivated using wastewaters media in a photobioreactor.

iv. To establish the chemical composition profile of bio-hydrocarbon production from microalgae biomass cultivated in domestic and food processing wastewater.

1.4 Scope of study

This study carried out using freshwater microalgae, *Botryococcus* sp., an indigenous microalga isolated from the tropical rainforest of Malaysia (Endau Rompin National Park, Johor). This microalga was employed in different wastewater (domestic wastewater and food processing wastewater) treatment. Physiochemical and heavy metals content measurement was conducted based on the Standard Methods and Examination of Wastewater (APHA, 2012). The phycoremediation study was conducted according to the maximum growth rate and biomass productivity of the algae according to the optimisation result. There are four basic environmental factors for optimisation such as temperature, light intensity, photoperiod and salinity were study using the synthetic medium. The phycoremediation was conducted with two culture conditions namely outdoor and indoor culture.

The outdoor culture was according to natural outdoor condition while indoor culture based on the optimisation value of environmental factors. The duration of phycoremediation also determined from optimisation study which is 18 days. The wastewater parameters were analysed at an interval of 3 days throughout the treatment duration. The growth mathematical model was developed and first order kinetic used to predict the removal of nutrient from wastewater. Flocculation technique was employed to harvest the microalgae biomass from the wastewaters. The inorganic (alum) and organic (chitosan) were used as flocculant was and the comparison was made in term of harvesting efficiencies via face-centered central composite design (FCCCD) analysis.

The universal oven was used for drying the algal paste biomass prior to solvent (95% n-Hexane) extraction using soxhlet apparatus. Lastly, crude algae oil is screened and characterised for hydrocarbon compound using Gas Chromatography and Mass Spectrometry) (GC-MS) and Fourier Transform Infrared Spectroscopy (FTIR).

1.5 Importance of study

In order to meet the environmental regulation, all types of wastewater need to be treated before it's discharged to the environment. The regulation wastewater treatment system prioritises the development of wastewater treatment technology employing environmental friendly and economical systems with the minimal use of chemicals. This can be achieved using microalgae in bioremediation technique (phycoremediation). Phycoremediation process releases less of greenhouse gases if compared to the conventional wastewater treatment. This is because the microalgae consume more CO_2 for growth and released O_2 as by–product of the process photosynthesis.

Since phycoremediation is bioremediation of wastewater using microalgae, then the novelty and inventiveness are the sources of microalgae species was utilised in this study. *Botryococcus* sp. was originally collected from Endau Rompin National Park and known as a wild type of microalgae that contain high resistance and capability in the metabolic process in their cell. These special characteristics due to the location of this type of algae lives.

This type of algae also known as indigenous species and have not been studied intensively yet in our country for phycoremediation treatment since it was locally collected from the tropical rainforest. The significant of this research is by combining the function of *Botryococcus* sp. in wastewater treatment and for further bio-hydrocarbon production.

Bio-hydrocarbon is very useful to produce a sustainable product from microalgae, for example, biofuel or biodiesel. As we known, the advantages of biodiesel are non-toxic and biodegradable thus it becomes an environmentally friendly biofuel production. Other than that, hydrocarbon derives from algal oil also potentially used as an other bio-based product such as bio-plastic, bio-fertilizer, fish feed, bioactive compound, pharmaceutical and value-added chemical feedstock in any industry. Thus, the novelty of this research lies in the double function of *Botryococcus* sp. to assimilate pollutants load in wastewaters and to produce sustainable bio-renewal hydrocarbon.

1.6 Dissertation overview

This dissertation consists of eight chapters. Chapter 1 describes the background of the study, problem statement, objectives and important of this research. Chapter 2 provides a critical review of the research about wastewater treatment, phycoremediation, microalgae cultivation and microalgae harvesting system. Meanwhile, Chapter 3 explains in details of the method development used in this study including wastewater examination, flocculation harvesting, extraction and hydrocarbon analysis. Chapter 4 is a result and discussion about the microalgae growth assessment under different environmental factors while Chapter 5 is discussing the phycoremediation study conducted in different culture conditions. Chapter 6 elaborates the production of microalgae biomass using photobioreactor and harvesting efficiency experiments via response surface methodology analysis and Chapter 7 explains about the establishment of hydrocarbon profile in microalgae oil cultivated using a different type of wastewaters. Chapter 8 is the conclusion for the overall objectives setup and further works to be recommended in the future.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Phycoremediation is one of the biological treatments that are considered sustainable and environmentally friendly method to eliminate contamination in wastewater. Other than biotransform the presence of the pollutant in wastewater, microalgae also well-known as an excellent source of hydrocarbon. Hydrocarbon from microalgae oil is considered sustainable since it is extracted from the biological plant. Nowadays, the increasing number of population and various types of industries in the world lead to the augmentation of wastewater disposal to the environment. Microalgae phycoremediation is one of the promising techniques that have high potential to assimilate the excessive pollutants in wastewater photosynthetically. Consequently, the discovery of new technologies to mitigate the adverse impact on the environment combined with sustainable hydrocarbon evaluation became one of the aims of this chapter. Therefore, this chapter provides a comprehensive review of wastewater phycoremediation studies and the potential of hydrocarbon extracted from microalgae. The cultivation system and harvesting approach of microalgae also discussed accordingly.

2.2 Characteristic of wastewaters

There are two types of wastewater discussed in this section. Among of them are domestic wastewaters and food processing wastewater. Each type of wastewaters is containing the different composition of biological, chemical and physical

characteristics. There have been several studies in the literature reporting about this wastewater characterization and method to reduce the pollutants load before discharged to the environments.

2.2.1 Domestic wastewater

In physical properties, Metcalf & Eddy (2003) said that wastewater temperature is important as it affects the chemical and biological reaction of aquatic organisms. Metcalf & Eddy (2003) also highlighted temperature was very important in the determination of various parameters such as pH, conductivity saturation level of gases and various form of alkalinity.

In term of chemical characteristic, Davis & Cornwell (2008) found that chemical compound in wastewater are almost limitless thus only a few general classes of compound are considered. According to Jorgensen & Weatherley (2003), organic material is a combination of carbon, hydrogen and oxygen and another important element such as ammonia. The occurrence of ammonia can be accepted as the chemical evidence of organic pollution. While common inorganic pollutant in wastewater is chloride, hydrogen, irons, nitrogen, phosphorus and amounts of heavy metal. The biological parameter is important to wastewater treatment because it contain a large amount of microscopic organisms. Within treatment facilities, wastewater provides an ideal medium for potential microbial growth, irrespective of being anaerobic or aerobic wastewater treatment (Mata *et al.*, 2012). Each contaminant has its own significance which the suspended solids can lead to the development of sludge deposits and anaerobic conditions when untreated wastewater is discharged in the aquatic life and when discharged on land, they also can contribute to the groundwater pollution.

Therefore, Table 2.1 shows the combination of the physical, chemical and biological characteristic of domestic wastewater done by the previous researcher before treatment. The different value of pollutant load in those wastewaters may due to the location of the domestic wastewater sampling. For example, Saeed *et al.* (2014) conducted the study using municipal wastewater collected from local outlet in Dhaka, Bangladesh while Thongtha *et al.* (2014) was using influent before treatment of domestic wastewater Bangkok, Thailand and Ahmad *et al.* (2013) collected

domestic wastewater from various sewage drain at Lahore city in Pakistan. Detail of the physiochemical composition each source summarised in Table 2.1 below.

Table 2.1: Domestic wastewater composition

No.	Parameter	References			Domestic effluent standard
		Saeed *et al.* (2014)	Thongtha *et al.* (2014)	Ahmad *et al.* 2013)	
1	pH	7.1	7.59	-	5.5 – 9[a]
2	DO (mg/L)	0.06	-	-	-
3	Turbidity	86.8	-	-	-
4	Total suspended solid (mg/L)	-	-	970	< 100[b]
5	Total Dissolved Solid (mg/L)	-	272	4650	< 5000[a]
6	COD (mg/L)	4048	77.8	721	< 100[b]
7	BOD (mg/L)	1903	20	407	< 50[b]
8	Total Phosphorus (mg/L)	23.1	1.04	22	0.1[a]
9	Total Kjeldahl Nitrogen (mg/L)	-	5.32	35	35[a]
10	Phosphate (mg/L)	-	-	10	-
11	Nitrate(mg/L)	115.5	-	-	-

[a]Thongtha *et al.* (2014)
[b] Environmental Quality Act of Malaysia 1974

2.2.2 Food processing wastewater

Generally, food processing wastewater can be categorised into four main sectors including fruit and vegetable, meat, poultry and seafood, beverage and bottling and dairy operations (Abdalla, 2014). Most of them consume or use a huge amount of water for their processing in the factory. Considerable parts of this wastewater are potential to be treated before released to the environment. Again according to Abdalla (2014), 50% of the water used in the fruit and vegetable sector is for washing and rising. The characterization of food processing wastewater is considered as nontoxic because it contains few hazardous compounds with the exception of some toxic cleaning products. Thus, wastewater discharged from food processing industry is organic due to high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Vanerkar *et al.*, 2013). The characterization of food wastewater described wide variation due to the type of product and also different raw material used.

Therefore, the data presented in Table 2.2 show there are many differences among the characteristic of food processing wastewater by different authors. Based on the study done by Cristian (2010), found that food wastewater contains high of BOD (4840.6 mg/L), COD (19251.2 mg/L) and TSS (5802.6 mg/L) if compared to

discharged limit. This amount of concentration may due to the wastewater come from dairy and milk processing. In general, wastewater from dairy processing industry contains a high concentration of organic material such as protein, carbohydrate and lipid. Cristian (2010) also reported that this kind of food wastewater high in chloride up to 616 mg/L which is significantly toxic to aquatic life. The reason why high in chloride because the presence of sodium chloride from salting the food product.

Table 2.2: Review of food processing wastewater pollutant

No	Parameter	Unit	References				Discharged Limit
			Cristian (2010)	Vanerkar *et al.* (2013)	Qasim & Mane (2013)	Ji *et al.* (2015)	
1	Biochemical oxygen demand (BOD)	mg/L	4840.6	6860	486.7	NA	< 50[a]
2	Chemical oxygen demand (COD)	mg/L	10251.2	11220	9720	NA	< 100[a]
3	Total suspended solid (TSS)	mg/L	5802.6	2210	538.8	Na	< 5000[c]
4	Chloride	mg/L	616	182	234.7	NA	-
5	pH	-	8.34	4.12–4.28	5.64	6.0	5.5 – 9[c]
6	Nitrogen (TN)	mg/L	663	16.4	95.2[b]	1385	35[c]
7	Phosphorus (TP)	mg/L	153.6	3.2	NA	108	0.1[c]
8	Total Organic Carbon (TOC)	mg/L	NA	NA	NA	14898	-
9	Zinc, Zn	mg/L	NA	0.62	NA	1.29	< 1.0[a]
10	Manganese, Mg	mg/L	NA	0.444	NA	97.01	< 1.0[a]
11	Ferum, Fe	mg/L	NA	12.63	NA	24.7	< 5.0[a]

[a] Environmental Quality Act of Malaysia (1974)
[b] Measured in TKN (Total Kjeldahl nitrogen)
[c] Thongtha *et al.* (2014)

Meanwhile, Vanerkar *et al.* (2013) also found that food processing wastewater they used in their study large concentration in BOD, COD, TSS, TN and chloride if compared to discharged limit. For example, BOD and COD could reach up to 6860 mg/L and 11220 mg/L, respectively. Other than that, metal elements also examined then contained Zn (0.62 mg/L), Mg (0.444 mg/L) and Fe (12.63 mg/L). The present of both organic nutrient and metal elements in this food processing wastewater is due to the additive used for a different food product. Besides, Qasim & Mane (2013) investigated the organic content of food processing wastewater from sweet snack factory lower that food wastewater studied by Cristian (2010) and Vanerkar *et al.* (2013). For example, BOD and COD are 486.7 mg/L and 9720 mg/L,

respectively but still significantly higher than acceptably discharged limit whereas pH value is in the range of discharged limit. While, for TKN, TSS and chloride are 95.2 mg/L, 538.8 mg/L and 234.7 mg/L, respectively. As a summary, effluent from food processing wastewater is very different in term of discharged concentration due to the season and type of process used in their industry respectively (Qasim & Mane, 2013).

2.3 Impact of wastewater on environment

The untreated wastewater discharged into the river degrades the quality of water. This problem normally causes the lack of clean water for human consumption. The untreated wastewater also one of the major contributor for the eutrophication process to take place. This situation can be explained where the water body receive an excessive amount of nutrient that causing a negative impact to the environment such as the depletion of oxygen level in the air and at once induces reductions in species of fish and other microbe populations (Sperling & Chernicharo, 2005).

In term of heavy metals, pollution was released from industrial and domestic sources causes serious changes in the aquatic ecosystem, resulting in a loss of biological diversity and the magnification and bioaccumulation of toxic agents in the food chain (Souza *et al.*, 2012). The aquatic such as a river, pond and lake are mainly affected by pollutant and heavy metal discharged in industrial effluents and represent a potential risk to human health and life (Souza *et al.*, 2012). Thus it is very significant to treat the wastewater before discharged either into the river or to the land, consumed or being used for other purposes.

2.4 Conventional wastewater treatment

In general, treatment of wastewater is the process of reduction or remove of excessive impurities present in wastewater. The impurities imply to the constituent concentration that more than the acceptable level for final discharge (Karia& Christian, 2013). Conventionally, wastewater treatment is a combination of physical and biological processes designed to remove organic matter and solids from solution (Hammer, 2004). Therefore, a wastewater treatment plant is designed for either of the following systems called preliminary treatment, primary treatment, secondary

treatment and tertiary treatment (Karia & Christian, 2013). The pictorial diagram in Figure 2.1 summarises the processes applied in conventional wastewater treatment.

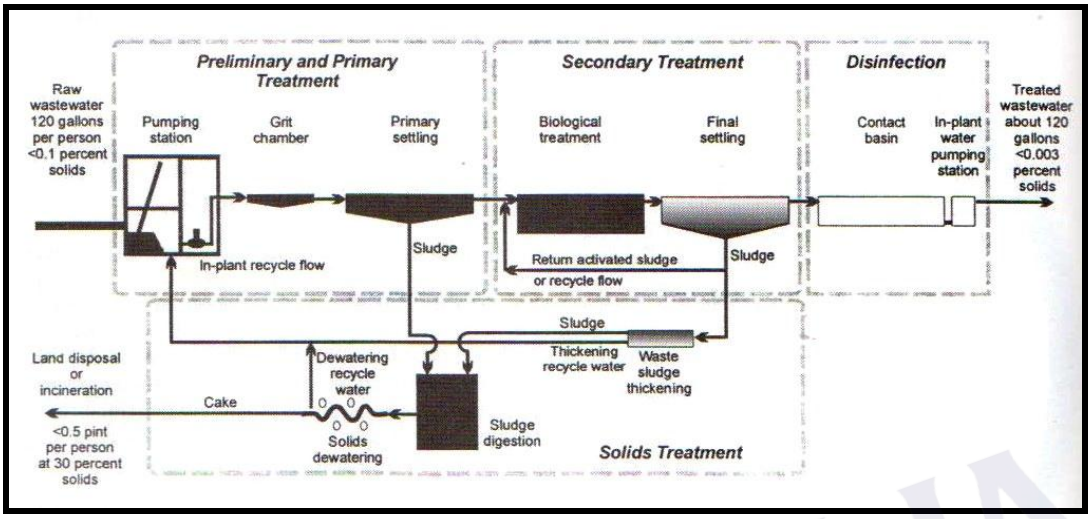


Figure 2.1: Schematic of conventional wastewater treatment processes (Hammer, 2004)

2.4.1 Preliminary treatment

The preliminary treatment is mainly to remove floating materials and large inorganic particulate contents of wastewater that usually cause maintenance or operational problems in the primary and secondary treatment of wastewater. It is also known as pre-treatment in the treatment system. The preliminary treatment includes sump and pump unit, approach channel, screen chamber, grit chamber and skimming tank. Screen and grit chamber are used to remove large floating materials and to remove up to 0.2µm size suspended solid, respectively. Meanwhile, skimming tank or typically named as oil and grease traps is used to remove or trap excessive oil and grease from raw influent (Karia & Christian, 2013).

According to Abdel-Raouf *et al.* (2012) described that preliminary treatment of sewage removes large solid materials delivered by sewers that could obstruct flow through the plant or damage equipment. These materials are composed of floating objects such as rags, wood, faecal material and heavier grit particles. Large floating objects can be removed by passing the sewage through bars spaced at 20 – 60 mm; the retained material is raked from the bars at regular intervals. Grit is removed by

reducing the flow velocity to a range at which grit and silt will settle, but leave the organic matter in suspension; this is usually in the velocity range of 0.2–0.4 m/s.

2.4.2 Primary treatment

Primary treatment includes all the unit in preliminary treatment and primary sedimentation tank, also known as primary settling or primary clarifier (Karia & Christian, 2013) (refer to Figure 2.1). It consists of temporary holding the sewage in a quiescent basin where heavy solid can settle to the bottom while oil, grease and light solid float to the surface (Rawat *et al.,* 2011). The settled and floating materials are removed and the remaining liquid may be discharged or subjected to secondary treatment.

The primary treatment can reduce about 60-70% of fine settleable suspended solid, which include about 30-32% of organic suspended solids (Karia & Christian, 2013). Meanwhile, Abdel-Raouf *et al.* (2012) reported that a well-designed sedimentation tank is capable of removing 40% of the BOD in the form of settleable solids and pathogen removal during primary treatment is highly varied with various removal rates reported for different organisms. However, colloidal and soluble organic content of wastewater is not removed at this stage.

2.4.3 Secondary treatment

The main purpose of secondary treatment is to reduce the soluble BOD that escapes from primary treatment by reducing the organic matter. Other than that, secondary treatment also to provide further removal of suspended solids (Abdel-Raouf *et al.,* 2012). Normally, biological processes are employed to remove the remaining organic and inorganic content in wastewater. Biological treatment processes are considered the most environmentally compatible and the least expensive of wastewater treatment method. These processes use microorganisms to break down or assimilate the chemical presents in wastewater (Rawat *et al.,* 2011). Microorganism converts the colloidal and soluble organic matter into various gases and into protoplasm. According to Cammack & Attwood (2006), protoplasm is the living contents of a cell that is surrounded by a plasma membrane. Since protoplasm has a specific gravity slightly greater than of water, it can be removed from the treated liquid by

gravity settling. Abdel-Raouf *et al.* (2012) found several investigators have pointed out that biological oxidation system can remove over 90% of pathogenic bacteria from sewage. In suspended growth reactors the intimate mixing of solid flocs and sewage gives 90% removal.

2.4.4 Tertiary treatment

Tertiary treatment process aims to remove all organic ions. It can be achieved biologically and chemically. The biological process appears to perform well when compared to the chemical processes which are in general too costly to be applied in most places and which may lead to the secondary pollution (Abdel-Raouf *et al.*, 2012). Tertiary treatment can be accomplished by the following stage such as granular filtration, membrane filtration, carbon absorption, phosphorus removal and nitrogen control (Davis & Cornwell, 2012).

Abdel-Raouf *et al.* (2012) stated that removal of ammonium, nitrate and phosphate was estimated four times more expensive than primary treatment in complete tertiary treatment. Typically, tertiary treatment combined with advanced treatment which is generally based on the complex technology as mentioned by (Davis & Cornwell, 2012). These techniques include a process to remove particular nutrient such as phosphorus and nitrogen which can stimulate eutrophication in certain situations.

2.5 Phycoremediation

Phycoremediation defined in a wider meaning as the use of microalgae or macroalgae for removal or biotransformation of pollutants, including nutrients and xenobiotics from wastewater and CO_2 from the waste air with concomitant biomass propagation (Phang *et al.*, 2015). There are numerous processes of treating water, industrial effluents and solid wastes using microalgae aerobically as well as anaerobically. Remediation is generally subject to an array of regulatory requirements, and also can be based on assessments of human health and ecological risks where no legislative standards exist (Rawat *et al.*, 2011).

As introduced by John (2000) where the term of phycoremediation was referred to the remediation carried out by algae. The use of algae to treat wastewater

has been in vogue for over 40 years, with one of the first descriptions of this application being reported by Oswald *et al.*, (1957) while Oswald (1988) found that the usage of microalgae for the treatment of municipal wastewater has been a subject of research and development for several decades. A lot of extensive work has been conducted to explore the feasibility of using microalgae for wastewater treatment, especially for the removal of nitrogen and phosphorus from effluents. It is simply a matter of allowing the consumption of nitrogen and phosphorus by microalgae in a controlled manner that benefits rather than deteriorates the environment. Concentrations of several heavy metals have also been shown to be reduced by the cultivation of microalgae, which has been explored and studied extensively by Muñoz & Guieysse (2006).

In order to improve the understanding about phycoremediation, Figure 2.2 below shows the biological treatment enhances the removal of nutrients, heavy metals and pathogens and furnish O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using, in turn, the CO_2 released from bacterial respiration.

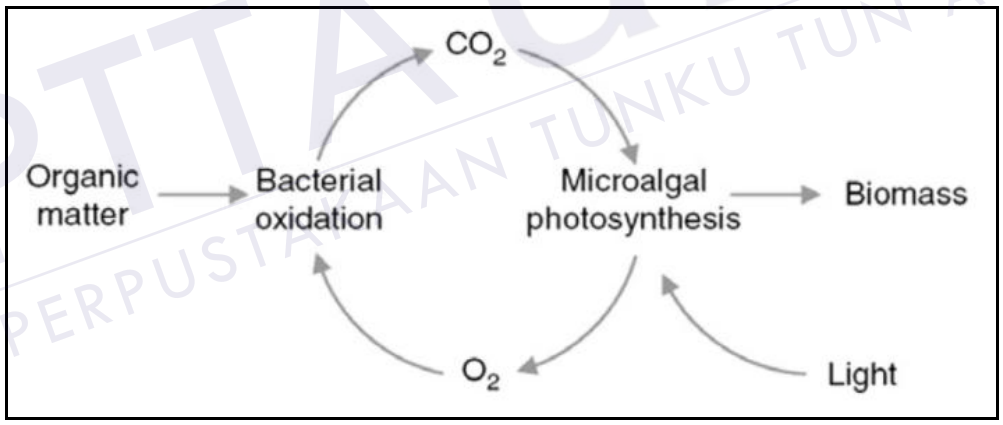


Figure 2.2: The principle of photosynthetic oxygenation in BOD removal process (Muñoz & Guieysse, 2006).

Photosynthetic aeration is therefore especially interesting to reduce operation costs and limit the risks for pollutant volatilization under mechanical aeration and recent studies have shown that microalgae can indeed support the aerobic degradation of various hazardous contaminants (Muñoz & Guieysse, 2006). Therefore, Rawat *et al.* (2011) have stated that the mechanisms involved in microalgae nutrient removal from industrial wastewaters are similar to that from

domestic wastewaters treatment. Phycoremediation comprises several applications: (i) nutrient removal from municipal wastewater and effluents rich in organic matter; (ii) nutrient and xenobiotic compounds removal with the aid of algae-based biosorbents; (iii) treatment of acidic and metal wastewaters; (iv) CO_2 sequestration; (v) transformation and degradation of xenobiotics; and (vi) detection of toxic compounds with the aid of algae-based biosensors. Nutrient removal with the aid of microalgae compares very favourably to other conventional technologies (Rawat *et al.,* 2011*).*

2.5.1 Advantages of phycoremediation

Microalgae play an important role in the tertiary treatment of domestic wastewater in maturation ponds or the treatment of small to middle-scale domestic wastewater in facultative or aerobic ponds (Rawat *et al.,* 2011). Nitrogen uptake could be increased if the microalgae were pre-conditioned by starvation. These hyper-concentrated algal cultures, called 'activated algae' were shown to decrease the land and space requirements for microalgae treatment of wastewaters. This process removed nitrogen and phosphorus within very short period of time. There is evidence that production of microalgae, given proper conditions, may be high enough even during colder periods to be of interest for wastewater treatment.

However, this is to be verified under the actual local environmental conditions, since many strongly variable factors are involved when defining microalgal growth and species composition. Microalgae can be efficiently used to remove a significant amount of nutrients because they require high amounts of nitrogen and phosphorus for protein, nucleic acid and phospholipid synthesis. Thus, Sivasubramaniam (2013) has listed several benefits when using phycoremediation in the process of treating the wastewater pollution which is:

i. Phycoremediation is a cost-effective, eco-friendly and a safe process.
ii. The microalgae employed are non-pathogenic photosynthetic organisms and they do not produce any toxic substances.
iii. Phycoremediation effectively reduces nutrient load thereby reducing total dissolved solid.
iv. Phycoremediation reduces sludge formation to a very large extent.

v. Phycoremediation increase dissolved oxygen levels through photosynthetic activity.
vi. Phycoremediation keeps the bacterial population under control.
vii. Algal growth in the effluent also removes waste CO_2 from the air thereby contributing to the reduction of greenhouse gases.
viii. The algal biomass has high nutrient value and can be suitable as a live feed for aquaculture.
ix. The algal biomass could also be used as a Bio-fertilizer and in EM (Effective Microbes).
x. Conventional chemical treatment of effluent results in concentrating the toxic waste in the form of sludge and requires landfill. Whereas phycoremediation detoxifies and removes it forever.
xi. Minimal odour compared to conventional methods of treatment.
xii. Simple operation and maintenance.
xiii. Construction and operation costs are typically less than half those of mechanical treatment plants (e.g. activated sludge, sequencing batch reactors).
xiv. Sustainable treatment solution with significant potential for energy and nutrient recovery.

2.6 Bioremediation of heavy metal by microalgae

Heavy metal is the pollutant that considered to be a significant environmental problem related to human health (Chekroun & Baghour, 2013). The contamination of water by toxic metals and organic pollutants recently increased due to anthropogenic activity. Thus, bioremediation techniques to assimilate that toxic have a high potential to be applied in wastewater treatment. Bioremediation is a process of using specific microorganisms to transform hazardous contaminations in water to nonhazardous waste products (Dwivedi, 2012).

Dwivedi also described there are two steps involved in the assimilation of heavy metals. First, the metal is adsorbed over the cell very quickly called physical adsorption (Dwivedi, 2012). Next, these metals are assimilated slowly into the cytoplasm in a process named chemisorption. However, absorption of heavy metal depends on the other parameter such as pH. As highlighted by Dwivedi (2012), surface charge studies showed that the availability of free sites depended on pH.

With increasing pH, the surface charged sites of calcium alginate became more negative, and then the uptake of metal increased with increasing pH.

Table 2.3: Summary of the microalgae in heavy metals bioremediation

No.	Microalgae	Heavy metal	Removal efficiency (%)	References
1	*Botryococcus* sp.	Chromium (Cr)	94	Onalo *et al.* (2014)
		Copper (Cu)	45	
		Arsenic (As)	9	
		Cadmium (Cd)	2	
2	*Synechocystis salina*	Chromium (Cr)	60	Worku & Sahu (2014)
		Iron (Fe)	66	
		Nickel (Ni)	70	
		Mercury (Hg)	77	
		Calcium (Ca^{2+})	65	
		Magnesium (Mg^{2+})	63	
		Total Hardness	78	
3	*Chlorella marina*	Zinc, Zn (Powder)	97	Kumar *et al.* (2013)
		Zinc, Zn (immobilised)	55.3	
4	*Porphyridium cruentum* (S.F. Gray)	Copper (Cu)	92	Soeprobowati & Hariyati (2013)
5	*Chlorella pyrenoidosa* (100% Conc.)	Chromium (Cr)	52.8	Ajayan & Selvaraju (2012)
		Copper (Cu)	77.1	
		Lead (Pb)	43.8	
		Zinc (Zn)	68.9	
	Scenedesmus sp. (100% Conc.)	Chromium (Cr)	52	
		Copper (Cu)	79.2	
		Lead (Pb)	47.8	
		Zinc (Zn)	66	
6	Indigenous microalgae	Barium (Ba)	91.2	Krustok *et al.* 2012)
		Iron (Fe)	94.6	
7	*Botryococcus* sp.	Zinc (Zn)	-	In this study
		Ferum (Fe)	-	
		Cadmium (Cd)	-	
		Manganese (Mg)	-	

Therefore, Table 2.3 shows the summary of microalgae in bio-remediate some of the heavy metals ions done by previous researchers. Worku & Sahu (2014) cultured *Synechocystis salina* in groundwater to reduce the heavy metals and total hardness within 15 days of treatment. At the end of the treatment, *Synechocystis salina* is able to remove of Cr 60%, Fe 66%, Ni 70%, Hg 77%, Ca^{2+} 65%, Mg^{2+} and total hardness 78%. Meanwhile, Kumar *et al.* (2013) had demonstrated to remove Zinc using immobilised and powder form from *Chlorella marina*. They found that

the highest removal fall to powder form of 97% compared to immobilise of 55.3%. At the same time, the optimum pH for heavy metal adsorption is at pH 8.

In bioremediation of industrial wastewater, Soeprobowati & Hariyati (2013) used *Porphyridium cruentum* isolated from brackish water to assimilate the Pb, Cd, Cu and Cr. During the experimental, pH, temperature, salinity and light were maintained to be on 7-8, 28-32°C, 32-34 ppt and 4200 lux, respectively. Thus, this red microalga was able to reduce Cu of 92 % from the wastewater. In a different study, Ajayan & Selvaraju (2012) examined two strain of microalgae; *Chlorella pyrenoidosa* and *Scenedesmus* sp. in tannery effluent. As mentioned in Table 2.3, they analysed that the highest removal using both microalgae were Copper, 77% and 79.2%, respectively. Whereas Krustok *et al.* (2012) were applying the Indigenous microalgae in wastewater collected from WWTP in Vasteras. Their finding was showing that this microalga very effective in removing of Barium 91.2 % and Iron 94.6 %.

In summary, most of the microalgae species as listed in Table 2.3 have their own advantages in bioremediation of heavy metal in water. Other than nutrient (phosphorus and nitrogen), microalgae also need a heavy metal element to build their cell, for example, iron and chromium (Dwivedi, 2012). In addition, major advantage using microalgae in bioremediation is that this process under the light condition and does not need oxygen; instead, they absorb CO_2 and release O_2. However, to the best author's knowledge, there is very limited information about *Botryococcus* sp. in treating the heavy metal except study done by Onalo *et al.* (2014) when they used *Botryococcus* sp. to reduce chromium, copper, arsenic and cadmium from textile industry wastewater. To address this gap, the application of *Botryococcus* sp. in wastewater treatment especially in domestic wastewater and food processing wastewater was the motivation to fulfil the knowledge in this bioremediation field.

2.7 Microalgae and wastewater treatment

Nowadays, microalgae have become an important or significant microorganism for biological purification and treatment of wastewater. This may due to they are able to accumulate and assimilate plant nutrients, heavy metal, pesticides, organic and inorganic pollutants and radioactive matter in their unicellular cells (Sahu, 2014). Microalgae cultivation combined with wastewater treatment system offer more

simple, convenient and economical technology or technique as compared to another environmental alternative system (Worku & Sahu, 2014).

Moreover, photosynthesis can be effectively exploited to generate oxygen from wastewater phycoremediation. Then, the choice of microalgae species used in wastewater pollutant biotransform is determined by their robustness against the contamination and also growth efficiency. Some of the selection of microalgae species and wastewater treatment are summarised in Table 2.4.

As shown in Table 2.4, Sahu (2014) studied the *Chlorella vulgari*s in organic and inorganic pollutant reduction using sewage from the treatment plant. His analysis revealed that 70% of BOD, 66% of COD, 71% of TN, 67% of phosphorus, 54% of volatile solid and 51% of dissolved solid was reduced. Meanwhile, industrial wastewaters had been treated using microalgal bacterial flocs was done by Hende *et al.* (2014) found that a significant removal of turbidity, BOD, TCOD, TOC, TC, TN and TP are 96%, 87%, 80%, 71%, 48%, 58% and 8%, respectively. They also observed the final effluent DO was 6.06mg/L and the average pH was a bit alkalinity.

Azarpira *et al.* (2014) compared two species of cyanobacteria namely *Oscillatoria limosa* and *Nostoc commune* in the removal of nutrients using polluted river water from Mula-Mutha, Pune. The average reduction efficiency was between 84% - 98%. Amongst the selected cyanobacteria, *Oscillatoria limosa* was the best as compare to *Nostoc commune*. Consequently, both algal also has very good potential for nitrogen fixation and biomass for paddy cultivation. In bioremediation of primary treated wastewater using *Chlorella minutissima, Nostoc* and *Scenedesmus* was attempted by Sharma & Khan (2013). The end result showed that these algae were very effective in reduction of the physiochemical parameter in sewage wastewater. Further, they observed that *Chlorella* was having the best phycoremediation which is able to remove of TDS 97%, Nitrogen 90%, Phosphorus 70%, BOD 95% and COD 90%. In spite of that, *Scenedesmus obliquus* study by Ji *et al.* (2013) in piggery wastewater treatment was capable of removing TN around 23-58% and TP 48-69% only and they suggested *Scenedesmus obliquus* is a promising candidate for environmental friendly bioenergy sources.

Table 2.4: Summary of the review concerning the selection of microalgae in wastewater treatment

No.	Microalgae species	Application	Type of wastewater	Author
1	*Botryococcus braunii*	Phycoremediation and biochemical composition analysis	Domestic wastewater	Raj GP *et al.* (2015)
2	*Chlorella vulgaris*	Organic and inorganic pollutant reduction	Sewage treatment plant	Sahu (2014)
3	Microalgal bacterial flocs	Wastewater treatment	Industrial wastewater	Hende *et al.* (2014)
4	*Oscillatoria* and *Nostoc commune*	Phycoremediation	Municipal wastewater	Azarpira *et al.* (2014)
5	*Chlorella minutissima*, BGA *Nostoc* and *Scenedesmus*	Wastewater bioremediation	Primary treated wastewater	Sharma & Khan (2013)
6	*Botryococcus braunii*	Wastewater treatment	Municipal wastewater	Can *et al*. (2013)
7	*Scenedesmus obliquus*	Nutrients removal	Piggery wastewater	Ji *et al.* (2013)
8	*Chlorella vulgaris Beijerinck* and *Scenedesmus quadricauda*	Wastewater bioremediation	Domestic wastewater	Kshirsagar (2013)
9	*Chlorella vulgaris*	Wastewater treatment	Drainage solution from greenhouse	Hultberg *et al.* (2013)
10	*Chlorella vulgaris, Rhizoclonium hieroglyphicum* and mixed algal	Phycoremediation	Sewage drainage	Ahmad *et al.* (2013)
11	*Chlorella* sp.	pH analysis	Tannery industry wastewater	Sivakumar & Rajendran (2013)
12	*Bortyococcus braunii*	Wastewater treatment	Greywater	Gokulan *et al.* (2013)
13	*Nostoc* sp.	Phycoremediation	Dairy wastewater	Kotteswari *et al.* (2012)
14	*Chlorella vulgaris*	Phycoremediation	Chemical industry wastewater	Rao *et al.* (2011)
15	*Oocystis* sp.	Wastewater treatment	Fish processing wastewater	Riaño *et al.* (2011)
16	*Cloacal* algae	Phycoremediation	Open drain wastewater	Sengar *et al.* (2011)
16	*Chlorella vulgaris*	Wastewater bioremediation	Textile wastewater	Lim *et al.* (2010)
17	*Chlorella vulgaris, Synechocystis salina* and *Gloeocapsa gelatinosa*	Phycoremediation	Industry wastewater	Dominic *et al.* (2009)
18	*Botryococcus braunii*	Wastewater treatment	Urban wastewater	Órpez *et al.* (2009)
19	*Pithophora* sp.	Phycoremediation	Thermal wastewater	Murugesan, & Dhamotharan (2009)
20	*Botryococcus* sp	Phycoremediation + Biomass + Bio-hydrocarbon production	Domestic wastewater and food processing wastewater	In this study

In a different study, Kshirsagar (2013) examined *Chlorella vulgaris* and *Scenedesmus quardricauda* in domestic wastewater. Thus, found that *Chlorella* effectively removes of COD 80.04%, BOD 70.91%, nitrate 78.08% and phosphate 62.73% while *Scenedsmus* able to remove COD 70.97%, BOD 89.21%, nitrate 70.32% and phosphate 81.34% on the 15th days of cultivation. In another microalgae species and different type of wastewater, Riaño *et al.* (2011) conducted an experimental investigation on *Oocyctic* sp. in fish processing wastewater treatment. Two photobioreactor inoculated with microalgae (*Oocystic* sp.) at 23°C and 31°C, respectively. They measured approximately 70% of TCOD and phosphate removal was achieved regardless of the temperature. Previously, in 2011, Sengar *et al*. revealed that *Cloacal* algae could change the pH of open drain wastewater from 8.1 to 7.1 and also increase the DO about 87.5% on the 25th days of cultivation. Bioremediation of thermal wastewater by *Pithosphora* sp. also showed a positive result which it is able to remove the physiochemical from 32% to 92% as done by Murugesan & Dhamotharan (2009).

Based on the microalgae selection in wastewater treatment where a lot of microalgae have a high potential for treating wastewater. In most of the microalgae as listed in Table 2.4, there are none research studies on *Botryococcus* sp. in phycoremediation using domestic and food processing wastewater. However, there are quite few investigation had been done specifically using *Botryococcus braunii* in wastewater treatment or phycoremediation. The *Botryococcus braunii* had been utilised in urban wastewater (Órpez *et al.,* 2009) and greywater (Gokulan *et al.,* 2013) phycoremediation. Meanwhile, Can *et al.* (2013) used *Botryococcus braunii* Kutz obtained from algae culture collection of Utex (The University of Texas) for municipal wastewater treatment. However, in 2015, Raj GP *et al.*, used *Botryococcus braunii* isolated from Chennai city, India for phycoremediation purpose and biochemical composition analysis. Then, realising the gap in the extant literature which is less extensive study had been conducted for the hydrocarbon production using domestic and industrial wastewater as culture media. More intensive research is needed for *Botryococcus* sp. in treating wastewater-coupled with hydrocarbon production especially domestic and food processing wastewater. Another motivation for this research is to employ the local isolated *Botryococcus* sp. from tropical rainforest for the phycoremediation effectiveness and future biofuel feedstock.

2.8 Microalgae and biomass production

The technology and innovation for the production of biomass from wastewater have been introduced since the 1950s where microalgae are very efficient in the removal of nutrients from wastewater. Hence, some of the microalgae species grow in wastewater due to a lot of carbon, nitrogen and phosphorus that act as nutrients for microalgae (Rawat *et al.*, 2011). Griffiths & Harrison (2009) suggested that growth of algae should focus on biomass productivity rather than lipid productivity as is the current thrust in the algal biofuels sector while Pittman *et al.* (2011) stated that large amounts of biomass produced will improve the viability on conversion of biomass to alternative biofuels.

Table 2.5: Summary of the review concerning the selection of microalgae in wastewater treatment and biomass production

No.	Microalgae species	Application	Type of wastewater	Author
1	*Desmodesmus* sp.	Biomass production and nutrient removal	Anaerobic digestion wastewater	Ji *et al.* (2014)
2	*Chlorella* sp.	Biomass production	Tannery industry wastewater	Sivakumar & Rajendran (2013)
3	*Chlorella vulgaris*	Wastewater treatment and biomass production	Greenhouse wastewater	Hultberg *et al.* (2013)
4	*Chlorella sorokiniana*	Nutrient removal and biomass production	Municipal wastewater	Hernández *et al.* (2013)
5	*Chlorella* sp.	Nutrient removal and biomass production	Municipal wastewater	Udom *et al.* (2013)
6	*Spirulina platensis*	Phycoremediatian and biomass production	POME	Hadiyanto& Soetrisnanto (2013)
7	*Phormidium sp., Chlorella vulgaris, Chlamydomonas reinhardtii* and *Scenedesmus rubescens*	Nutrient removal	Clarifier tank of WWTP	Su *et al.* (2012)
8	Indigenous microalgae	Nutrient removal and biomass production	Dairy wastewater	Asmare *et al.* (2013)
9	*Botryococcus* sp.	Phycoremediation and biomass production	Domestic & food processing	In this study

Production of biomass from wastewater requires, similar production of biomass on artificial media, depends on a number of factors. However, heavy metal contamination factors require greater attention than in conventional production from

media. Besides that, algal biomass has the ability to collect heavy metals and may require desorption of metal before further processing after lipid extraction. Wastewater often contains nitrogen in the form of ammonia, which in high concentrations may be inhibitory to algal growth (Rawat *et al.*, 2011). For that reason, Table 2.5 presents the option or selection of microalgae in wastewater treatment and biomass production.

As reported by Ji *et al.* (2014), the highest growth of *Desmodesmu*s sp. for 7 days and 14 days were 0.21g/L and 0.412 g/L, respectively when they cultivated the algae anaerobic digestion wastewater. That result similar to the study done by Wang *et al.* (2010) upon using *Chlorella* sp. in dairy manure. Meanwhile, Sivakumar & Rajendran (2013) examined the growth by spectrophotometer and found that 30 grams of dry biomass per day from 10 litres of water. Then, these 30 grams contains 10 grams of lipid.

However, Hultberg *et al.* (2013) also used *Chlorella* to produce the biomass from greenhouse effluent. Their experiment was performed in a greenhouse with 16h/8h day/night and temperature at 20°C. Conversely, results showed that the amount of biomass obtained significantly lower than in another experiment. A possible reason is an intense proliferation of bacteria that inhibited the growth of *Chlorella vulgaris* and also probably caused by the presence of grazing organisms in the nutrient solution. Another *Chlorella* species studied by Hernández *et al.* (2013) found that pig manure liquid wastewater produced 26.3 mg/L of dry weight while potato processing wastewater produces 18.8 mg/L dry weight of biomass. Higher biomass growth due to the higher phosphorus concentration. In addition, Riaño *et al.* (2011) also observed the increasing in biomass productivity concomitantly with the increment of nitrogen and phosphorus rate. Other than that, carbon and nutrients contained in wastewater were used by microalgae to build their cell and to biotransform the pollutant into non-toxic.

Generally, to obtain the biomass from algae, harvesting process can be done in many techniques. One of them is flocculation method. Therefore, Udom *et al.* (2013) revealed that the most effective coagulant was Zetag 8819 compared to Ferric Chloride and Alum. They conducted the harvesting of the biomass of *Chlorella* sp. in municipal wastewater. Nevertheless, Su *et al.* (2012) discovered *Scenedesmus rubescens* had the highest biomass (6.56 $g/m^2/d$), followed by *Chlorella vulgaris* (6.28 $g/m^2/d$) and *Chlamydomonas reinhardtii* (6.06 $g/m^2/d$). According to their

results, it was clear indicated that algae suitable for wastewater treatment and biomass productivity.

It can be concluded that different study that has done by the previous researcher showed the different biomass amount production. It may due to the different type of wastewater used, algae type, climate condition and also environmental factor. As summarised in Table 2.5, the researcher to date has more tended to focus on *Chlorella* sp. rather than *Botryococcus* sp. in wastewater treatment combined with biomass production. In fact, studies relating to *Botryococcus* sp. have been relatively few and that there is no study focusing on the combined function of wastewater phycoremediation and biomass production. Recognising the lack of it, wastewater treatment using microalgae *Botryococcu*s sp. should be studied more detail to find the potential of phycoremediation technology in our country.

2.9 Algae

Algae are an extremely diverse group consisting predominantly of aquatic plants showing relatively little differentiation of tissue and organs as compared to bryophytes and tracheophytes. It includes both prokaryotic and eukaryotic photosynthesis organisms with chlorophyll a and other photosynthetic pigments, releasing O_2. Algal plant body ranges from unicellular to colonial, filamentous, siphonous and even parenchymatous and never contains roots, stems or leaves (Sharma, 2011).

From the physiological and biochemical point of view, algae are more or less similar to other plants in many respects. Algae possess almost the same biochemical pathways as of higher plants. Resembling the higher plants, all algae possess chlorophyll a and have almost the same carbohydrate and protein. Meanwhile, from the long fossil records of both prokaryotic and eukaryotic algal forms, it is evident that the prokaryotic algae were the first photosynthesis cellular plants (Sharma, 2011).

According to Sharma (2011), it is very difficult to give a precise definition of algae because they are mainly belonging to the varied nature of the plants comprising the group. According to Salleh (1996) said algae is lower plant possess own characteristics and unique. Features owned by most of the species of algae because

algae are a plant similar to organism's autotrophs, which can synthesise their own food source through the process of photosynthesis. Algae can be categorised into microalgae and macroalgae based on the classification of the species. The classification of the algae species according to following character (Sharma, 2011): (1) Pigments; (2) External form; (3) chromatophore; (4) Reserve food products; (5) Flagella; (6) Cell wall; (7) Nucleus; (8) Chromosome; (9) Algal physiology; (10) Ecological data that exist in the GenBank.

2.9.1 Macroalgae

Macroalgae is well known as seaweed belongs to the lower plants, which does not have roots, stems and leaves. Instead, they are composed of a thallus (leaf-like) and sometimes a stem and a foot. Some species have gas-filled structures to provide buoyancy. They are subdivided into three groups, the red, green and brown macroalgae (Rajkumar *et al.,* 2014). They can grow very fast and in sizes of up to tens of meters in length. Macroalgae differ in various aspects, such as morphology, longevity, and ecophysiology. Based on their pigmentation, they can be classified into Phaeophyta (brown), Rhodophyta (red), and Chlorophyta (green) algae (Rajkumar *et al.,* 2014).

In their natural environment, macro-algae grow on rocky substrates and form stable, multi-layered, perennial vegetation capturing almost all available photons. Because of the fact that seaweeds are fixed to their substrate, values for maximum productivity capable of achieving 10 times higher for a seaweed stand than for a plankton population, and can be as high as 1.8 kg C m^{-2} y^{-1}(Carlsson *et al.,* 2007). Therefore, it is estimated that around 200 species of macroalgae are used worldwide, about ten of which are intensively cultivated, such as the Phaeophyta, *Laminaria japonica* and *Undaria pinnatifida*, the Rhodophyta, *Eucheuma, Gracilaria, Porphyra* and *Kappaphycus,* and the Chlorophyta, *Enteromorpha* and *Monostroma*.

2.9.2 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure (Mata *et al.,* 2010). Their photosynthetic mechanism is similar

to land-based plants, but due to a simple cellular structure, and submerged in an aqueous environment where they have efficient access to water, CO_2 and other nutrients, they are generally more efficient in converting solar energy into biomass (Carlsson *et al.*, 2007). At present, microalgae species are divided into four groups, namely diatoms (Bacillariophyceae), green algae (Chlorophyceae), blue-green algae (Cyanophyceae), and golden algae (Chrysophyceae) (Rajkumar *et al.*, 2014) and Table 2.6 shows some species of microalgae according to their group.

Table 2.6: Microalgae species (Sharma, 2011)

Chlorophyceae	**Green**
	*Chlamydomonas, Chlorogonium, Brachiomonas, Pteromonas, Haematococcus, Lobomonas, Phacotus, Pyramimonas, Pyrobotrys, Eudorina, Pandorina, Gonium, Stephanosphaera, Volvox, Chlorella, Oocystis, Chodatella, Tetraedron, Ankistrodesmus, Characium, Actinastrum, Micractinium, Scenedesmus, Stichococcus, **Botryococcus**, Spirogyra* etc.
Chrysophyceae	**Blue Green**
	Chroococcus, Gloeocapsa, Aphanothece, Merisanopedia, Gomphosphaeria, Microcystis, Chamaesiphon, Oscillatoria, Anabaera, Nostoc, Rivularia, Stigonema etc.
Bacillariophyceae	**Diatoms**
	Melosira, Cyclotella and stephanodiscus, Tabellaria, Diatoma, Meridion, synedra, Fragilaria, Asterionella, Cocconeis, Navicula, Pinnularia, Surirella etc.
Cyanophyceae	**Golden Brown**
	Ochromonas, Mallamonas, Dinobryon, Synura, Uroglena etc.

In other word, microalgae are single cell microscopic organisms or plant which naturally can be found in either freshwater or marine environment. Kumar *et al.* (2013) stated more than 50,000 microalgae species exist in the world but estimated only 30,000 were analysed. Furthermore, microalgae are considered to be one of the oldest living organisms on our planet. They are thallophytes - plants lacking roots, stems, and leaves that have chlorophyll as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells. In addition, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2, and other nutrients (Kumar *et al.*, 2013).

2.9.3 *Botryococcus* sp.

Based on the original findings by Prescott (1978), *Botryococcus* sp. showing an expression in which a colonial complex is formed by interconnecting strands of tough mucilage. Colonies frequently appear solitary and as a yellowish-brown lump in which individual cells can scarcely be seen, if at all. The colour of the colony lays mostly in the mucilage. This species often forms "blooms", especially in hard water lakes. Belcher & Swale (1976) reveal that *Botryococcus* sp. capable grew in colonies up to 0.5mm across, with numerous green cell 5-10 μm long embedded in an oily matrix which varies in colour from a clear yellow to totally opaque orange or brick red. Orange oil pigmented with carotene might be squeezed from it under a cover slip. Plankton at ponds and lakes, often floating on the water.

Botryococcus sp. is a green colonial microalga (Chlorophyceae) that is an unusually rich renewable source of hydrocarbons and other chemicals. This colonial microalga is widespread in fresh and brackish waters of all continents. *Botryococcus* sp.is regarded as a potential source of renewable fuel because of its ability to produce large amounts of hydrocarbons (Qin & Li, 2006). Depending on the strain and growth conditions, up to 75% of algal dry mass can be hydrocarbons. The chemical nature of hydrocarbons varies with the producer strain. Amaro *et al.* (2011) had stated that *Botryococcus* sp.is more suitable for biodiesel production due to its high proportion of oleic acid.

Botryococcus sp. has been studied for several decades not just for its potential as a source of biofuel but for its ability to sequester carbon. The optimum growth *Botryococcus* sp. has been shown to grow best at a temperature of 23ºC, a light intensity of 30-60 W/M^2, with a light period of 12 hours per day and a salinity of 0.15 Molar NaC1 (Qin & Li, 2006). This tested with one strain and others certainly vary to some degree. In the laboratory, *Botryococcus* sp. is commonly grown in cultures of Bold Basal's medium (culture medium used in microbiology for the growth of certain algae species).

2.10 Environmental factors effect on microalgae culturing

Microalgae growth rate and biomass accumulation may be highly affected by photoperiod, light intensity, temperature, and salinity. Since microalgae are a plant

and grow similarly to a terrestrial plant by utilising light energy for photosynthesis process to produce biomass. In the middle of the photosynthesis process, the influence of other parameters such as photoperiod, temperature and salinity are very important in order to maximise and optimise the growth and biomass productivity. Figure 2.3 below illustrates the mechanisms involved during microalgae photosynthesis as influenced by basic environmental factors such as light, photoperiod, temperature, and salinity in the culture, besides absorbing carbon dioxide and release oxygen as a by-product.

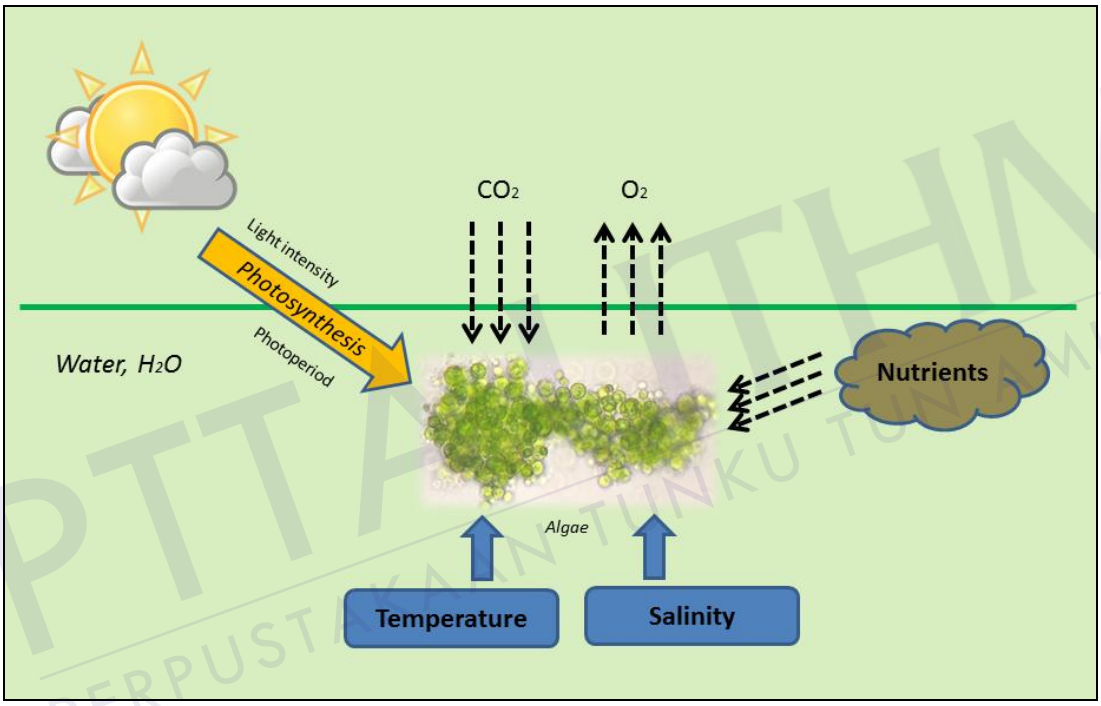


Figure 2.3: Photosynthesis mechanisms of microalgae influenced by light intensity, photoperiod, temperature, and salinity

2.10.1 Photoperiod

Generally, photoperiod refers to light exposure in terms of duration with minimum and maximum are 0:24 hour and 24:0 hour, respectively. Photoperiod is as important as light intensity because it directly influences the efficiency of photosynthesis of microalgae in the culture. However, the optimum or ideal photoperiod of microalgae is depending on the species and strain. Other than that, the natural habitat of microalgae also may contribute to differences in required optimal photoperiod.

Photoperiod is also important if the economic aspect is taken into account since algae biomass is produced with the light from artificial sources.

There have been few reports on the influence of photoperiod on the productivity and growth rate of *Neochloris conjuncta, Botryococcus braunii, Scenedesmus* sp., *Eustigmatophyte Nannochloropsis* sp. and *Chlorella vulgaris* as stated in Table 2.7. According to Krzemińska *et al.* (2014), continuous illumination (24:0) stimulated the growth of *Botryococcus braunii* and *Scenedesmus obiquus* more effectively while *Neochloris conjuncta* more tolerant with 12:12 hours in terms of growth rate and biomass production. Apart from that, Wahidin *et al.* (2013) studied the influence of photoperiod on *Nannochloropsis* sp. and found that the best light regime is on 18:6 hours with a maximum cell concentration of 6.5×10^7 cells/mL after 8 days of examination. They also report that a reduction of photoperiod duration to 12:12 hours lead to decreasing of growth rate value of *Nannochloropsis* sp. (Wahidin *et al.*, 2013).

However, optimum photoperiod for *Chlorella vulgaris* is totally different from other microalgae species as stated in Table 2.7, as they maximised their biomass when subjected to a photoperiod of 16:8 hours with 62.5 µmol $m^{-2}s^{-1}$ of illumination (Khoeyi, Seyfabadi, & Ramezanpour, 2012). This might due to the natural habitat of the *Chlorella vulgaris* since it was collected from Anzali wetland in International Sturgeon Research Institute if compared to *Nannochloropsis* sp. (Wahidin *et al.*, 2013) was obtained and isolated from Borneo Marine Research Institute, University Malaysia Sabah. Thus, Table 2.7 reveals that the most favourable light exposure for microalgae ranges between 12 hours to 24 hours because of the photosynthesis process needed. Indeed, a light and dark exposure allows for either an increase in final concentration or lowering of production cost (Khoeyi *et al.*, 2012).

2.10.2 Light Intensity

Principally, there are basic characteristics of light that influence biological plant growth, namely quantity, quality and duration of exposure (Khoeyi *et al.*, 2012; Meseck, Alix, & Wikfors, 2005; Wahidin *et al.*, 2013). Light is one of the major energy input sources for the photosynthesis of microalgae. Thus, light quantity refers to the intensity of the illumination either from natural or artificial light. Expectedly,

the growth of microalgae biomass in the culture is increased by increasing the light intensity until reach at a saturation point where the photosynthesis rate is at the maximum level (Cheng & He, 2014). However, too much light intensity exposure or oversaturation of light may lead to photoinhibition. Photoinhibition occurs due to the formation of reactive oxygen species which is harmful to microalgae cell and indirectly decrease the biomass productivity. Therefore, difference microalgae species and strain have difference requirement of light intensity for their growth at the optimal condition as stated in Table 2.7.

According to Sforza *et al.* (2014), green microalgae *Scenedesmus obliquus* 276.7 was grown optimally at a light intensity of 150 µmol $m^{-2}s^{-1}$ in the BG11 medium at a temperature of 23°C. They found that the growth rate increases linearly with light intensity increment until the saturation point (Sforza *et al.*, 2014). Meanwhile, *Spirulina platensis* strain SZ100 preferred intensity of light between 1500Lux (20.3 µmol $m^{-2}s^{-1}$) to 2500Lux (33.8 µmol $m^{-2}s^{-1}$) for their reaching to the optimal growth rates with pH level between 7-9 during cultivation (Mustafa *et al.*, 2013). Light intensity examination shows that the growth rates significantly increased with the time and the maximum growth was achieved at the end of 20 days of cultivation time (Mustafa *et al*., 2013). In 2008, Da-Cong *et al.* published a paper in which they investigated two strain of *Botryococcus* sp., namely *Bortyococcus braunii* UTEX 572 and UTEX 2441 in China. As a result, they found that both *Bortyococcus braunii* strain have a different optimum light intensity which is 800 µmol $m^{-2}s^{-1}$ and 400 µmol $m^{-2}s^{-1}$, respectively.

However, in 2006, Qin and Li studied optimisation of another *Bortyococcus* sp. strain called *Bortyococcus braunii* strain CHN 357 obtained from Chinese Academy of Sciences, Wuhan. Thus, they discovered that the *Bortyococcus braunii* strain showed optimum growth in 60 Wm^{-2} light intensity when cultured in soil extraction medium at a temperature of 23°C. Other species such as *Selenastrum minutum, Coelustrum microporum* and *Cosmarium subprotumidum* have a light intensity of 420 µmol $m^{-2}s^{-1}$, 400 µmol $m^{-2}s^{-1}$ and 400 µmol $m^{-2}s^{-1}$, respectively (Bouterfas *et al.*, 2002). These findings as stated in Table 2.7 show that most microalgae have different light intensity requirements due to their natural habitat, species, strains and culture conditions.

2.10.3 Temperature

Temperature is also one of the essential environmental parameters which influence the growth of microalgae. Similar to the effect of light intensity, microalgae growth increases linearly to an optimal point, after which cell growth gradually declines (D. Cheng & He, 2014). Other than growth, temperature factor also has an effect on the cell size and biochemical composition of microalgae (Juneja *et al.*, 2013). Temperature above the optimal range cause the growth decline even is able to kill some of the microalgae cells. However, a temperature below the optimal range and above frozen level will not kill microalgae but cause them to become inactive for growing (Zhu, 2014). Normally, the ideal temperature range for microalgae growth at optimal condition is between 20°C to 35°C, as shown in Table 2.7.

Based on Munir *et al.* (2015), the optimal temperature range for the three species of freshwater microalgae called *Spirogyra* sp., *Oedogorium* sp. and *Chlorella* sp. was between 24°C to 28°C when they cultivated them using Blue-Green medium and Bold's Basal medium. All studied microalgae sample were collected from different location of Lahore including ponds and damp soil places (Munir et al., 2015). Marine algae were found to have a tolerance of temperatures between 20°C to 30°C as studied by Rai and Rajashekhar (2014). There are six species of marine phytoplankton examined for the effect of temperature ranging between 20°C to 50°C under 1000 Lux illumination with 8:16 hours of light and dark regime (Rai & Rajashekhar, 2014). Another study by Mustafa *et al.* (2013) found that the best temperature range for the growth of *Spirulina platensis* strain SZ100 is within 25°C to 30°C.

Mustafa *et al.'s* (2013) findings are almost in line as obtained by the Rai and Rajashekhar (2014) study. In 2008, Da-Cong *et al*. reported the optimal temperature for *Botyococcus braunii* (UTEX 572 and 2441) to be 30°C, but it is still tolerant at temperatures between 25°C to 35°C. Similarly, *Rhodomonas* sp. also had optimal temperature almost the same like other microalgae as stated in Table 2.7 which is between 25°C to 27°C (Renaud *et al.*, 2002). It can be concluded that all optimum temperatures of microalgae in Table 2.7 are at the average level of the natural outdoor conditions in Malaysia (24°C to 35°C).

2.10.4 Salinity

Another basic environmental factor that influences the growth of microalgae is salinity. Salinity refers to the presence of salt content in the water for microalgae grow. Expectedly, marine algae use or consume higher salinity concentration if compared to freshwater algae (Juneja *et al.*, 2013). Salinity is also a critical parameter to be tested as the presence of salinity may influence the growth of algae and biochemical composition of algae cell (Juneja *et al.*, 2013).

Exposing algae to high salinity is harmful to freshwater algae cells since it might transform their cell structure (Mata *et al.*, 2010). Excess salinity inhibits photosynthesis process reduces the biomass productivity of microalgae (Cheng & He, 2014). Therefore, Table 2.7 indicates the optimal salinity concentration acceptable to some microalgae species. Most microalgae are tolerant to salinity within 10 psu- 30 psu except for Artic-sea-ice algae with a salinity of between 4 psu - 74 psu. Meanwhile, *Dunaliella* sp. grew at optimally when 25% NaC1 was added to the medium culture (AbuSaraa *et al.*, 2011).

Another study by Qin and Li (2006) on *Botryococcus braunii* Strain CHN 357 found that 0.15M of NaC1 was the greatest growth in the culture. In summary, different microalgae species require different concentrations of salinity for proper growth and biochemical composition.

Table 2.7: Systematic review condition of environmental factors for different microalgae species

Environmental factors	Microalgae species	Optimal condition	References
Photoperiod	*Neochloris conjuncta*	12:12 hours	Krzemińska *et al.* (2014)
	Botryococcus braunii	24:0 hours	
	Scenedesmus sp.	24:0 hours	
	Eustigmatophyte	18:6 hours	Wahidin *et al.* (2013)
	Nannochloropsis sp.		
	Chlorella vulgaris	16:8 hours	Khoeyi *et al.* (2012)
Light intensity	*Scenedesmus obliquus* 276.7	150 µmol $m^{-2}s^{-1}$	Sforza *et al.* (2014)
	Spirulina platensis strain SZ100	1500 lux - 2500 lux	Mustafa *et al.* (2013)
	Dunaliella sp.	61 µmol $m^{-2}s^{-1}$	AbuSaraa *et al.* (2011)

Table 2.7 (continued): Systematic review condition of environmental factors for different microalgae species

Environmental factors	Microalgae species	Optimal condition	References
Light intensity	*Botryococcus braunii* UTEX 572	800 µmol $m^{-2}s^{-1}$	Da-Cong *et al.* (2008)
	Botryococcus braunii UTEX 2441	400 µmol $m^{-2}s^{-1}$	
	Botryococcus braunii strain CHN 357	30 - 60 Wm^{-2}	Qin & Li (2006)
	Selenastrum minutum	420 µmol $m^{-2}s^{-1}$	Bouterfas *et al.* (2002)
	Coelustrum microporum	400 µmol $m^{-2}s^{-1}$	
	Cosmarium subprotumidum		
Temperature	*Spirogyra* sp.	24 - 28°C	Munir *et al.* (2015)
	Oedogonium sp.		
	Chlorella sp.		
	Marine algae (*Chroococcus turgidus, Lyngbya conferroides, Nostoc commune, Chaetoceros calcitrans, skeletonema costatum* and *Nannochloropsis oceanica*)	20 - 30°C	Rai & Rajashekhar (2014)
	Spirulina platensis	25 - 30°C	Mustafa *et al.* (2013)
	Botryococcus braunii (UTEX 572 & 2441)	30°C	Da-Cong *et al.* (2008)
	Rhodomonas sp.	25 - 27°C	Renaud *et al.* (2002)
Salinity	*Thalassiosira weissflogii*	25 psu	Garcia *et al.* (2012)
	Dunaliella sp.	2.5% of NaC1	AbuSaraa *et al.* (2011)
	Artic-sea-ice algae	4 - 74 psu	Zhang *et al.* (1999)
	Isochrysis sp.	25 psu	Renaud & Parry (1994)
	Nannochloropsis oculata	20 - 30 psu	
	Nitzschia (frustulum)	10 - 15 psu	

2.11 Cultivation system of microalgae

Growing microalgae either for commercial or research purposes can be done in both open and closed system. Their cultivation would require a huge volume of media containing necessary nutrients for their growth, though conditions may vary. Small-scale cultivations, for example, would utilise synthetic mediums, while large-scale cultivations normally utilise wastewater instead, especially from domestic sources, as the medium replacement. Domestic wastewater has been found to be a promising medium able to provide enough nutrients for their growth, while simultaneously allowing for the wastewater to be treated through phycoremediation (Can *et al.*, 2013). In spite of the nutrients provided, successful cultivation of microalgae still relies significantly on the culturing system used, which may be either through an

open system, or a closed system, each of which is discussed further in the following sections.

2.11.1 Open system

Open pond system is the most favourable method of microalgae biomass production, particularly in commercial projects to provide nutrition and produce biofuel. Several types of open pond system are available such as the raceway, shallow big, and circular tank systems (Borowitzka & Moheimani, 2013). Raceway pond is the most famous cultivation system among these three, where typically, a fully closed loop and an oval shape circulation channel are constructed (Costa & de Morais, 2014a). A raceway pond's shape resembles a race track and it is usually between 0.2 m – 0.5 m deep. The culture is stirred using paddlewheels for homogenisation, which would promote algal growth and biomass productivity. The pond can be made of concrete, glass fibre or membrane (Brennan & Owende, 2010). Compared to other open pond systems, the raceway is the most economic option for large-scale microalgae cultivation (Costa & de Morais, 2014b). Moreover, it is very easy to construct and operate and has a very low power consumption (Ugwu, Aoyagi, & Uchiyama, 2008). This cultivation technique that is exposed to sunlight, however, is susceptible to water loss due to naturally high evaporation rate. In addition, the temperature and pH level are difficult to control, and the open system meant exposure to cross-contamination by undesired microorganisms. Figure 2.4a and Table 2.8 show the schematic diagram and advantages/disadvantages of open raceway pond, respectively.

There are a few studies utilising open raceway pond to culture microalgae for many kinds of the application as stated in Table 2.9. According to Hende *et al.* (2014), for example, used this technique to cultivate microalgae bacterial flocs in the outdoor condition in Kortrijk, Belgium, for the purpose of aquaculture wastewater treatment and biomass production. They found that scaling up the culture decreases nutrients removal efficiencies and biomass productivity compared to a lab-scale system (Hende *et al*., 2014).

Another example of open raceway pond usage was by Ashokkumar & Rengasamy (2012) in Tamil Nadu, India, where *Botyrococcus braunii* was grown for biofuel production. A total 1800L of modified Chu 13 medium was prepared as the

medium culture in the experiment, which was conducted in a concrete raceway pond lined with porcelain tiles having a total working volume of 2000L. After 15 days of cultivation, up to 1.8 g/L, *Botryococcus braunii* biomass was able to be produced (Ashokkumar & Rengasamy, 2012). Yet another example of the open raceway pond usage was by Lim, Chu, & Phang (2010), in which wastewater from the textile industry was bio-remediated using *Chlorella vulgaris* cultivated in High Rate Algal Pond. The ponds were installed on the rooftop of the Institute of Postgraduate Studies, University of Malaya; with each single-loop raceway stirred using a paddlewheel at 15 rpm. A total 40L of textile wastewater was used for each pond and 10 days were allowed for treatment. There was a reduction of ammonium (44.4 – 45.1%), phosphate (33.1 – 33.3%), and COD (38.3 – 62.3%) while colour removal ranged from 41.8 to 50%. Biomass productivity ranged from 0.17 to 2.26 mg chlorophyll a/L in the textile wastewater (Lim *et al.,* 2010).

Over in Rio-Grande, Brazil, *Spirulina platensis* was cultivated using the same system by Radmann, Reinehr, & Costa (2007), for the purpose of investigating its growth and biomass concentration. Cultivation was carried out in a 6L acrylic open raceway pond containing 5L of Zarrouk culture medium, which was agitated using paddlewheels rotating at 18 revs/min and illuminated with 3000 Lux of light intensity. A Box-Behnken experimental design was used for the analysis, and the *Spirulina platensis* productivity was found to be at 0.028 to 0.046 g/L/day, with maximum specific growth rate being 0.038 to 0.138/day. Further in 2014, Pawlowski *et al.* (2014) demonstrated the cultivation of microalgae to address the effective utilisation of flue gases with the proper pH control in an open raceway pond. The raceway was operated at constants depth of 0.2m, mixing was done using 1.2 m diameter paddlewheels with eight blades while flue gases were injected through membrane diffusers at the bottom of the sump. It was found that evaluated control algorithm significantly improves the pH control accuracy, which in turn has a direct influence on biomass productivity (Pawlowski *et al.,* 2014).

2.11.2 Closed system

The drawbacks of the open pond application as stated in Table 2.8 have motivated intensive investigation into closed system especially for vertical photobioreactor (Figure 2.4b). This particular system is used for the cultivation of

microalgae for high-quality products, such as pharmaceuticals and food supplement, where there is a strict requirement against contamination risk that is present in the exposed open system (Rawat *et al.*, 2013). In a closed system's photobioreactor, there is no direct exchange of gases or contaminants between the cultivation system and the outside environment. Instead, gas exchange, which is essential for the mixing of algae in the culture, is provided using sterilised gas to avoid and minimise contamination inside the culture system. Another fundamental principle of photobioreactors' development is the total amount of illumination received by algae cells (Wang, Lan, & Horsman 2012). Closed photobioreactors are more flexible than open systems on this as they can utilise artificial lighting to further increase the intensity given by natural sunlight, which enters the system and illuminates the microalgae culture inside through the transparent walls of the vertical photobioreactor tube. A gas inlet would be installed at the bottom of the reactor to supply CO_2 and to allow for mixing (Figure 2.4b). This naturally leads to a typical disadvantage of photobioreactors, which is highly cost to build and operate. The advantages of a closed photobioreactor, as shown in Table 2.8, are the availability of larger surface area exposed to sunlight; higher biomass productivity, and higher mass-transfer rate with good mixing. Meanwhile, the drawbacks are the possibility of cell sedimentation during cultivation and the potential of microalgae growth on the photobioreactor's wall due to inconsistencies in mixing.

Several attempts have been made to cultivate microalgae in closed photobioreactors for various types of application as stated in Table 2.9. In 2014, Batista *et al.* (2015) have demonstrated the use of photobioreactors for different microalgae species cultivated using urban wastewater collected from Aguas de Figueira, Portugal. A 150L tabular vertical photobioreactor was used to grow the microalgae under natural sunlight and outdoor temperature until nutrients depletion. This reduction in nutrients was done *Scenedesmus obliquus* for ammonium, phosphate and COD at 97.9%, 100%, and 54%, respectively. The microalgae species also produced the highest $BioH_2$ (56.8 mL H_2/gvs) compared to other species tested in the study (Batista *et al.*, 2015). Bilad *et al.* (2014) meanwhile cultivated *Chlorella vulgaris* in a photobioreactor using Wright's cryptophytes medium to produce biomass, as well as to conduct a pre-harvesting investigation using different photobioreactors and membrane photobioreactors. The 25L cylinder photobioreactor operated continuously under different dilution rates for 45 days, after which a

filtration system was added to the system, turning it into a membrane photobioreactor. It was found that the membrane photobioreactor was able to operate at higher dilution rate and thus increased growth rate compared to regular photobioreactors.

Another variation, the bubble column photobioreactor, was used by Valdes, et al. (2012) to cultivate the *Nannochloropsis oculata* in modified seawater with the f/2 medium. The 1.7 m tall and 0.14 m wide photobioreactor was made of transparent PVC, with an estimated volume of about 25L, and it was placed outdoors in Alicante in September. The aim of their study was to investigate the behaviour of microalgae cultured in photobioreactor system in relation to CO_2 net balance using analysis of pH profiles. Mixing was done by injecting air using a microperforated circular pipe located at the bottom of the photobioreactor. A maximum photosynthesis active radiation (PAR) value of close to 55% was recorded at noon when the concentration of the culture was 0.14 g/L.

In an earlier study by Riaño, Molinuevo, & García-González (2011), two photobioreactors were set up with a total working volume of 3L, where *Oocystis* sp. was cultivated using fish processing wastewater for phycoremediation potential. Each photobioreactor was exposed to light at 12,000 Lux for 24 hours and mixed using magnetic stirrers, though different temperatures were maintained for each reactor (23°C and 31°C). Similar TCOD and phosphate removal were achieved (about 70%) while ammonium concentration was completely exhausted in both photobioreactors. However, higher biomass productivity was recorded in the reactor with the higher temperature (about 55%) compared to the other one (Riaño *et al.,* 2011).

Feng, Li, & Zhang (2011) investigated lipid production of *Chlorella vulgaris* cultured in sterilised artificial wastewater using four 2.2 L aerated column photobioreactors. The wastewater was inoculated with *Chlorella vulgaris* at 30°C and continuously illuminated at 3000 Lux intensity. The highest lipid productivity of *Chlorella vulgaris* was about 147 mg/L/day with nutrients removal of COD, ammonium and TP of 86%, 97%, and 96%, respectively. It was their conclusion from the research that the findings would lead to an economical technology of algal lipid production (Feng *et al.,* 2011).

Table 2.8: Advantages and disadvantages of open raceway pond and closed tubular photobioreactor (Lam & Lee, 2014).

Raceway (Open Pond)	Closed photobioreactor (Vertical tubular)
Advantages: • Low-cost technique for large-scale cultivation. • Low power consumption/ requirement. • Easy to maintain and clean. • Capture atmosphere CO_2 effectively.	*Advantages:* • The large surface area that is exposed to natural light. • Higher productivity and easy to control. • Suitable for most microalgae species. • High mass-transfer rate with good mixing. • Compact, easy to operate, and low-cost, relatively. • Less water evaporation. • Low cross contamination risk.
Disadvantages: • Easily contaminated by other microorganisms. • Cell density is low due to shadowing of their cells. • Loss of water due to evaporation. • Uncontrolled temperature and pH level.	*Disadvantages:* • Small illumination surface area. • Cells sedimentation may occur during cultivation. • The growth of microalgae on the wall of photobioreactor due to inconsistent mixing. • Limit on the length of the tubes due to inefficient gaseous exchange.

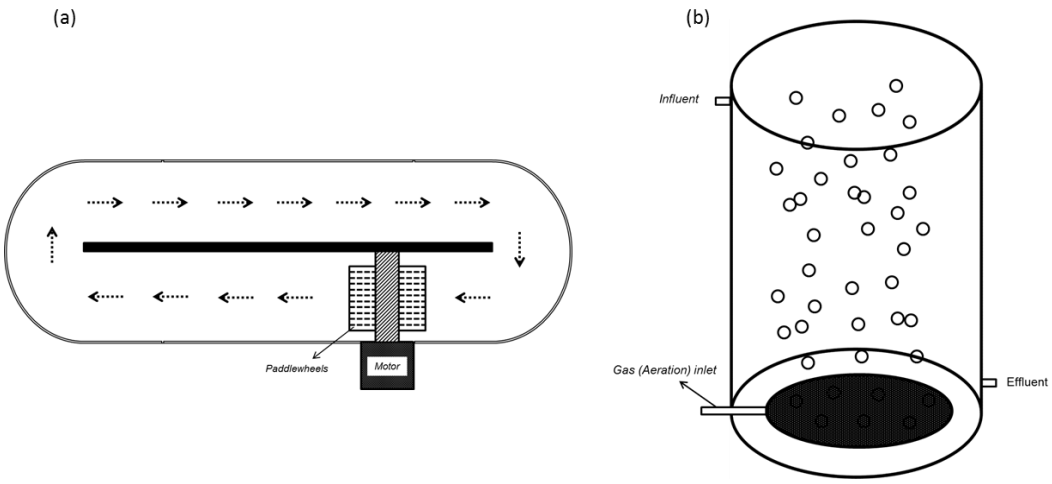


Figure 2.4: Schematic diagram of (a) raceway open pond and (b) closed tubular photobioreactor.

Table 2.9: Utilisation of raceway and photobioreactor system in previous studies.

Type of cultivation system	Microalgae species	Location of study	Application	References
Raceway (Open Pond)	Microalgal bacterial flocs	Kortrijk, Belgium	Aquaculture wastewater treatment and biomass production.	Hende *et al.* (2014)
	Botryococcus braunii Kutz.	Tamilnadu, India.	Biofuel production	Ashokkumar & Rengasamy (2012)
	Chlorella vulgaris	Kuala Lumpur, Malaysia	Textile wastewater bioremediation	Lim *et al.* (2010)
	Spirulina platensis	Rio-Grande, Brazil	Biomass concentration and growth investigation.	Radmann *et al.* (2007)
	Species not specify	Madrid, Brazil	Investigation of effectiveness utilisation for flue gases.	Pawlowski *et al.* (2014)
Photobioreactor (Vertical)	*Chlorella vulgaris*, *Scenedesmus obliquus* and *Consortium C.*	Lisbon, Portugal	Wastewater treatment with Bioenergy potential for bio-hydrogen production.	Batista *et al.* (2015)
	Chlorella vulgaris	Kortrijk, Belgium	Biomass production and pre-harvesting of microalgae.	Bilad *et al.* (2014)
	Nannochloropsis oculata	Alicante, Spain	To obtained behaviour of microalgae/photobioreactor system related to the CO_2 net balance.	Valdes *et al.* (2012)
	Oocystis sp.	Valladolid, Spain	Phycoremediation of fish processing wastewater.	Riaño *et al.* (2011)
	Chlorella vulgaris	Nangang, China	Lipid production of algae cultivated in artificial wastewater.	Feng *et al.* (2011)

2.12 Harvesting of microalgae

Harvesting is another significant part of the biotechnology industry related to microalgae. In fact, the cost of harvesting is estimated to be up to 20% – 30% of the total cost of cultivation (Hattab, Ghaly, & Hammoud, 2015). Many types of harvesting method are available and widely used presently, such as centrifugation, filtration, sedimentation, and flocculation. The selection of harvesting method depends on several factors, including the application of the microalgae biomass, culture condition, and microalgae species. Each method has its advantages and disadvantages, as discussed further in the following section. Some previous studies in the literature reported that no single harvesting method is suitable for every microalga species since the particular strain and final product to be produced vary so widely (Granados *et al.,* 2012). This makes the choice of harvesting technique to become more complex, particularly for large scale productions due to the need for optimisation, and the magnitude of laborious work to be carried out.

2.12.1 Centrifugation

Centrifugation is a common method used in lab-scale microalgae harvesting due to several advantages and accompanying disadvantages. While it is the fastest method to recover microalgae biomass, as well as the most efficient harvesting method compared to others due to its ability to recover the biomass of most microalgae species, it consumes very high amount of energy, leading to its higher cost (Barros *et al.,* 2015). The process may also cause damage to microalgae cells due to its high spinning speed (Harun *et al.,* 2010). Due to this, the technique is neither recommended nor deemed feasible for large scale productions. Shah *et al.* (2014) reported that 80% - 90% of microalgae recovery can be achieved when laboratory centrifugation tests were conducted on pond effluent at 500-1000xg speed. Harun *et al.* (2010) also recorded 88% - 100% cell viability and around 95% - 100% harvesting efficiency by centrifugation at 13000xg. Table 2.10 shows the advantages and disadvantages of application of centrifugation in microalgae harvesting.

2.12.2 Filtration

Filtration is a method of harvesting with the aid of porous media in which algae paste retains on the media while the water passes through. This conventional and competitive method of harvesting is sustainable for harvesting long length microalgae or those forming large colonies. It is also the preferable method to harvest algae cells of very low density (Show & Lee, 2014). The filter media can be categorised according to their pore size, viz. microfiltration (pore size of 0.1-10 µm), macrofiltration (pore size of >10 µm), ultrafiltration (pore size of 0.02-0.2 µm), and reverse osmosis (pore size of <0.001 µm) (Hattab *et al.*, 2015). The main problem of this method, however, is the limitation on fluid flow volume, and clogging/fouling of the filter by deposited cells. The cost of filtration is mostly due to the frequent need to replace or clean the filter medium, leading to an increase in maintenance cost, and which may make it not cost-effective for small scale projects (Barros *et al.*, 2015). Tabulation of these advantages and disadvantages are shown in Table 2.10.

2.12.3 Sedimentation

Sedimentation is a process of separating suspended solid, such as microalgae, from the liquid that is typically used in wastewater treatment plants (Shah *et al.*, 2014). In microalgae harvesting, suspended particles are separated from the culture gravitationally, the effectiveness of which depends on the density of the microalgae. Larger densities will result in faster sedimentation rate while the opposite would require a longer time to settle. Normally, flocculation is always used to increase the efficiency of gravity sedimentation (Hattab *et al.*, 2015). As stated in Table 2.10, sedimentation is very straightforward, costs less, efficient and requires less energy. On the downside, time consumption due to slow sedimentation may lead to the deterioration of algae biomass.

2.12.4 Flocculation

Flocculation is done with the help of flocculants that cause the coagulation of microalgae cells into small clumps or formations, known as flocs (Shah *et al.*, 2014). It serves as a preparatory step before other advanced harvesting methods such as

sedimentation. As a harvesting method by itself, flocculation possesses certain advantages and disadvantages as well. It is easy to do and reliable in terms of cost. However, unsuitable chemical flocculants may be toxic to certain microalgae biomass, and reusability of the media is very limited as stated in Table 2.10. Two types of flocculation method are available, i.e. chemical flocculation and auto-flocculation. Auto-flocculation occurs due to the precipitation of algal cells, while chemical flocculation requires the addition of a chemical coagulant (inorganic or organic) to the microalgae culture (Hattab *et al.,* 2015).

Several studies have revealed that microalgae harvesting efficiency using chemical flocculants depend highly on three basic characteristics; pH, coagulant dosage and coagulant type (Hamid *et al.,* 2014). Since microalgae carry a negative surface charge, which prevents them from self-aggregation from the suspension, the coagulant is added to counter it. Flocculants normally used for harvesting include $A1_2(SO_4)_3$ (Aluminium sulphate), $FeC1_3$ (Ferric chloride) and $Fe_2(SO_4)_3$ (Ferric sulphate). Organic chitosan flocculants have also been successfully applied in microalgae harvesting (Kurniawati, Ismadji, & Liu, 2014).

pH sensitivity also affects the clumping ability of microalgae at the maximum efficiency. Flocculation of microalgae *Scenedesmus quadricauda* and *Chaetoceros muelleri* at pH 11.6 and 11.5 was observed to have the maximum flocculating activity of around 94.7% and 100%, respectively (Huo *et al.,* 2014). Another microalgae species, *Nannochloropsis oculata* obtained from India was cultivated in Walnes's medium, and maximum flocculation was observed at 93.8% and 87.33% when $FeC1_3$ and $Fe_2(SO_4)_3$ were used at the concentration of 0.4 g/L and 0.6 g/L, respectively (Surendhiran & Vijay, 2013).

In another study, an unspecified species of microalgae grown on wastewater was also harvested using flocculation (jar test) with the help of metal salts, a cationic polymer, anionic polymer and natural coagulant. Alum, ferric chloride and cationic polymers were found to be able to achieve about 90% algal recovery at the optimal dosage (Udom *et al.,* 2013). The green microalgae *Tetraselmis tetrahele* was also tested for use as a potential biodiesel feedstock. Two out of five flocculant types used, NOaH and $A_2(SO_4)_3$ showed the highest flocculating efficiencies at 96.15% and 98.65%, respectively, at the concentration of 200 mg/L (Marco *et al.,* 2012). In this study, flocculation method was chosen because it offers a lot of advantages compared to other methods. Flocculation is a very cost effective method, faster and

easy to be conducted either in the laboratory or in the field. In addition, RSM analysis has been employed to intensively optimise flocculation efficiency to attain the best harvesting process for microalgae biomass.

Table 2.10: Advantages and disadvantages of microalgae harvesting technique (Barros *et al.*, 2015)

Type of harvesting	Advantages	Disadvantages
Centrifugation	• High biomass recovery. • Quick method. • Suitable for most microalgae species.	• Needs more energy to operate. • Not cost effective. • Cell damage due to high speed.
Filtration	• Able to harvest microalgae cells of very low density. • High recovery efficiency.	• Filter medium easily clogged/fouled by algae cells. • The medium should be regularly cleaned. • Medium replacement and pumping are major contributors to its cost.
Sedimentation	• Very simple and low-cost method. • Requires less energy.	• Time-consuming due to slow sedimentation and possibly leads to deterioration. • Low algae paste concentration.
Flocculation	• Reliable and cost-effective. • Easy to conduct and very fast method.	• Chemical flocculants may be toxic to microalgae biomass. • Reusability of the media is limited.

2.13 Microalgae and hydrocarbon production

Hydrocarbon always refers to the production of lipid content and lipid productivity. As discussed earlier, the hydrocarbon is a combination of two elements namely hydrogen and carbon. The composition of hydrocarbon exists in many products and materials. Currently, the popular sources of hydrocarbon come from fossil fuel and expectedly to diminish from time to time. In addition, it also considered unsustainable and give negative impact to our environment. But, it is different from hydrocarbon made of the biological plant. Since the microalga is photosynthesis organisms and the hydrocarbon obtained called as bio-hydrocarbon. Hydrocarbon

from biological plant expected given the discovery new renewable energy more valuable. So, it is necessary to find alternative renewable hydrocarbon which is more sustainable and environmentally friendly using potential microalgae biomass.

Nowadays, the production of hydrocarbon by previous author normally utilised for the production of biofuel. Biofuel can be categorised into primary and secondary. Then biodiesel is a secondary as 3rd generation of biofuel from microalgae (Dragone *et al.,* 2010). The potential of microalgae as a source of renewable energy has received considerable interest, but if microalgae biofuel production is to be economically viable and sustainable, further optimisation of mass culture conditions are needed (Pittman *et al.,* 2011).

As shown in Table 2.11, microalgae appear to be the only source of biodiesel that has the potential to completely displace fossil diesel. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Oil content in microalgae can achieve 30 - 70% by weight of dry biomass.

Table 2.11: Comparison of biodiesel sources (Gill *et al.,*2013)

No.	Sources	Oil yield L/Ha
1	Corn	172
2	Soybean	446
3	Canola	1190
4	Jatropha	1892
5	Coconut	2689
6	Palm oil	5950
7	Microalgae (70% oil in biomass)	136900
8	Microalgae (30% oil in biomass)	58700

Depending on species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils (Banerjee *et al.,* 2002; Metzger and Largeau, 2005; Guschina and Harwood, 2006). Most importantly, they do not compete with food crop and can be produced using non-arable land, wastewater in the bioreactor (Gill *et al.,* 2013). Table 2.12 shows the oil content of some microalgae species and clearly state that *Botryococcus sp*. is a species of microalgae that contains the most oil content of 25 - 80%. Therefore, cannot doubt that *Botryococcus* sp. has a very high potential to produce hydrocarbon combined with the application of phycoremediation technology. Through this literature review summarised in Table 2.12, the *Botryococcus* sp. was selected for this study compare to other species. The

Botryococcus sp. was rank as a priority of selection for lipid content of microalgae in dry biomass.

Table 2.12: The summary of lipid content by species in dry biomass.

No	Microalgae	Lipid content (% of dry biomass)	References
1	*Botyococcus braunii*	25-80	Wu *et al. (*2012); Gill*et al.* (2013)
2	*Dunaliella primolecta*	23	
3	*Euglena gracilis*	14-20	
4	*Nannochloris sp.*	30-50	
5	*Nannochloropsis sp.*	31-68	
6	*Pleurochrysis carterae*	30-50	
7	*Prymnesium parvum*	22-38	
8	*Spirulina maxima*	6-7	
9	*Spirulina platensis*	4-9	
10	*Phormidium sp.*	6.2-11.5	Ramachandra *et al.* (2013)
11	*Spirogyra sp.*	18.4-20	
12	*Euglena sp.*	24.6-31	
13	*Chlamydomonas mexicana*	33	Abou-Shanab *et al.* (2013)
14	*Scenedesmus obliquus*	31	
15	*Chlorella vulgaris*	29	
16	*Neochloris vigensis*	19.29	Aravantinou *et al.* (2013)
17	*Scenedesmus rubescens*	14.91	
18	*Chlorococcum sp.*	6.93	
19	*Scenedesmus acutus*	28.3	Sacristán de Alva *et al.* (2013)
20	*Chlamydomonas polypyrenoideum*	59	Kothari *et al.* (2013)
21	Indigenous microalgae	4.9-11.3	Woertz *et al.* (2010)

2.14 Biofuel

Biofuels are normally referred to solid, liquid or gaseous fuel originated from organic matter (Nigam & Singh, 2011). Based on Figure 2.5, biofuels are classified into nature biofuels, primary biofuels and secondary biofuels. Natural biofuels are generally derived from organic sources and include vegetable, animal waste and landfill gas. On the other word, primary biofuels are fuel-wood used mainly for cooking, heating, brick kiln or electricity production. The secondary biofuels are bioethanol and biodiesel developed by biomass process and used in the transport sector (Nigam & Singh, 2011). Whereas the secondary biofuels are divided into three generation, namely first generation, second generation and third generation biofuels

depended on their different features such types of processing, feedstock and their development levels (Dragone *et al.*, 2010).

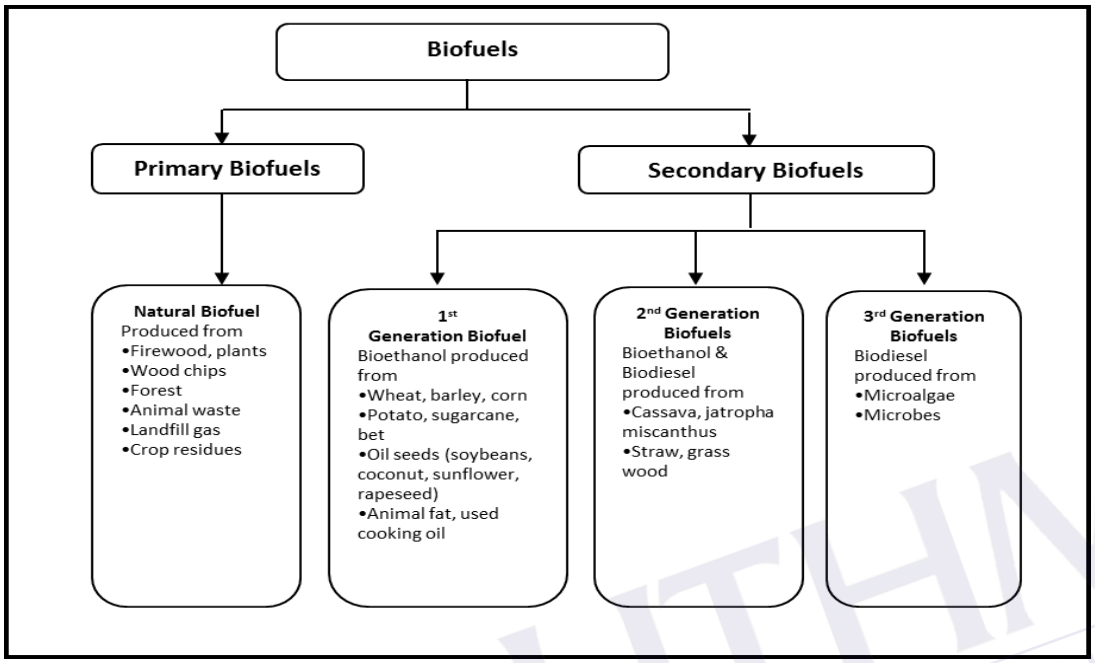


Figure 2.5: Biofuel production sources (Dragone *et al.*, 2010)

Hence, the aim of the research is drawn to third generation biofuels. The main component of third generation biofuels is microalgae as shown in Figure 2.5. It is currently considered to be a feasible alternative renewable energy resource for biofuel production overcoming the disadvantages of first and second generation biofuels (Alam *et al.*, 2012). The potential for biodiesel production from microalgae is 15 to 300 times more than traditional crops on an area basis (Dragone *et al.*,2010). Additionally, the microalgae generally have higher productivity than land-based plants as some species have doubling times of a few hours and accumulate very large amounts of triacylglycerides (TAGs).

However, it is well known that the production of biofuel is a complex process. Thus, Figure 2.6 shows a schematic of biofuel production from microalgae. The process consists of following several stages: a) stage 1 - microalgae cultivation, b) stage 2 - harvesting, drying & cell disruption (cells separation from the growth medium), c) stage 3 - lipid extraction for biodiesel production through transesterification and d) stage 4 - starch hydrolysis, fermentation & distillation for

bioethanol production. These processes are complex, technologically challenges and economically expensive (Alam *et al.*, 2012).

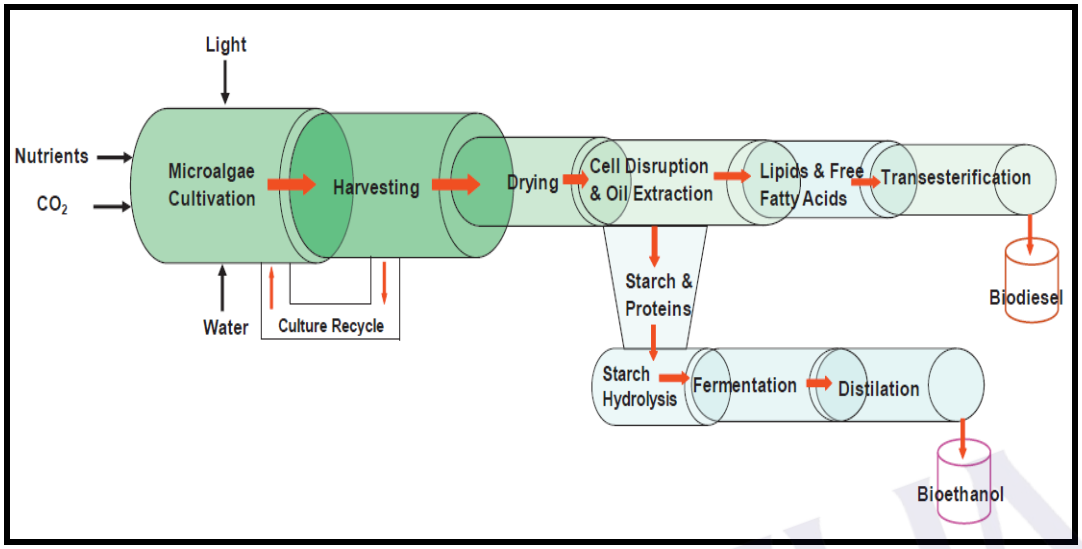


Figure 2.6: The biofuel production process from microalgae (Dragone *et al.*, 2010)

2.15 Biodiesel and its advantages

Biodiesel or fatty acid methyl ester (normally known in the scientific word) is derived from plant or animal oil or fat. Biodiesel is produced or created by a combine reaction among three kinds of ingredients such as oil, alcohol and chemical catalyst. Normally, vegetable or animal oil serves as the oil component while methanol or ethanol is used as the alcohol and lye or potassium hydrochloride is used as a catalyst. After processing, the result is 100 percent of biodiesel. Biodiesel can be used without mix with diesel or blend together with petrodiesel in varying combinations.

The enthusiasm of the researchers in arguing about the quality of biodiesel production due to the several advantages that cannot be disputed to our environment (Purcella, 2009). Firstly, biodiesel burns cleaner than petrodiesel, with the level of carbon dioxide, hydrocarbons, carbon monoxide and particulates. It contains no sulfur dioxide- the substance that's implicated in acid rain. Even when mixed with petrodiesel, biodiesel substantially lowers emissions. Other than that, biodiesel does not contribute to the global warming due to the biodiesel emits only the CO_2 it originally contained as a plant. Next, biodiesel is biodegradable liquid. If it spills, it will degrade naturally without harming the environment. This makes it a potentially

valuable fuel for marine use because it will simply break up without damaging the water and aquatic life. Lastly, biodiesel is considered nontoxic, as it contains no substances that are harmful to people or the environment. The most potentially toxic aspect of biodiesel is during manufacturing process only, since the caustic substance often used as a catalyst but it can be avoided with extra careful handling or process (Purcella, 2009).

2.15.1 Biodiesel sources

According to the book written by Purcella (2009) which is stated biodiesel can be made from many oil feedstock plants. Soybeans are currently the most commonly used and many other plants have a great potential as a biodiesel feedstock. Another oil with great promise is the jatropha plant. Jatropha oil is vegetable oil produced from the seed of the Jatropha Curcas, a plant that able to grow in the wasteland. The Jatropha plant grows almost everywhere, even on poor sandy soils, gravelly soil, or other soils. After the oil is extracted from the plant, the remaining biomass can be used to power electricity plants. Another the most talked about feedstock for biodiesel is algae where almost directly related to this study. According to Purcella (2009), microalgae have much faster growth rates than terrestrial crops. This gives algae huge potential as a biodiesel feedstock. However, the investigation is still going on to the microalgae species which is can produce the highest and quality of hydrocarbon. Commercially, vegetable oils are evaluated for use as a biofuel and rated on criteria such as:

i. Suitability as fuel, based on flash point, energy content, viscosity, combustion products and another factor.
ii. Cost, based in part on yield, the effort required growing and harvest, and postharvest processing cost.
iii. Cold-weather performance, some oils will produce biodiesel that gels at a temperature as high as 18.3°C while others will be suitable for below 4.4°C.

2.15.2 Advantages of using microalgae for biodiesel production

Many research reports and articles described many advantages of using microalgae for biodiesel production in comparison with other available feedstock. From a

practical context, they are easy to cultivate, can grow with little or even no attention, using water unsuitable for human consumption and easy to obtain nutrients *(*Mata *et al.*, 2010).

In addition, they can grow almost anywhere, requiring sunlight and some simple nutrients, although the growth rates can be accelerated by the addition of specific nutrients and sufficient aeration (Aslan & Kapdan, 2006). According to Delucchi (2003) where algae biodiesel contains no sulfur and performs as well as petroleum diesel while reducing emissions of particulate matter, CO_2 and hydrocarbons.

The utilisation of microalgae for biofuels production can also serve other purposes. Some possibilities currently being considered are listed below.

i. Removal of CO_2 from industrial flue gases by algae bio-fixation, reducing the GHG emissions of a company or process while producing biodiesel (Wang *et al.*, 2008).
ii. Wastewater treatment by removal of NH_4^+, NO_3^-, PO_4^{3-}, making algae to grow using these water contaminants as nutrients (Wang *et al.*, 2008).
iii. After oil extraction the resulting algae biomass can be processed into ethanol, methane, livestock feed, used as an organic fertiliser due to its high N:P ratio, or simply burned for energy cogeneration (Wang *et al.*, 2008).
iv. Microalgae can potentially revolutionise a large number of biotechnology areas including biofuels, cosmetics, pharmaceuticals, nutrition and food additives, aquaculture, and pollution prevention (Raja *et al.*, 2008).

2.16 Summary

The potential of microalgae to be used to treat wastewater in removing the chemical and organic contaminants and heavy metal is high. Moreover, wastewater provided a conducive growth medium for microalgae to assimilate those contaminations. The resulting biomass is energy rich which can be further processed to make biofuel, biodiesel, and other bio-hydrocarbon. Other than that, algae biomass also can be used to obtained product called as a bio-based product such as bio-plastic, fertiliser, micro-beads, animal food and much more. It should be noted, however, most of previous studies of phycoremediation and hydrocarbon production that are reported in the open literature using different type of microalgae species (*Demodesmus* sp.,

Chlorella sp. and *Spirulina platensis*) rather than our study and yet the application of wastewater treatment (Hadiyanto *et al.*, 2013; Ji *et al.*, 2014; Sivakumar & Rajendran, 2013). There is very limited use of *Botryococcus braunii* in hydrocarbon production coupled with wastewaters phycoremediation such as piggery wastewater, soybean curd wastewater and greywater (An *et al.*, 2003; Gokulan *et al.*, 2013; Yonezawa *et al.*, 2012). Previously, researchers preferred using synthetic media to investigate the production of hydrocarbon from *Botryococcus braunii* (Ashokkumar *et al.*, 2014; Cheng *et al.*, 2013; Furuhashi *et al.*, 2016; Manchanda *et al.*, 2016; Sakamoto *et al.*, 2012; Samorì *et al.*, 2010; Talukdar *et al.*, 2013). To the best of author's knowledge, the phycoremediation of domestic and food processing wastewater by *Botryococcus* sp. locally isolated from our tropical rainforest along with metals removal and valuable biomass for hydrocarbon extraction has not been studied by the researchers in the past. Nevertheless, this microalgae species (*Botryococcus* sp.) had been examined only for the phycoremediation of different wastewaters such as greywater and meat processing wastewater (Atiku *et al.*, 2016; Latiffi *et al.*, 2016) and this motivated to the present study. Next chapter presents the research methodology development used in this study to address the gaps as stated above.

CHAPTER 3

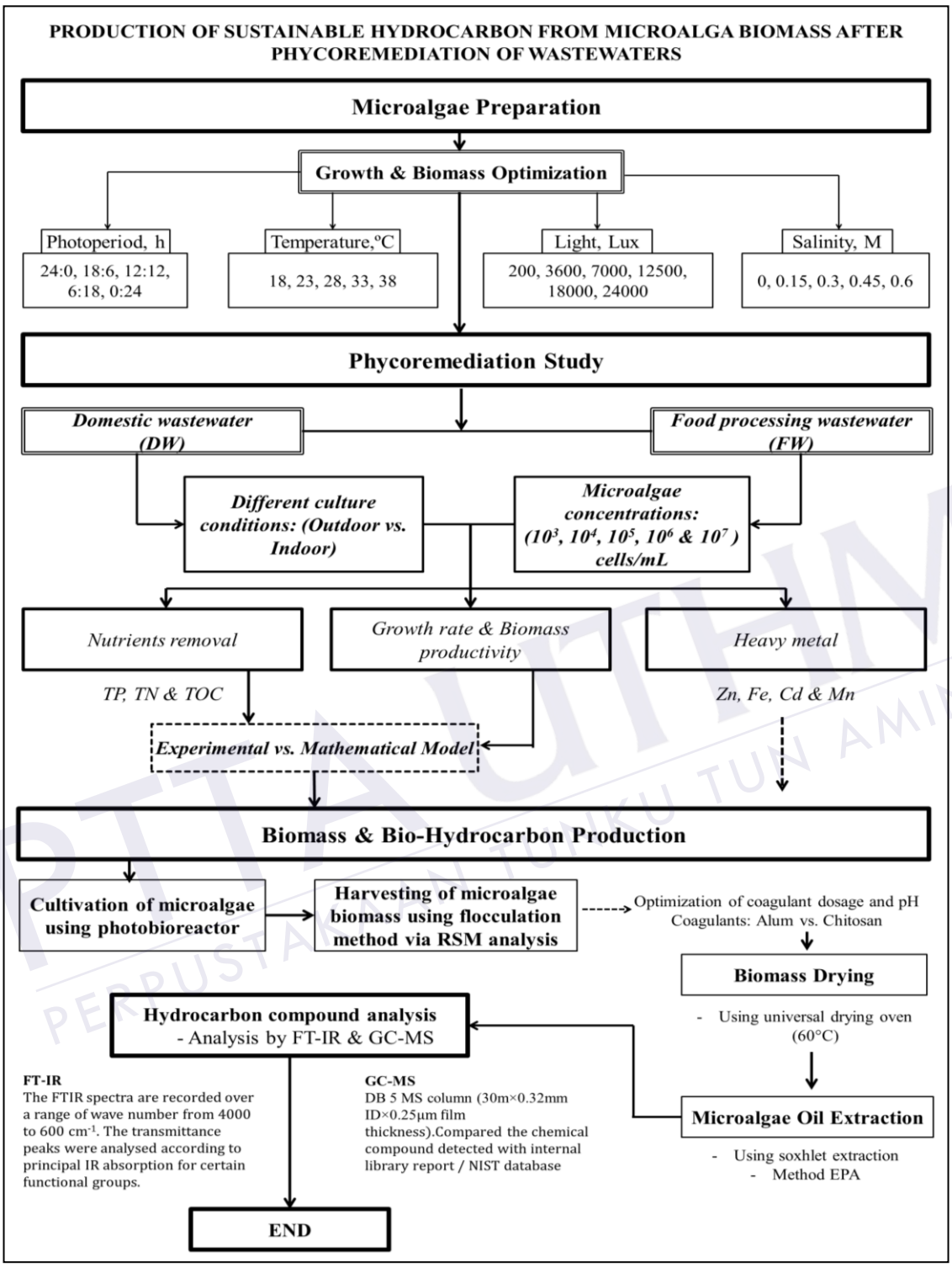
METHODOLOGY

3.1 Introduction

This study carried out with a systematic work plan to achieve the outline goals and objectives successfully. In this chapter there are several stages of work that has been designed to facilitate the work for study. There are including the information on the laboratory experimental procedure, process and analysis. Other than to develop a technique of phycoremediation, the objectives of this study are also to analyses the effectiveness of microalgae to treat the wastewater and determine the growth of *Botryococcus* sp. in wastewaters and Bold's Basal Medium (BBM). Then, the last stage of this experimental is to specify the hydrocarbon production from this microalga after carried out phycoremediation process. Therefore, experimental and method development needs to clarify clearly to ensure this research achieves the main objective. Experimental and research method development is shown in Figure 3.1 and the explanations of all technique and analysis method are discussed further in this chapter.

3.2 Experimental and method development

Generally, there are several parts involved in this study (refer to Figure 3.1). First part is microalgae preparation which is mostly concerned with the biological part. Microalgae samples collected from the field and isolated before *Botryococcus* sp. identification.



Objectives:

i. To optimize the specific growth rate and biomass productivity of *Botryococcus* sp. at different situation of environmental factors.
ii. To develop a new technique employing microalgae *Botryococcus sp.* in treating wastewater (domestic and food processing wastewater).
iii. To evaluate the potential of biomass production from microalgae biomass cultivated using closed photobioreactor.
iv. To establish the chemical composition profile of bio-hydrocarbon from microalgae biomass cultivated in domestic and food processing wastewater.

Figure 3.1: Flow of research methodology development.

After done isolation and identification, microalgae cultured and maintained as a stock in synthetic medium. Before carrying out the phycoremediation process, an environmental factor of microalgae to growing need to be determined first. There is four environmental factor need to optimise which is photoperiod, temperature, light intensity and salinity (Qin & Li, 2006). After done the optimisation of environmental factor, then phycoremediation study conducted using wastewaters in the laboratory. Statistical analysis (SPSS) was used to validating the environmental factor and also compared with a mathematical model. The length of the phycoremediation period determined as well.

The second part is a phycoremediation process. This process is very important and should be emphasised in more detail since it's related to environmental engineering field. In this part, phycoremediation study both indoor and outdoor undertaken. For the indoor study, phycoremediation did according to a controlled environmental factor which obtained from growth rate and biomass productivity of *Botryococcus* sp. when preliminary studied using synthetic media. While the outdoor phycoremediation implemented is based on the natural conditions outside. Both phycoremediation conditions were compared in term of pollutants removal, biomass productivity and specific growth rate. The best conditions of environmental factor proceed for upscale culture using photobioreactor system.

Then, the third part of this research method development is up-scale biomass and hydrocarbon production by photobioreactor. Several factors need to be taken into account in designing this photobioreactor. Among them are light penetration, CO_2, water and temperature. Before obtaining the hydrocarbon, microalgae have to harvest after phycoremediation by using flocculation method. In this stage, coagulant dose used and optimum pH determined according to Jar Test standard method in wastewater. Once done the harvesting, biomass of *Botryococcus* sp. dried with the help of universal drying oven instead of freeze dryer. In this process, biomass estimated based on the volume of the wastewaters used and harvesting efficiency can be found. The crude oil from *Botryococcus* sp. will be extracted using an organic solvent with the help of soxhlet apparatus. The soxhlet extraction is a common method to obtain the lipid from the biological plant. After that, the purity of microalgae oil was analysed and measured using Gas Chromatogram – Mass Spectrometry (GC-MS) and Fourier Transform Infrared Spectrometry (FTIR) to detect the hydrocarbon compound profile.

3.3 Microalgae preparation

There are three common works used in microalgae preparation of this study. The first step is freshwater microalgae sampling, second isolation process and the last one is the identification of microalgae species. All these processes need to be done properly to ensure the only particular that microalgae species obtained to carry out this study.

3.3.1 Microalgae sampling

The type of freshwater algae employed in this study is green microalgae come from genus *Botryococcus* sp. (Figure 3.2) and categorised as a microscopic plant. This microalga obtained from a tropical rainforest in the Southern region of Peninsular Malaysia (between N 02° 30.711" E 103° 20.984" and N 02° 30.740" E 103° 20.996"). Sterilised glass bottles used to collect fresh algae from the field before conducting the isolation process. Firstly, fill about 1/3 of the collection bottles with the water of the habitat and then put the collected fresh indigenous algae in them. On reaching the laboratory, the cork of the bottles should be opened and cover using the cotton wool ball. Avoid using tab water of the laboratory for storing collected fresh algae because it is usually chlorinated and may toxic to the algae.

3.3.2 Microalgae isolation

Isolation is a compulsory process to obtain the pure cultures and presents the second step after sampling. The method that applied in this study is as same as described by Andersen (2005). Firstly, wash the sample using sterile distill water three times. After that, dilute the sample in five times (ratio, 100 µL:900 µL). Three methods of isolation was employed which is pour plate, streaking and bold's basal medium (BBM). For the pour plate method, 2% sterile nutrient agars used was mixed with BBM and pours it into a sterile petri dish. Put the entire sample in room condition with exposure to the sunlight. Let them grow about 14 days before. After that, observed the sample under the microscope to ensure that the thallus is clean and worth isolation. Repeating the process until pure single culture obtained. Then, maintained the free suspension pure algal in BBM.

3.3.3 Microalgae identification

A sample of isolated algal culture examined under morphologically in a light microscope for preliminary identification and confirmation that the culture is unialgal using an OLYMPUS CX22LED microscope (Olympus Corporation Japan) attached to digital camera connected to the computer (Abdelaziz *et al.*, 2014). Preliminary identification also made using a field guide as recommended by Prescott (1978). Lastly, species confirmation done according to DNA sequence and then blasted using GenBank securely database online to identify the species and phylogenic tree.

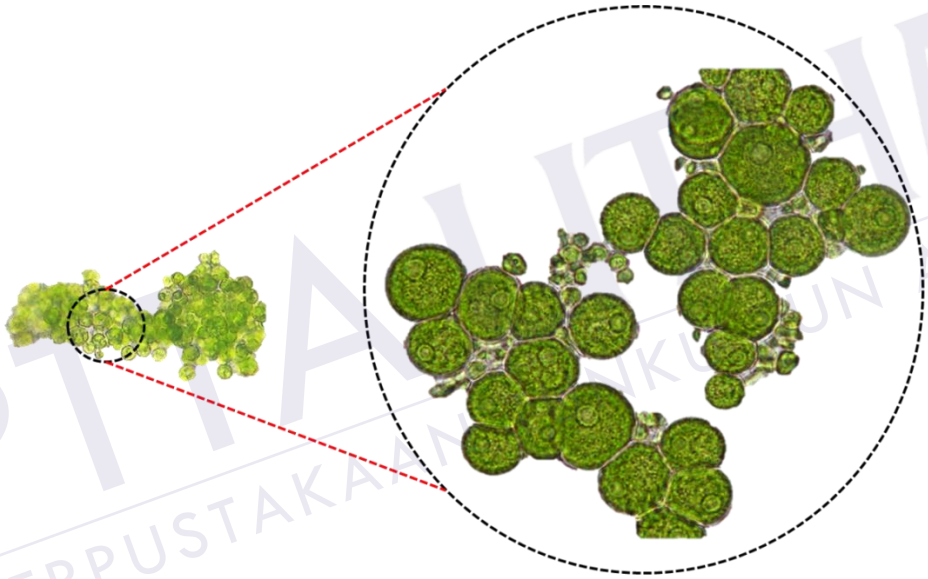


Figure 3.2: The morphology of *Botryococcus* sp. (× 40) observed under a light compound microscope.

The DNA extraction of isolated species was performed using cetyl trimethyl ammonium bromide. After extraction, 5 µL of the DNA sample was run on test gel. Then, one DNA product from the species was obtained. The nuclear-encoded 18rRNA genes were amplified by polymerase chain reaction (PCR) using a primer published by Medlin *et al.* (1988). The PCR program was run as follows: 95°C, 5 min; 94°C, 45 s (denaturation); 55°C, 30 s (annealing); 72°C, 2 min (DNA synthesis, elongation); repeated for 30 cycles; 72°C, 10 min; and 4°C hold. The PCR product was purified with a purification kit supplied by Sigma and was again checked on 1% agarose gel to prepare the sequencing. The sequence obtained was matched with

those available in GenBank using a basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The BLAST database found that a high similarity to *Botryococcus* sp. at about 92%, with an accession number of JQ585723.1 (refer to **APPENDIX A**).

3.3.4 Bold's Basal Medium (BBM) stock culture

The isolated microalgae stock is cultivated in Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) with the stock solutions compositions are shown in Table 3.1. Approximately 10 ml of each of the stock solution and 1 ml of micronutrients stock solutions are added to 940 ml of distilled water. After that stock solution prepared it placed in the autoclave for 15 minutes (121ºC) before inoculated with *Botryococcus* sp. cell. Then, the media are exposed to the direct sunlight for 14 days to observe the cultivation of microalgae.

Table 3.1: The composition of Bold's Basal Medium

Composition of stock solutions	Per Liter distilled water (dH_2O)
NaNO3	25.0 g
$CaCl_2.2H_2O$	2.5 g
$MgSO_4.7H_2O$	7.5 g
K_2HPO_4	7.5 g
KH_2PO_4	17.5 g
NaCl	2.5 g
EDTA	50.0 g
KOH	31.0 g
$FeSO_4.7H_2O$	4.98 g
H_2SO_4	1.0 ml
H_3BO_3	11.42 g
Micronutrients	g.L-1
$ZnSO_4.7H_2O$	8.82 g
$MnCl_2.4H_2O$	1.44 g
MoO_3	0.71 g
$CuSO_4.5H_2O$	1.57 g
$Co(NO_3)_2.6H_2O$	0.49 g

3.4 Estimation and measurement of microalgae growth

In this study, there was two type of method was used to estimate the growth of microalgae. Initially, *Botryococcus* sp. growth was measured using haemocytometer with the help of microscope for the preliminary growth assessment and lab scale of

phycoremediation. Meanwhile, optical density (OD) measurement was employed at the stage of scale-up of study using closed photobioreactor for biomass production. Details for both methods were discussed intensively in the following section.

3.4.1 Determination of cell concentration using Haemocytometer

Cell count method is a method to estimate the growth rate and biomass productivity of microorganisms. In this research, a Haemocytometer is used to calculate the cell number of *Botryococcus* sp. in media and determine the growth rate of *Botryococcus* sp. (Andersen 2005). The Haemocytometer is a device used to count cells. The Haemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber (Figure 3.3). This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is, therefore, possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall (refer to Equation 3.1). All the standard procedure is done according to a standard method (APHA, 2012).

Figure 3.3: Compound light microscope and haemocytometer grid chamber

Total 25 smaller squares in the middle of the grid marked 5, each small square is 0.2 × 0.2 mm^2, and the volume is thus 0.004 mm^3. For small cells or organelles, the particles/ml equals the average number of particles per small square times 25 × mm^4 times any sample dilution.

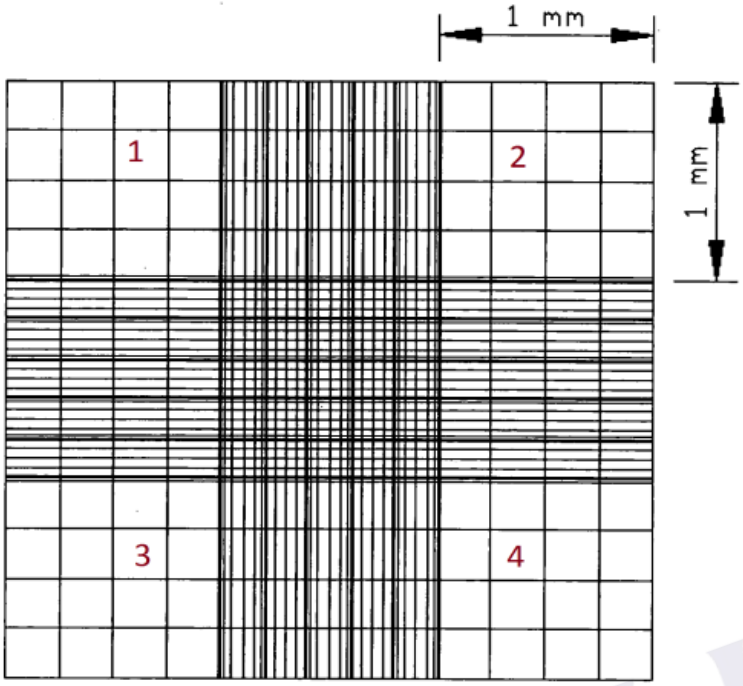


Figure 3.4: Areas in Haemocytometer

Grids 1-5 are all size of 1 mm^2. Meanwhile, grids 1-4 are divided into 16 smaller squares (0.25 mm on each side) grid 5 are divided into 25 smaller squares (0.2 mm on each side). Grid 5 is further subdivided into 16 of the smallest squares found on the haemocytometer grid chamber. A number of cells per cm^3 is also number per ml. The calculation is referred to Equation 3.1 (Gani *et al.*, 2016b).

Number of cells/ml = Average number of cells per 1 mm^2/ 10^{-4} x sample dilution (3.1)

3.4.2 Measurement of growth using UV-Vis spectrometry

The growth of *Botryococcus* sp. was observed by determining the OD at 680nm using spectrophotometer (DR6000, UV-Vis Spectrophotometer – HACH, USA). The dry weight (mg/L) was determined according to total suspended solid (TSS) according to a standard method (APHA, 2012). The plotted against the corresponding OD_{680} to obtain a calibration curve (Figure 3.5) was described by Equation 3.3.

$$\text{Dry weight (mg/L)} = 287.46x - 2.221 \quad (R^2 = 0.999) \tag{3.3}$$

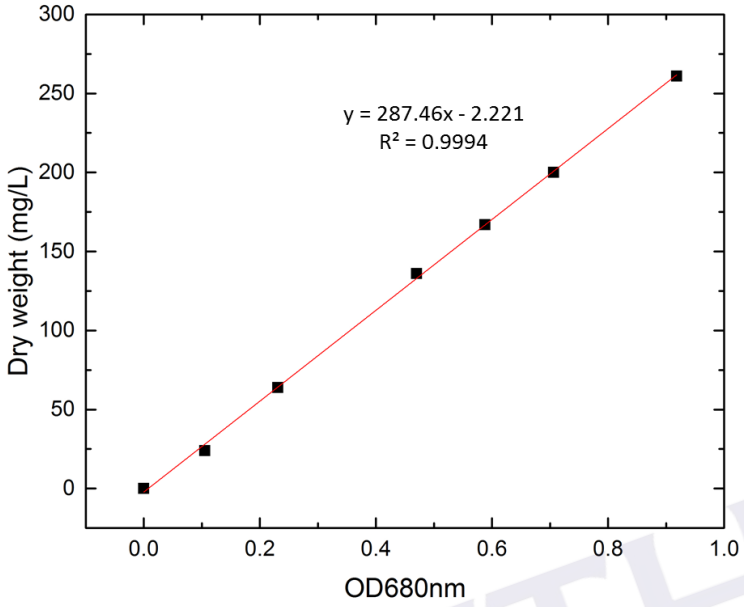


Figure 3.5: Calibration curve (Dry weight vs. OD_{680}).

3.5 Growth optimisation

Optimisation of *Botryococcus* sp. growth measured based on the environmental factors. According to Qin & Li (2006), there are several environmental factor need to be optimised such as photoperiod, temperature, light intensity and salinity. The optimisation of these factors depended on the rate of specific algal growth and biomass productivity. Synthetic medium (BBM) as a culture media and examined based on the preliminary study of daily temperature and rate of light intensity presented in Table 3.2.

Total seven days of preliminary study was conducted at daily temperature is 28.2°C with maximum 35.5°C and minimum 25.8°C. However, maximum temperature used in this study was extended to 38°C and the minimum reduced up to 18°C (Table 3.4). The reason is to identify the survival of this microalga when subjected to the changes of unexpected temperature. For instance, growth abilities of this microalga to be adopted in the cool area and high temperature water especially in the highlands and hot springs. While, for maximum and minimum outdoor light intensity was 18000 Lux and 200 Lux, respectively. The lowest of intensity due to

the early morning of the day and the highest was because of the afternoon daylight. But, this experiment was conducted with the highest light intensity up to 24000 Lux due to the biomass still increase high on 18000 Lux (Table 3.5).

Table 3.2: Environmental preliminary study of temperature and daylight intensity

No.	Environmental factors	Average (n=7)	Minimum	Maximum
1	Temperature (°C)	28.2	25.8	35.8
2	Light Intensity (Lux)	7000	200	18000

The environmental factor optimisation was conducted by using 500ml Erlenmeyer flask in the culture room (dark room) which is not interrupted by other light illumination. The temperature adjustment was controlled by using refrigerated water bath as an alternative to plant growth chamber (Belcher & Swale, 1982).

3.5.1 Photoperiod

Total five photoperiods (24h light: 0h dark, 16h light: 8h dark, 12h light: 12h dark, 6h light: 18h dark and 0h light: 24h dark) used in this study with three replicates, respectively (Qin & Li, 2006). Photoperiod experiment had been done using electric socket timer (Brand: Eurosafe, Model: ES-24HT). Each flask contained 350ml BBM medium inoculated with 1000 cell/ml of *Botryococcus* sp. then cultured at 28ºC and 7000 Lux irradiance. The algal growth measured by cell counting using haemocytometer. Then, homogeneous algal liquid is sampled from each flask daily. Summary of the photoperiod optimisation as described in Table 3.3.

Table 3.3: Photoperiod for optimisation study

Photoperiod (H)	24:0	16:8	12:12	6:18	0:24
Temperature (ºC)	28	28	28	28	28
Light Intensity (Lux)	7000	7000	7000	7000	7000
Salinity (M)	0	0	0	0	0

3.5.2 Temperature

Total five set of temperature used in these experiments: 18, 23, 28, 33 and 38ºC controlled with water bath equipment. Three replicates are used in each examination. At all temperature, the microalgae are cultured under the following conditions: manual shaking, 7000 Lux (Model: LX-101, Made of Taiwan), photoperiod of 12 h light: 12 h dark and 0 M of salinity. A volume of 350 ml algal liquid is bath watered in each 500 ml Erlenmeyer flask. The algal growth measured every day and Table 3.4 shows the detail of this experimental.

Table 3.4: Temperature for optimisation study

Temperature (ºC)	18	23	28	33	38
Photoperiod (H)	12:12	12:12	12:12	12:12	12:12
Light Intensity (Lux)	7000	7000	7000	7000	7000
Salinity (M)	0	0	0	0	0

3.5.3 Light intensity

Total six light intensities (200, 3600, 7000, 12500, 18000 and 24000 Lux) with three replicate conducted. The unit of Lux had been converted to µmol $m^{-2}s^{-1}$ by multiply the Lux value with 0.1035 (cool daylight lamp coefficient) for the further discussion in Chapter 4. The cool daylight bulb (Dimmable LED Bulb, 12W) had been used to illuminating the algae culture. Each flask contains 350 ml of algal culture inoculated with 1000 cell/ml of *Botryococcus* sp. manually shaking and exposed to the photoperiod 12 h light: 12 h dark. Temperature constant within 28ºC and the salinity which is 0 M. Table 3.5 shows the condition of this experiment.

Table 3.5: Light intensity range for optimisation study

Light Intensity (Lux)	200	3600	7000	12500	18000	24000
Photoperiod (H)	12:12	12:12	12:12	12:12	12:12	12:12
Temperature (ºC)	28	28	28	28	28	28
Salinity (M)	0	0	0	0	0	0

3.5.4 Salinity

Total five different of salinity levels are involved: 0, 0.15, 0.3, 0.45, 0.6 M of NaCl with three replicates (Qin & Li, 2006). The determination of salinity based on molarity calculation (Stephenson, 2010). Each flask contains as same as photoperiod, temperature and light intensity algal liquid. The algal was cultured under 28ºC, 7000 Lux and 12 h light: 12 h dark photoperiod. The sampling schedule is the same as in light experiment and Table 3.6 shows the detail.

Table 3.6: The salinity range for optimisation study

Salinity, M (%)	0 (0%)	0.15 (3.07%)	0.3 (6.14%)	0.45 (9.20%)	0.6 (12.27%)
Light Intensity (Lux)	7000	7000	7000	7000	7000
Photoperiod (H)	12:12	12:12	12:12	12:12	12:12
Temperature (ºC)	28	28	28	28	28

3.6 Growth rate, biomass productivity, and mathematical model measurement

3.6.1 Maximum growth rate determination

The kinetic growth of *Botryococcus* sp. was determined according to the maximum growth rate (day^{-1}); meanwhile, division per day (D_d) and doubling time (t_d) were calculated according to Equation 3.4 and Equation 3.5, respectively (Andersen, 2005). Division per day in microalgae growth refers to the situation of algae growth undergoes cell division process per day meaning that each of algae cell divides once in a day.

Maximum specific growth rate was obtained from the slope of the exponential stage of the growth curve (Zwietering *et al.*, 1990). Normally, this parameter is estimated by deciding subjectively which part of the curve is approximately linear and then determining the slope of this curve section, eventually by linear regression (Figure 3.6). This method describes the number of cells (N) or the logarithm of the number of cells [Log(N)] as a function of time (Zwietering *et al.*,

1990). At least three-time points were considered to satisfy or confirm the exponential stage (Andersen, 2005).

$$\text{Division per day } (D_d) = \frac{\mu_{max}}{\ln(2)} \quad (3.4)$$

$$\text{Doubling time } (t_d) = \frac{1}{D_d} \quad (3.5)$$

μ_{max} = Maximum growth rate (day^{-1})
D_d = Division per day

3.6.2 Biomass productivity measurement

Biomass productivity measurement is a very important parameter to be evaluated for microalgae cultivation (Gani *et al.*, 2016b). In this study, biomass productivity was determined volumetrically based on growth kinetic parameter using Equation 3.6, in which $\mu_{max,}$ X*m* and X*o* were defined as maximum specific growth rate, maximum cell concentration in the culture, and initial cell concentration, respectively (Figure 3.6).

In this case, we consider that the time spent in the lag phase and in the late stationary phase of the cultures must not be included in calculations, in order to reduce sources of variation that can hide productivities (initial biomass concentration of the inoculum or its preservation conditions), as can be seen in Figure 3.6. Then, we arrive at the following expression considering only the biomass generated once initial biomass has increased in a 10% and until 90% of the maximum biomass is reached (refer to **APPENDIX B**).

$$\text{Biomass productivity} = \frac{\mu_{max}\,(0.9X_m - 1.1X_0)}{\ln \frac{9(X_m - 1.1X_0)}{1.1X_0}} \quad (3.6)$$

μ_{max} = Maximum growth rate (day^{-1})
X_m = Maximum cell concentration (cell/mL)

X_0 = Initial cell concentration (cell/mL)

3.6.3 Growth mathematical model

The Verhulst logistic model was used to predict *Botryococcus* sp. growth in the culture compared with an experimental curve (Arbib *et al.*, 2014). Therefore, a logistic equation was selected for growth mathematical model per Equation 3.7, where dx/dt is the microalgae growth rate and X is the cell concentration of microalgae in the medium. By integrating equation 3.7, we obtained Equation 3.8. When t = 0, the *Botryococcus* sp. concentration may be derived via initial the cell concentration value (X = Xo). The complete calculation of growth mathematical model was described in **APPENDIX C**.

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_m}\right)X \tag{3.7}$$

$$X = \frac{X_0 . X_m . e^{\mu_{max} t}}{X_m - X_0 + X_0 . e^{\mu_{max} t}} \tag{3.8}$$

X = Cell concentration

X_m = Maximum cell concentration

X_0 = Initial cell concentration

μ_{max} = Maximum growth rate

t = Time

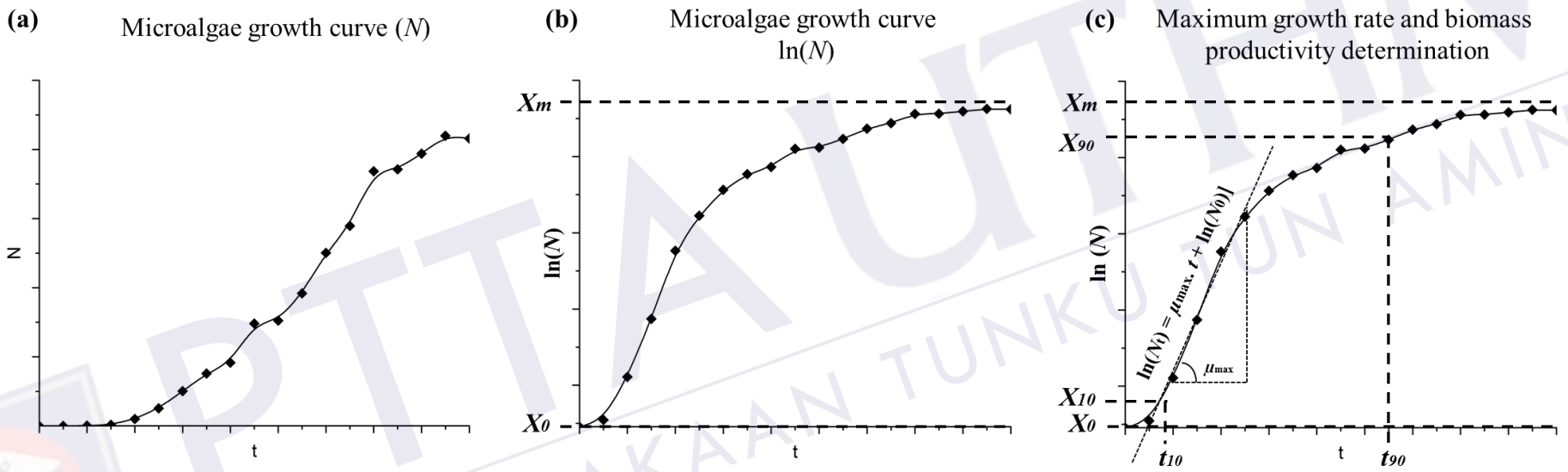


Figure 3.6: Demonstration of maximum growth rate (day^{-1}) measurement used in this study adapted from Zwietering *et al.* (1990) and biomass productivity calculation as proposed by Álvarez-Díaz *et al.* (2014). (a) Typical microalgae growth curve (*N*), (b) growth curve in the form of ln(*N*) and (c) maximum growth rate and biomass productivity determination.

3.7 Wastewater sampling and processing

There are two types of wastewater collected and to be used to carry out this research which is domestic (DW) and food processing (FW) wastewater. The DW used in this study was effluent wastewater obtained from the wastewater treatment plant located in the Universiti Tun Hussein Onn Malaysia campus (N 01° 51' 55.224" E 103° 5' 21.183"), Johor, Malaysia. This treatment plant receives DW generated from the students' residential college and cafeteria on the campus. Meanwhile, the FW was collected from local manufacturing industry located in Rengit Town of Johor, Malaysia (N 01° 41' 13.2" E 103° 07' 43.6"). Basically, this industry carries out processing activities and the production of snack foods such as potato chips, banana chips, and cookies. The main reason the usages of different types of wastewater are to evaluate the efficiency of the phycoremediation technology. Besides, it is to compare a different kind of hydrocarbon production by used the different wastewater.

All the preparation of samples conducted accordingly to the established standard method (Water and Wastewater Standard Method 22nd Edition, 2012). A sampling of each site or location conducted during the dry season (April to October). Samples stored in the HDPE bottle and duran bottle (heavy metal analysis) for transportation to the laboratory. Once in the laboratory, the wastewaters characterised in term of physiochemical and heavy metal content based on the standard method. Before the phycoremediation process, wastewater was filtered twice. First, filtered using cloth sieve to remove course material including zooplankton then secondly, filtered by membrane filter 0.45um pore size to remove all suspended solid and another microorganism that expected potentially give competitive to microalgae in term of food or nutrient consumption. Therefore, unwanted microbes have to be removed before phycoremediation and this process called as pre-treatment of wastewater.

3.8 Examination of wastewater quality

All wastewater involved in this research examined in physiochemical and heavy metal content based on the laboratory standard method (Water and Wastewater Standard Method 22nd Edition, 2012). There are several physiochemical only

analysed for the removal efficiency as listed in Table 3.7. Similarly, selected heavy metal element (Zn, Fe, Cd and Mn) had been choosing for removal analysis since they were detectable in the sample.

3.9 Phycoremediation setup

Two culture conditions (outdoor and indoor) were established for the phycoremediation conducted in the present study (Figure 3.7). Both cultures were subjected to five different initial concentrations: 10^3, 10^4, 10^5, 10^6, and 10^7cells/mL. The experiments in the outdoor condition were conducted outside of the laboratory (wastewater engineering laboratory) in the Faculty of Civil and Environmental Engineering, Universiti Tun Hussein Onn Malaysia (N 01° 51' 46.9" E 103° 04' 59.8").

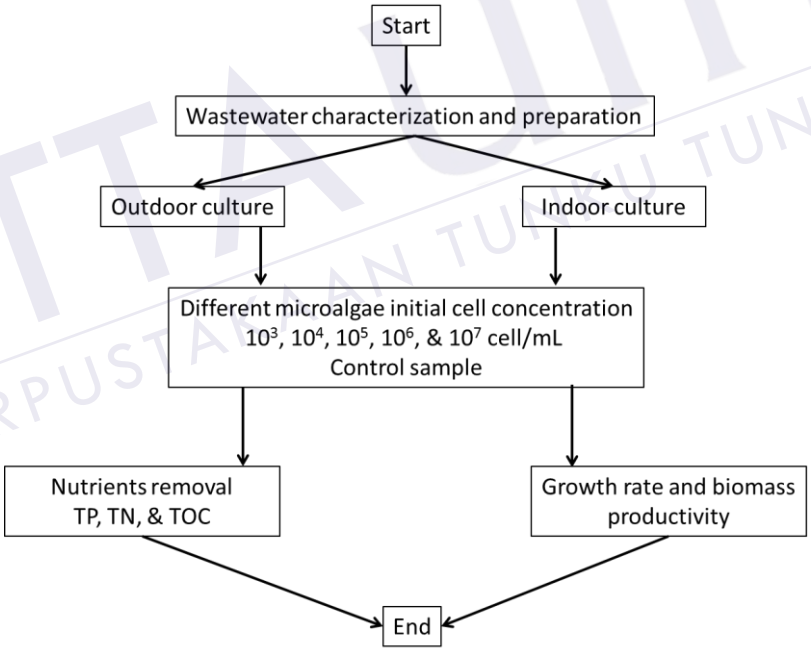


Figure 3.7: Experimental design carried out in the phycoremediation study.

Table 3.7: Wastewater parameters

Description	Unit	Characterization	Removal analysis	Method
Physiochemical		√	-	DR6000 (Method 8000)
Chemical oxygen demand	mg/L	√	-	DR6000 (Method 8000)
Biochemical oxygen demand	mg/L	√	-	APHA, 2012 (Method 5210)
Total phosphorus	mg/L	√	√	DR6000 (Method 10127)
Total nitrogen	mg/L	√	√	TOC Analyzer
Total suspended solid	mg/L	√	-	APHA, 2012 (Method 2540D)
Total dissolved solid	mg/L	√	-	APHA, 2012 (Method 2540C)
Total organic carbon	mg/L	√	√	TOC Analyzer / DR6000 (10129)
Total carbon	mg/L	√	-	TOC Analyzer
Inorganic carbon	mg/L	√	-	TOC Analyzer
Nitrate	mg/L	√	-	Ion Chromatography
Phosphate	mg/L	√	-	Ion Chromatography
Chloride	mg/L	√	-	Ion Chromatography
Dissolved oxygen	mg/L	√	-	APHA, 2012 (Method 4500O)
pH	-	√	-	pH Meter
Turbidity	NTU	√	-	Turbidity Meter
Salinity	%	√	-	Multimeter
Heavy metals				
Zinc, Zn	ppb	√	√	ICP-MS
Ferum, Fe	ppb	√	√	ICP-MS
Cadmium, Cd	ppb	√	√	ICP-MS
Manganese, Mn	ppb	√	√	ICP-MS

The outdoor temperature ranged from 28 – 38°C while the light intensity ranged from 200 (2.7 μmol $m^{-2}s^{-1}$) – 18000 lux (243 μmol $m^{-2}s^{-1}$). Meantime, the indoor condition was conducted in an environmental chamber (Wisd – ThermoStable SWGC) with a controlled temperature (33°C) and light intensity (243 μmol $m^{-2}s^{-1}$) that was maintained throughout the photoperiod. These factors were considered to be optimal for this microalgae species according to our preliminary growth assessments (Data are shown in Chapter 4.).

Total 36 Erlenmeyer flasks (500 mL) were filled with 350 mL of prepared domestic wastewater in triplicate including the control sample (wastewater without microalgae). A total of 18 flasks were exposed to the outdoor condition and the other 18 flasks were exposed to the indoor condition. The experimental flasks were inoculated with different initial cell concentrations of microalgae *Botryococcus* sp. inoculum (Gani *et al.*, 2016). The flasks were covered with sterile cotton plugs and were shaken from time to time to ensure homogenised cells in the wastewater. Samplings for nutrient analysis were collected at intervals of three days. Total nutrient removal was calculated in accordance with Equation 3.9. Meanwhile, the microalgae growth measurement was conducted daily with the help of a haemocytometer (Improved Neubauer grid chamber). The experiment was carried out for a period of 18 days. The optimum day of culture is revealed to be 18 days to achieve the maximum of growth under controlled condition.

$$\text{Nutrient removal, \%} = \frac{\text{Initial conc. (mg/L)} - \text{Final conc. (mg/L)}}{\text{Initial conc. (mg/L)}} \times 100 \qquad (3.9)$$

3.9.1 Phycoremediation mathematical model

The data obtained from the phycoremediation experimental were used to generate the phycoremediation prediction using the mathematical model – assume P as the phycoremediation potential of *Botryococcus* sp. over time, which can be written as Equation 3.10 where k and t are the coefficient rate constant and time, respectively (Mamun *et al.*, 2015):

$$\frac{-d[P]}{dt} = k[P] \quad (3.10)$$

Rearrange Equation 3.10:

$$\frac{d[P]}{[P]} = -k\,dt \quad (3.11)$$

Then integrate Equation 3.11:

$$\int \frac{d[P]}{[P]} = k \int dt$$

$$\ln [P]_t - \ln [P]_0 = -k(t-0)$$

$$\ln [P] = -kt + C \quad (3.12)$$

Where C is the constant integration. To evaluate the value of C using the boundary condition, when t = 0, [P] = $[P]_0$ where $[P]_0$ is the initial concentration of P. Substituting into Equation 3.12, we obtain:

$$\ln [P]_0 = -k(0) + C \quad (3.13)$$

Then, $C = \ln[P]_0$

Rewrite the integrated form for the first order kinetic as follows:

$$\ln[P] = -kt + \ln[P]_0 \quad (3.14)$$

Rearrange Equation 3.14 to obtain Equation 3.14:

$$[P] = [P]_0 \exp(-kt)$$

$$[P] = [P]_0 e^{-kt} \quad (3.15)$$

The mathematical model derived is valid for t > 0 days. The value of *k* will be positive if the curve between the parameter against time is increasing, otherwise negative. The decreasing curve is due to the removal of nutrients in wastewaters by microalgae.

3.10 Biomass and hydrocarbon production (Scale-up)

According to Rawat *et al.* (2013), there are four types of microalgae cultivation techniques that available, namely; photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic. Of these, the most dominant method commonly used for microalgal cultivation is phototrophic cultivation (Chen *et al.* 2011).

In this study, biomass and hydrocarbon production have been scaled up using photobioreactor (refer to **APPENDIX D**). The construction of photobioreactor according to Yen *et al*. (2014) with some modifications such as diameter and height of photobioreactor tube. Four tabular vertical closed photobioreactors were designed and constructed using transparent acrylic materials with the total working volume of 25L. The photobioreactors were washed using Decon 90 detergent for surface disinfection, followed by three times washes with sterile distilled water. A total of 22L of filtered domestic wastewater was transferred to each of the three photobioreactors (triplicates) while the remaining photobioreactor was filled up with autoclave-distilled water as a control sample. Cultures were continuously mixed by sparging using air pumps (maximum output: 3L/min) from the bottom of the photobioreactors. The air pumps maintained the gas exchanges (CO_2 and O_2) in the culture during the photosynthesis process. The photobioreactors were situated outside of the laboratory and outdoor natural sunlight and temperatures (24 - 33°C) were employed. The microalgae were allowed to grow for 12 days before the flocculation experiments.

3.11 Harvesting of microalgae

Recovery of microalgae biomass is one of the most challenging works and need to be given full attention for hydrocarbon production due to the size, density and the value target product. According to Brennan & Owende (2010), there is two stage of the process involve in the harvesting of biomass namely bulk harvesting and thickening. Bulk harvesting is a separation of biomass from the bulk suspension. There are several methods used in this stage that is including flocculation, flotation or gravity sedimentation. Meanwhile, thickening is to concentrate the slurry through techniques such as centrifugation, filtration and ultrasonic aggregation.

In this study, bulk harvesting focuses on the flocculation method while thickening stage only using filtration method. In flocculation process, externally added compound causes the suspended algae to form flocs, which if of the correct size, will freely sediment (Leite *et al*., 2013). Based on Leite *et al.* (2013), desirable flocculants should be non-toxic, recyclable, inexpensive and efficient at low concentrations. Thus, Rawat *et al.* (2013) proposed flocculation may be achieved by use of alum as generally use in conventional wastewater treatment. After done flocculation process, the floc of microalgae was collected as sediment in the bottom of jar test beaker; after that the filtration process was continued. Filtration is a method commonly used for solid-liquid separation. One of the alternative filtration methods to be used is nylon mesh strainer due to be more efficient and suitable for harvesting fragile microalgae. Upon completion of filtration, biomass was stored in a beaker and available for the drying process or dewatering by using universal drying oven or freeze dryer.

3.11.1 Flocculation procedure

Flocculation was conducted on the last day of cultivation when the cultures reached the stationary phase. The standard jar test according to ASTM (1995) was modified for the flocculation of microalgae. There was two type of coagulant had been used in this study, namely inorganic (alum) and organic (chitosan). For coagulant solution preparation, a total of 3% of alum and chitosan solution was prepared separately. Approximately 30 g of aluminum sulfate ($Al_2(SO_4)_3$) was dissolved in 1000 mL of distil water while 30 g of chitosan was dissolved in 900 mL of distilling water containing 10 mL of glacial acetic acid (M.W: 60.05 g/mol) and heated at 60°C with the aid of a magnetic stirrer until completely dissolved. Each 0.5mL of these stock solutions was equal to 30 mg/L when added to 500 mL experiment beaker to be tested.

After adding coagulant agent and pH adjusting, the sample in the tested beaker was vigorously stirred at 80 rpm for three minutes and then reduced to 30 rpm for 20 minutes. After that, the sample was allowed to settle for about 20 minutes before pipetted out about ten mL at a height of two-thirds from the bottom of the beaker and optical density (OD) was measured at 680 nm (Kim *et al.,* 2013). The harvesting efficiency was expressed as Equation 3.16 where A is the OD after

flocculation and B are OD before flocculation. All jar test experiments were conducted at room temperatures of between 24 and 26°C (Gani *et al.*, 2016d).

$$\text{Harvesting efficiency}, \% = \left(1 - \frac{A}{B}\right) \times 100 \quad (3.16)$$

3.11.2 Response surface methodology (RSM) design

Design Expert Software (version 7.0.0) was used for the statistical design of experiments and data analysis for flocculation efficiency. A face-centered central composite design (FCCCD) via response surface methodology (RSM) was applied to optimise and obtain the relationship between the variables and the response (Gani *et al.*, 2016d). For statistical calculations, the variables were coded based on the Equation 3.17, where X_i is the coded value of the i^{th} independent variables; X_o is the value of X_i at the centre point of the investigated area, and δx is the value of step change.

$$Xi = \frac{Xi - Xo}{\delta x} \quad (3.17)$$

The pH and coagulant dosage were chosen as two independent variables in the flocculation process. Their range and levels are stated in Table 3.8. The preliminary experimental considerations were applied in the selection of the range in which they were varied. The different type of coagulant used usually influence the effect of pH to attain the best of microalgae harvesting. For instance, pH 12 had been chosen due to the significant effect on the biomass recovery since both organic and organic coagulants were able to flocculate the microalgae biomass in wastewater. Therefore, this study led the pH value had been ranged from 5 to 12. This proved by the highly significant statistical analysis obtain *via* RSM. The response obtained was fitted by a second-order model in the form of quadratic polynomial equation (Equation 3.18) as follows (Pérez *et al.*, 2016):

$$Y = \beta o + \sum_{j=1}^{k} \beta j\, Xj + \sum_{j=1}^{k} \beta jj\, X^2 j + \sum_{i} \sum_{<j=2}^{k} \beta ij\, Xi\, Xj + \ell i \quad (3.18)$$

According to Equation 3.18, where Y is the predicted response, βo is the constant coefficient, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the interaction coefficient. The interactive effects of the independent variable on the dependent one were illustrated by 3D response surface and contour plots. Triplicates experiments were performed for each coagulant to verify the validity of the experimental statistical strategies.

Table 3.8 Experimental factors and their set up levels

Variables	Range and level		
	-1 (Min)	0 (Medium)	1 (Max)
pH	5	8.5	12
Coagulant dosage (mg/L)	30	105	180

3.12 Microalgae oil extraction (EPA Method 90718B)

Extraction of microalgae oil is central to the production of hydrocarbon from microalgae. Oil extraction is performed by chemical methods in the form of solvent extractions, physical methods or a combination of the two (Rawat *et al.,* 2011). The solvents are widely used to extract metabolites such as astaxanthin, b-carotene and fatty acids from algal biomass (Brennan & Owende, 2010). An example of solvents that usually applied used such as hexane, ethanol (96%), or a hexane–ethanol (96%) mixture which being possible to obtain up to 98% quantitative extraction of purified fatty acids (Mata *et al.*, 2010).

In this study, the type of solvent to be used is n-Hexane (95%). This is because Hexane is the most commonly used to extract the biological lipids from a biological plant such as microalgae. The advantage of using these solvents for oil extraction is that they are inexpensive and very efficient for oil extraction. Extraction and refining oil from microalgae biomass with some modification using n-hexane as a solvent is now being explored for its efficiency in recovering oil from algal cells at industrial scale (Rawat *et al*., 2013).

Therefore, n-Hexane extractable material for sludge, sediment and solid samples method that used in this research. This technique is adopted from United State Environmental Protection Agency (EPA Method 9071B). This method is also applicable to the extraction of non-volatile hydrocarbon, vegetable oils, animal fats,

waxes, soaps, grease, biological lipids and related material. A solid sample is mixed with anhydrous sodium sulfate, placed into an extraction thimble or between two plugs of glass wool, and extracted using an appropriate solvent in a Soxhlet extractor. The procedure of this extraction divided into 3 phase; (1) Preparation for extraction, (2) Sample preparation and (3) Extraction process (Gani *et al.*, 2017).

3.12.1 Soxhlet extraction procedure

There is three main stage of the soxhlet extraction analytical procedure that used in this study. Firstly, preparation for extraction; secondly, sample preparation and third, extraction process. In preparation for extraction, place 3 or 4 boiling chips into the solvent vessel. Dry the solvent vessel in a drying oven to constant weight (about 1 hour) at 103ºC. Put the solvent vessel after drying in the desiccators and allow it to cool to room temperature (about 30 minutes). Weigh the solvent vessel containing the boiling chips to an accuracy of ±1mg. Next, sample preparation which is dry the algae paste in the universal oven about 24 hours.

After that homogenise the sample with mortar and pestle then blend it with anhydrous sodium sulphate and surrogate standard spiking solution onto the sample. Transfer the homogenise paste to an extraction thimble. Fill the solvent into the vessel about 250 ml and attach the vessel to the soxhlet apparatus. Extract the sample at a temperature of 100ºC - 130ºC for 6 hours until the solvent to become minimise. Continue to heat the solvent vessel until all of the solvents has been evaporated and condensed in the soxhlet extractor. Care must be taken not to heat the oil residue to decomposition. Place the vessel containing the oil residue in a drying oven at 103°C and heat to constant weight. Allow the vessel containing the oil to cool to room temperature by put it in the desiccators for about 30 minutes. Weigh the vessel containing the boiling chips and oil residue.

3.13 Hydrocarbon compound analysis of microalgae oil

3.13.1 Fourier Transform Infrared Spectrometry (FT-IR) analysis

Fourier Transform Infra-Red (FTIR) Spectrometer of Thermo-Scientific (Nicole 6700) was used to analyse the extracted bio-oil samples. This method was adopted

from the previous study by Kothari *et al.* (2013). The FTIR spectrophotometer has been supplemented with an accessory device called Attenuated Total Reflectance (ATR) to enhance the use and application of the instrument in a way that materials like filmy/papery; liquid nature for further test. The Zinc Selenium (ZnSe) pellets are taken for correction of background spectrum. The FTIR spectra are recorded over a range of wave number from 4000 to 600 cm^{-1}. The transmittance peaks were analysed according to principal IR absorption for certain functional groups. Each sample was analysed in triplicate (Gani *et al.*, 2016e; Kothari *et al.*, 2013).

3.13.2 Gas Chromatogram – Mass Spectrometry (GC-MS) analysis

The compound analysis is to determine the chemical hydrocarbons that contained in the extracted *Botryococcus* sp. oil. This analysis is called as a qualitative analysis using GC-MS equipment to screen the possibility chemical compound available in this microalgae oil. From this analysis, the chemicals or hydrocarbons found in this oil was determined the potential purpose or use that may be adopted by the microalgae oil. Therefore, the sample was analysed using DB 5 MS column (30 m x 0.32 mm ID x 0.25 µm film thickness) using GC–MS. The conditions are used as per Dayananda *et al*.,(2005). The initial temperature of the oven is at 130°C for 5 min which will be increased to 200°C at the rate of 8°C per minute. After maintaining at 200°C for 2 min, the temperature was increased to 280°C at the rate of 5°C/min and maintained for 15 min. The injector port and the detector temperatures are 240°C and 250°C, respectively. The peaks are tentatively identified based on library search report or NIST (National Institute of Standard and Technology) database (Gani *et al.*, 2017).

3.14 Summary

This chapter presents the method development of the study including materials and equipment to be employed. Generally, this chapter describes the main part of the method development in which divided into three parts. The first part is about the preparation of microalgae, optimisation of environmental factors (light, photoperiod, temperature and salinity) including growth and biomass assessment using synthetic media. The second part performs the capability of microalgae to treat the

wastewaters known as phycoremediation in different culture condition along with difference microalgae concentrations. Lastly, biomass and hydrocarbon potential production has been fully developed using photobioreactor cultivation system. The method of harvesting microalgae via flocculation also discussed. After that, extracted algae oil has been analysed to determine the fraction of hydrocarbon compound present in microalgae cultivated in different types of wastewaters media. All methods and materials are successfully applied and employed according to the standard method precisely and some are adopted from other previous researchers with some modification.

CHAPTER 4

GROWTH OF MICROALGAE

4.1 Introduction

This chapter gives the analysis data obtained from the first part of the study. Generally, there are four basic environmental factors used to determine the maximum specific growth rate and biomass productivity of *Botryococcus* sp. that are photoperiod, light intensity, temperature, and salinity (Qin & Li, 2006). Every algal species differ in terms of nutritional and light requirements, life cycles and modes of reproduction. The conditions of culture have a different effect on the growth rate and biomass production (Krzemińska *et al*., 2014). Therefore, next sub-sections discuss the effect of these environmental factors on the maximum growth rate and biomass productivity of *Botryococcus* sp. cultivated in Bold's Basal Medium.

4.1.1 Photoperiod analysis

The growth curves of various light photoperiods were analysed as shown in Figure 4.1. Then, computation of the growth rate and biomass productivity was carried out as stated in Table 4.1 and illustrated in Figure 4.2. According to the Figure 4.1, *Botryococcus* sp. grew well when exposed to the light for more than 12 hours. However, the best photoperiod was observed on continuous light exposure (24:0 hours) with 40.55×10^4 cell/ml/day of biomass productivity and 1.18 day^{-1} of maximum growth rate. Meanwhile, the highest daily cell concentration (39.7×10^5 cells/mL) was occurred on the day 16 of culturing when illuminated with continuous photoperiod. This was followed by the second best of biomass productivity

36.20×10^4 cell/ml/day, which is 18:6 hours with a maximum growth rate of 0.99 day^{-1}. These values are slightly higher than 12:12 hours (0.96 day^{-1}). Even though there was not much difference in terms of maximum specific growth rate between 12:12 hours and 18:6 hours, results varied in terms of biomass production, as 12:12 hours was able to produce up to 25.84×10^4 cell/ml/day compared to 18:6 hours (36.20×10^4 cell/mL/day).

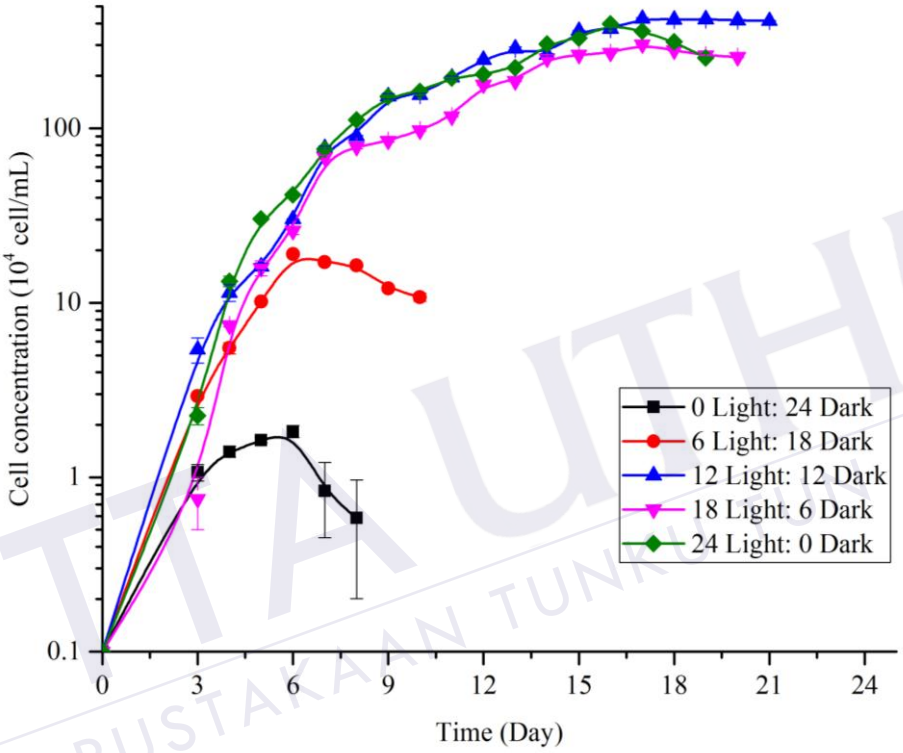


Figure 4.1: The growth of *Botryococcus* sp. at different photoperiod.

However, there is the less significant difference in maximum growth rate among 6:18 hours, 12:12 hours and 18:6 hours' photoperiod (Table 4.1 and Figure 4.2. These findings are also comparing the doubling or generation time of *Botryococcus* sp. which the highest maximum specific rate generates the lowest doubling time as stated in Table 4.1. According to Andersen (2005), generation or doubling time refers to the length of time needed by the microalga to double of their cell numbers in the culture as calculated using Equation 3.5 (stated in Chapter 3.6.1). In this study, the lowest doubling time was 0.59 days, which is a 24:0 hour photoperiod as compared to Krzemińska *et al.* (2014) which reached 0.78 days. This indicates that *Botryococcus* sp. is able to grow subjected to light for 6 hours or more

but that it is quite interrupted when cultivated without light. This indicates that algae are the type's phototropic microalga which is they need light to growth. Light exposure also had an essential effect on specific growth rate and microalgae biomass productivity.

Table 4.1: Computation of growth and biomass productivity in different photoperiod.

Photoperiod (Light:Dark)	Maximum growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity, cell/mL/day (10^4)
0:24	0.59±0.01	0.85±0.01	1.18±0.01	0.18±0.01
6:18	0.95±0.01	1.37±0.00	0.73±0.00	2.19±0.01
12:12	0.96±0.00	1.38±0.00	0.72±0.00	25.84±0.04
18:6	0.99±0.01	1.43±0.01	0.70±0.01	36.20±0.12
24:0	1.18±0.01	1.70±0.01	0.59±0.01	40.53±0.44

Data are expressed as mean ± SE (n=3)

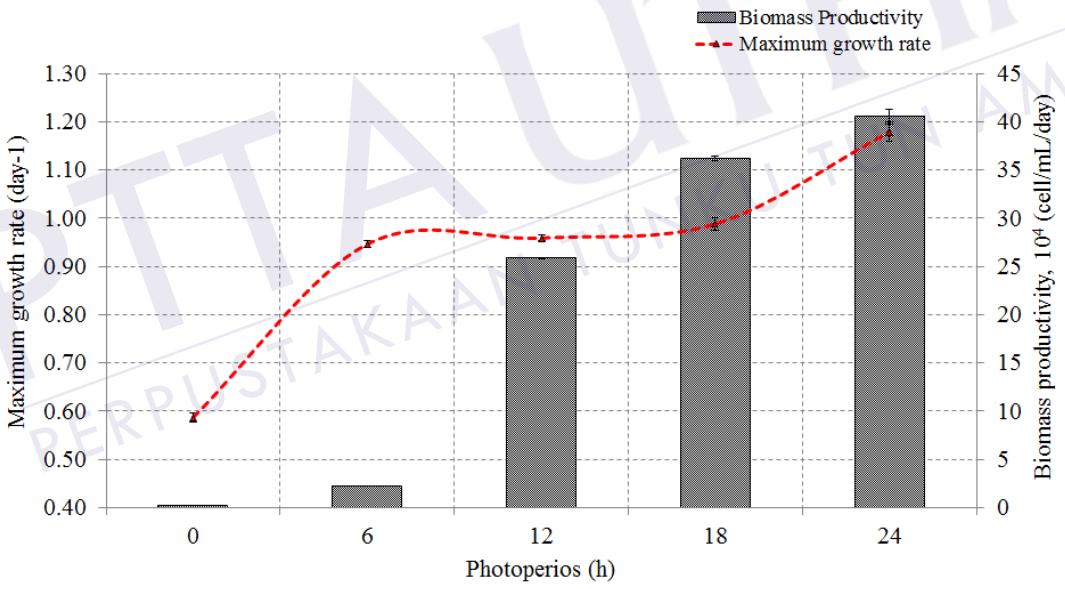


Figure 4.2: Growth rate and biomass productivity in different photoperiod

The differences in the photoperiod also may change the biochemical composition such as protein, pigments, and fatty acid content in microalgae biomass (Krzemińska *et al.*, 2014). In economic terms, a photoperiod is necessary if algal biomass is cultivated with a supply of artificial light sources because continuous light definitely used more electricity energy and leads to higher cost. Harun *et al.* (2014) reported that 12 to 15 hours illumination duration is generally considered as an economically optimal balance for algal growth between cost and productivity.

4.1.2 Light intensity analysis

The photosynthetic utilisation of light as energy sources is necessary for microalgae grown in water. The best growth was when exposed to 243 µmol $m^{-2}s^{-1}$ with biomass productivity and maximum specific growth rate are 81.52 × 10^4 cell/ml/day and 1.307 day^{-1}, respectively (Table 4.2). Thus, the day 19 showed the maximum daily cell concentration (76.6×10^5 cells/mL) could be reached under of this light intensity (Figure 4.3). Meanwhile, 94.5 µmol $m^{-2}s^{-1}$, 176 µmol $m^{-2}s^{-1}$ and 324 µmol $m^{-2}s^{-1}$ showed almost the same curve trend but different values for growth rate and biomass production.

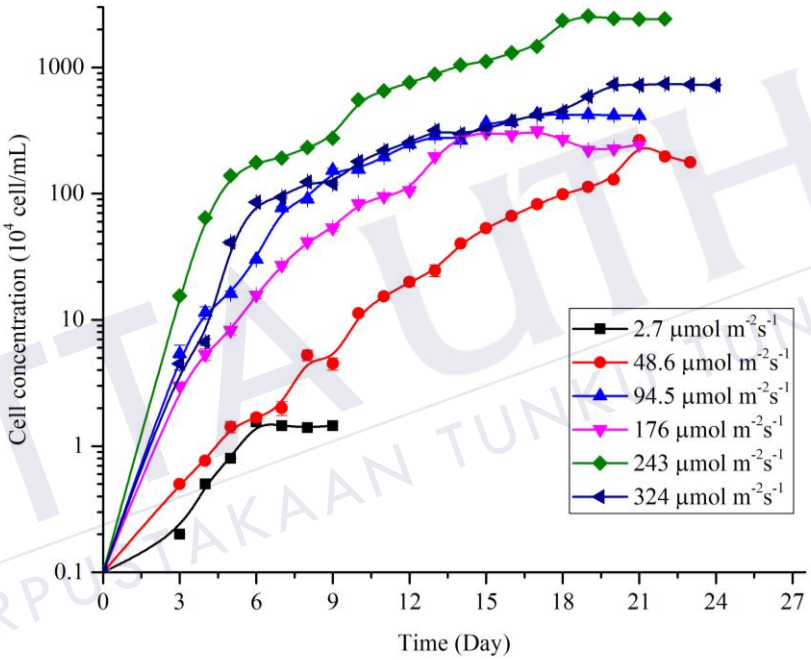


Figure 4.3: The growth of *Botryococcus* sp. at the different light intensity.

For example, on 94.5 µmol $m^{-2}s^{-1}$, 176 µmol $m^{-2}s^{-1}$ and 324 µmol $m^{-2}s^{-1}$ had 0.96 day^{-1}, 1.03 day^{-1} and 1.16 day^{-1} of maximum specific growth rate, respectively and biomass productivity were 35.12×10^4 cell/ml/day, 28.62×10^4 cell/ml/day and 70.35×10^4 cell/ml/day, respectively (Figure 4.4). However, 2.7 µmol $m^{-2}s^{-1}$ and 48.6 µmol $m^{-2}s^{-1}$ intensity is only able to produce a biomass productivity 0.11× 10^4 cell/ml/day and 10.98×10^4 cell/ml/day, respectively, and are considered not efficient enough to produce massive biomass due to the lower growth rates, which are 0.41 day^{-1} and 0.46 day^{-1}, respectively.

Table 4.2: Computation of growth and biomass productivity in different light intensity

Light intensity ($\mu mol.m^{-2}s^{-1}$)	Maximum specific growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity, cell/mL/day (10^4)
2.7	0.41±0.00	0.59±0.00	1.68±0.00	0.11±0.00
48.6	0.46±0.01	0.67±0.01	1.50±0.01	10.97±0.18
94.5	0.96±0.00	1.38±0.00	0.72±0.00	35.08±0.38
176	1.03±0.01	1.48±0.01	0.68±0.01	28.60±0.34
243	1.31±0.00	1.89±0.00	0.53±0.00	81.52±0.22
324	1.16±0.00	1.67±0.00	0.59±0.00	70.34±0.19

Data are expressed as mean ± SE (*n*=3)

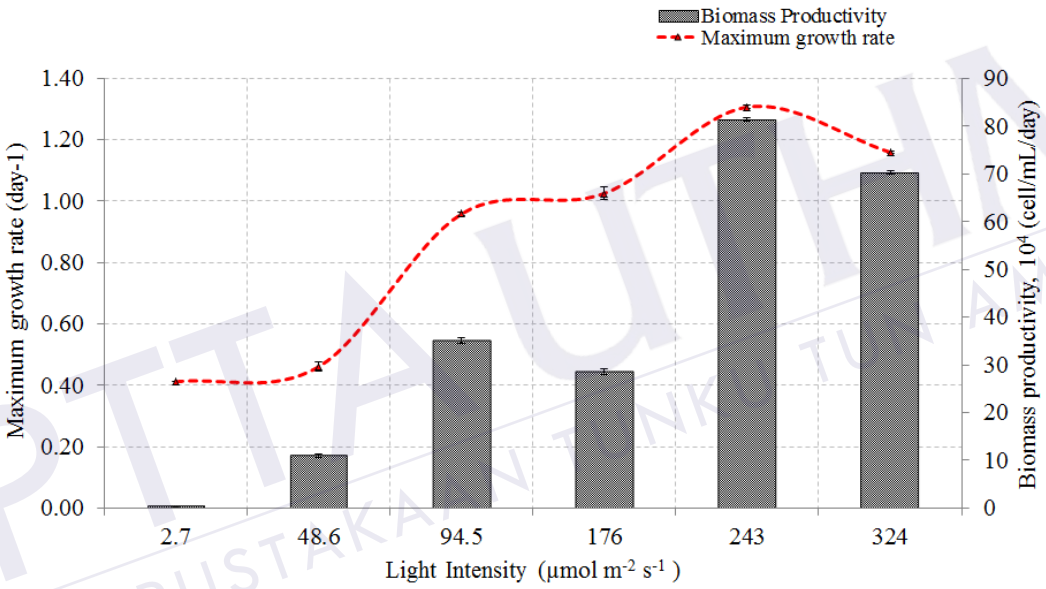


Figure 4.4: Growth rate and biomass productivity in different light intensity

In terms of doubling time, 243 µmol $m^{-2}s^{-1}$ also still lead to the best generation time because their cells population could be double about 0.530 day which is within 12.72 hours. This value very differs from a study done by Ruangsomboon (2012) where 200 µmol $m^{-2}s^{-1}$ and continues illumination were the most optimum for the cultivation of *Botryococcus braunii* KMITL2 isolated from freshwater reservoir in central Thailand. Since light intensity plays an important role in microalgae photosynthesis, increasing light intensity levels would increase the biomass productivity and growth rate at an optimal level, but production decreased if exposed to very high light intensity, as in the present study. Figure 4.4 shows that this *Botryococcus* sp. was unable to accommodate the excess concentration of light

intensity on 324 µmol $m^{-2}s^{-1}$ and causes a decrease in biomass productivity and maximum specific growth rate. This may be due to the higher light intensities can lead to photoinhibition (Wahidin *et al.,* 2013) and normally optimum light intensity would depend on the alga's photosynthesis capability to fully capture photon energy (Harun *et al.,* 2014).

4.1.3 Temperature analysis

The growth curve for experimental of *Botryococcus* sp. in five different temperatures (18°C, 23°C, 28°C, 33°C, and 38°C) were tested using constants light intensity (94.5 µmol $m^{-2}s^{-1}$), salinity (0M) and photoperiod (12:12) as plotted in Figure 4.5. The constant environmental factors resemble the natural outside of the origin habitat of this microalga. According to Figure 4.5 and 4.6, *Botryococcus* sp. was observed more productive at a temperature between 23°C to 33°C. The highest biomass productivity is on 33°C (43.91×10^4 cell/ml/day) follows by 23°C (39.3×10^4 cell/ml/day) then 28°C (35.1×10^4 cell/ml/day). This is slightly different from maximum specific growth rate, for which the best growth rate was 23°C (1.1146 day^{-1}). Meanwhile, the trend of maximum specific growth rate did not follow the biomass productivity trend (Figure 4.6 and Table 4.3). The maximum specific growth rate of 33°C was 0.93 day^{-1} which are lower than 23°C and 28°C (0.96 day^{-1}). For significant results between 23°C and 33°C, if compared the maximum specific growth rate and biomass productivity, the *Botryococcus* sp. cell at 23°C grew faster but produced lower biomass production than 33°C. This is because *Botryococcus* sp. cells under 33°C conditions have a slow growth rate but are excellent in biomass production compared to high growth rates. This situation may be due to the level of cell maturity much faster in 23°C causing the productivity was not as much as in 33°C. With that, the highest daily cell concentration (56.2×10^5 cells/mL) happened on the day 21 for 33°C (Figure 4.5). These results reveal the *Botryococcus* sp. is able to adapt when subjected to sudden temperature changes with different specific growth rate and biomass production. This finding is almost in line with that reported for *Botryococcus braunii*, with a suitable growth temperature in a range of 25°C to 35°C and optimal temperature of growth at 30°C (Da-Cong *et al.,* 2008). Other species of microalgae such as *Nannochloropsis oculata*, *Isochrysis aff. galbana, Chaetoceros muelleri* and *tetraselmis chui* were reported to possess different optimal

temperatures of 26°C, 28°C, 33°C and 25°C, respectively (Chen *et al.*, 2012). The optimal growth temperature served microalgae cell to undergo photosynthesis process without change or modify any biochemical and physiological parameters (Ras *et al.*, 2013). Obviously, the temperature is an essential environmental factor that influences algal growth rate, cell size, biochemical composition and nutrient requirements.

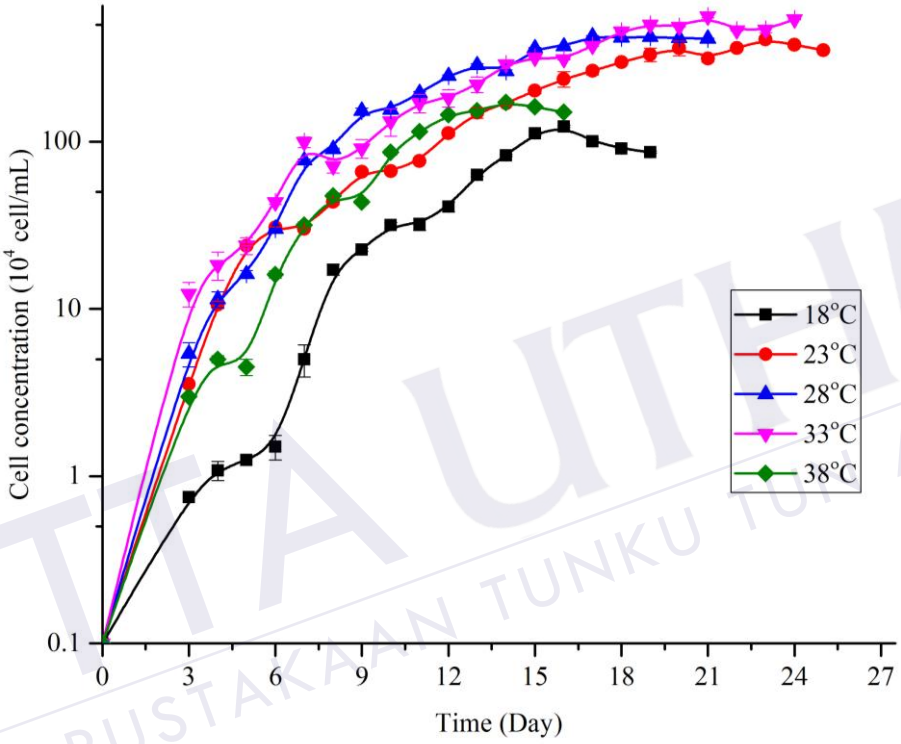


Figure 4.5: The growth of *Botryococcus* sp. at a different temperature.

Moreover, temperature also plays an important role in photoinhibition which is well-known to have an impact on algal growth rate (Juneja *et al.*, 2013). From these findings, the optimal temperature of algal growth was revealed to be the same as the environment of the microalgae collected. The results of these investigations have indicated that *Botryococcus* sp. enjoys healthy living at a temperature about 23°C to 33°C, the same as the natural outdoor condition climate in tropical rainforest located at Taman Negara Endau Rompin (Upeh Guling), Mersing Johor. Nevertheless, any batch bioreactor of microalgae operated indoors at a controlled temperature is also suitable for the optimal temperature provided in this study.

Table 4.3: Computation of growth and biomass productivity in different temperature

Temperature (°C)	Maximum specific growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity, cell/mL/day (10^4)
18	0.57±0.01	0.83±0.01	1.21±0.01	6.89±0.16
23	1.12±0.00	1.61±0.00	0.62±0.00	39.32±0.44
28	0.96±0.03	1.38±0.00	0.72±0.00	35.08±0.38
33	0.93±0.01	1.35±0.01	0.74±0.01	43.94±0.73
38	0.80±0.01	1.16±0.01	0.86±0.01	12.99±0.04

Data are expressed as mean ± SE (n=3)

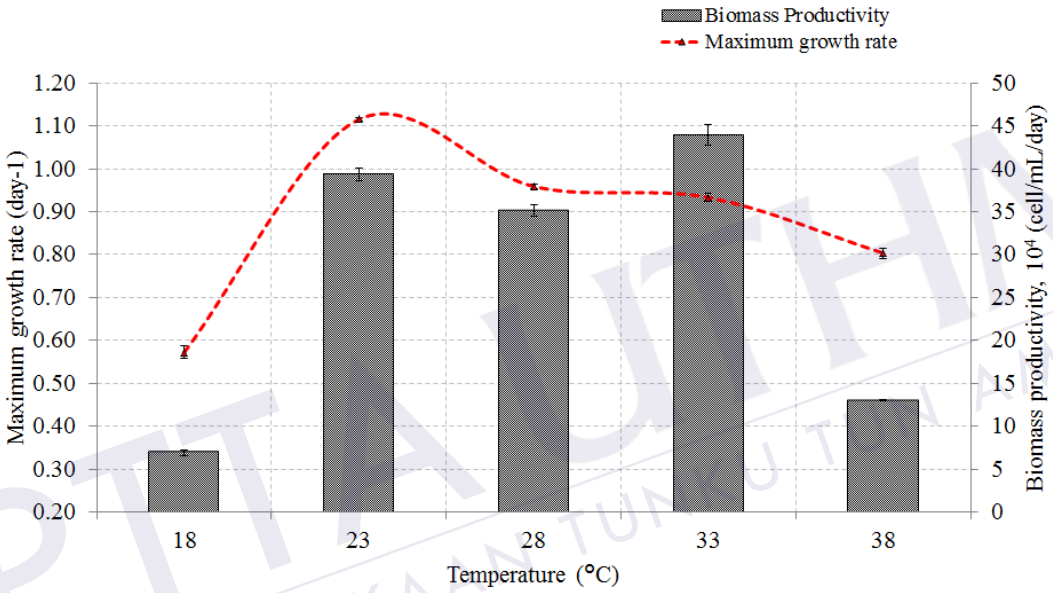


Figure 4.6: Growth rate and biomass productivity in different temperature

4.1.4 Salinities concentration analysis

The growth effects of different salinities concentration for experimental of NaC1 are presented in Figure 4.7. The microalgae exhibited low resistance to higher salinity, with a decrease in their growth when sodium chloride was added, compared to those with no sodium chloride. The most abundant growth occurred without the addition of NaC1 and decreased when mixed with 0.15M (3.07%) and 0.3M (6.14%), while insignificantly increasing at 0.45M (9.20%) and 0.6M (12.27%). This growth curve (Figure 4.7) also shows that 0M (0%) of NaC1 was better for a maximum specific growth rate (0.96 day^{-1}) and biomass productivity (35.1×10^4 cell/ml/day), as

illustrated in Figure 4.8 and computed in Table 4.4. In fact, culture without additional salinity showed the highest daily cell concentration (42.6×10^5 cells/mL) was occurred on the day 17 (Figure 4.7). Apart from that, this microalga remains tolerant to a range of salinities, i.e. 0.15M (3.07%) – 0.3M (6.14%) (Figure 4.7 and Figure 4.8).

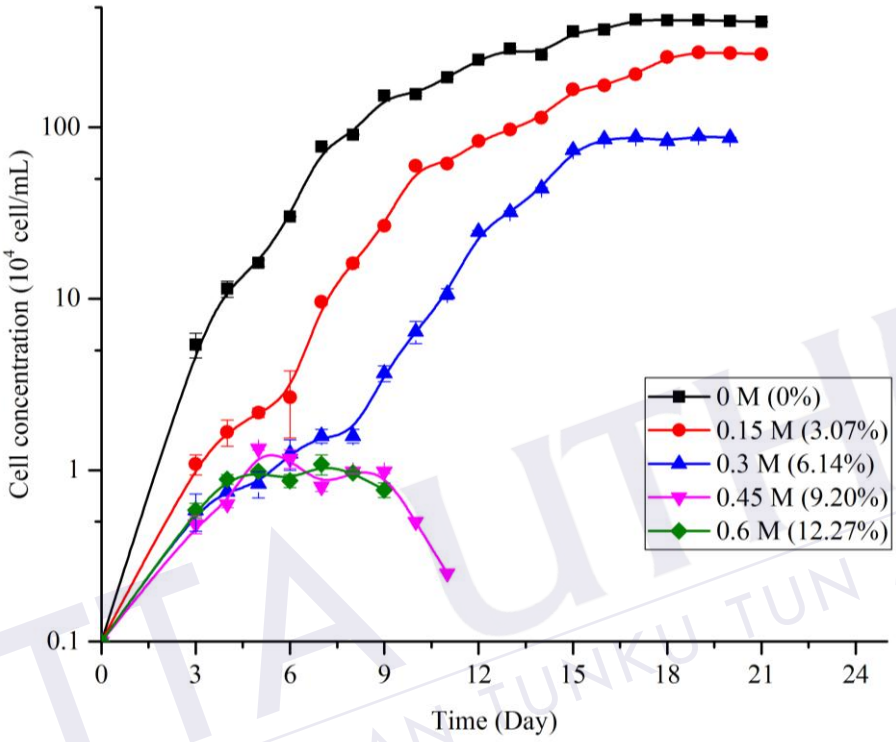


Figure 4.7: The growth of *Botryococcus* sp. at different NaC1 concentrations.

The study has gone some way towards enhancing understanding the optimal growth of *Botryococcus* sp. in media (BBM) without the addition of sodium chloride (NaC1). Table 4.4 shows the lowest doubling time at 0M salinity (0.723 days) and the highest at 0.3M salinity (1.621 days). However, this finding differs from another study where the greatest growth of *Botryococcus braunii* is at 0.15M salinity concentration (Qin & Li, 2006). This might due to the different strains and location adaptation used, as they obtained the *Botryococcus* sp. from Wuhan, China. The present study used a local strain collected from Malaysia's tropical rainforest. According to Juneja *et al.* (2013), salinity is an essential factor that changes the biochemical composition of microalgal cells, including such lipids, proteins, chlorophylls, and carbohydrates.

Table 4.4: Computation of growth and biomass productivity in NaC1 concentrations

Sodium chloride concentration M (%)	Maximum specific growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity, cell/mL/day (10^4)
0 (0%)	0.96±0.01	1.383±0.01	0.72±0.01	35.08±0.38
0.15 (3.07%)	0.61±0.01	0.875±0.01	1.14±0.01	14.91±0.12
0.3 (6.14%)	0.43±0.01	0.617±0.01	1.62±0.01	3.84±0.06
0.45 (9.20%)	0.38±0.01	0.686±0.02	1.46±0.02	0.11±0.00
0.6 (12.27%)	0.25±0.01	0.799±0.01	1.25±0.01	0.11±0.01

Data are expressed as mean ± SE (n=3)

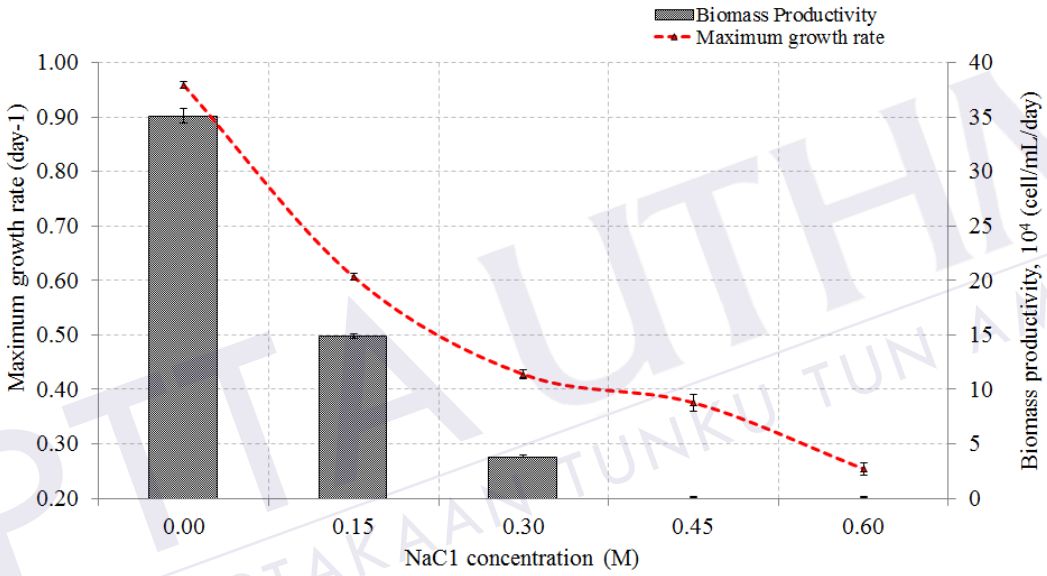


Figure 4.8: Growth rate and biomass productivity in different NaC1 concentrations

Exposing algal to lower or higher salinity levels than their natural habitat, however, can transform growth rate and change biochemical composition (Rai & Rajashekhar, 2014). Similarly to the conditions of this study, the natural habitat of collected *Botryococcus* sp. is living in the freshwater river. Therefore, volumetric biomass productivity (Table 4.4 and Figure 4.8) decreased with an increase in the concentration of NaC1. Other than that, different salinities also have a considerable effect on the morphology characteristic of microalga (Latala, 1991) due to the inability of the alga to adapt to high salinity since they belong to the freshwater microalgae (Juneja *et al.*, 2013).

4.2 Summary

One of the more significant findings to emerge from this chapter is that differences in growth rate and biomass productivity of *Botryococcus* sp. were highly dependent on the environmental factors applied. The main findings are summarized as follows: 1) Growth rate and biomass production increased when exposed much longer to light in terms of either duration exposure or light intensity; 2) the growth rate decreased when exposed to too much light intensity but increase in term of biomass productivity; 3) the growth rate tolerated temperatures between 23°C and 33°C and the samples grew well without any addition of salinity concentration. This chapter has led to more questions and a need for further investigation. Further work conducted to establish the phycoremediation process and sustainable biomass production for the future bio-based feedstock industry. Thus, next chapter presents the analysis of the phycoremediation study and potential biomass production done in outdoor and indoor culture condition using domestic and food processing wastewater where the indoor culture was implemented according to the optimal environmental factors that successfully obtained in this chapter. In addition, the period of phycoremediation was determined according to the average of the maximum day of daily cell concentration for each optimum environmental factor obtained which is about 18 days.

CHAPTER 5

PHYCOREMEDIATION OF WASTEWATERS

5.1 Introduction

This chapter discusses the results of the laboratory scale experiments performed for phycoremediation, heavy metal bio-removal and analysis of growth and biomass productivity from domestic wastewater (DW) and food processing wastewater (FW) using microalgae. Generally, this chapter is divided into two main sections. The first section describes the details of the results of the experiments conducted using DW. Meanwhile, the second section gives the details of the results of the FW phycoremediation experiments with the effect of different culture condition. The results and discussion of the main experiments performed to investigate the phycoremediation efficiencies for both DW and FW are explained in detail in section 5.2.2 and 5.3.2, respectively. The effect of microalgae concentration and different culture condition on biomass productivity is discussed in the section 5.2.4 for DW while for FW in section 5.3.4. Lastly, the findings of this chapter were summarised as stated in section 5.4 and suggestion of the next study is mentioned accordingly.

5.2 Phycoremediation of domestic wastewater

5.2.1 Characteristic of domestic wastewater

Wastewater characterization is compulsory and essential for determining the organic and inorganic nutrient supplements required for microalgae growth during the phycoremediation process. The collected domestic wastewater (DW) was dark grey

in colour. Table 5.1 shows the physiochemical and heavy metal parameters of DW compared to effluent standard limits set by the Environmental Quality Act of Malaysia 2009.

In general, the characteristics of the DW (Table 5.1) were highly variable but comparable to the range reported by previous studies. The concentration of COD and BOD were 129 mg/L and 71.08 mg/L, respectively; this concentration was different from that used in other research paper. For instance, Órpez *et al.* (2009) used untreated DW containing 49.7 mg/L to cultivate green microalgae *Botryococcus braunii*. Zhang *et al.* (2013) cultivated mixotrophic microalgae strain in DW containing 142 mg/L of COD while Mostafa *et al.* (2012) used DW containing 50 mg/L of COD and 15 mg/L of BOD to grow cyanobacteria and *Chlorella vulgaris*. However, the concentration of both COD and BOD in this study was found to be above the permissible limits according to Environmental Quality Act 9174 (Table 5.1). The DW contained much higher amounts of TSS compared to TDS, indicating high contents of large and small suspended solid. Therefore, TSS (2158 mg/L) was found to be higher than Standard A and Standard B of permissible limits (Table 5.1). The wastewater used also contained 8.99 mg/L of TP, which was below than Standard A, while TN was 15.89 mg/L. Both parameters were compared to a study conducted by Zhang *et al.* (2013), who used that TP and TN were 1.59 mg/L and 27.7 mg/L, respectively. Other than that, the pH value showed acceptable concentration compared to the effluent standard and suitable enough for microalgae cultivation (Creswell, 2010). Nevertheless, the heavy metal contents of the DW were still within the allowable limit of effluent standard limit (Table 5.1).

Result obtained from the analysis of the raw wastewater in this study was a clear indication and in fact evident that wastewater coming from domestic usage need to be properly treated prior to discharge into environmental. A few researcher (Can *et al*., 2013; Ji *et al*., 2013; Teles *et al*., 2013; Zhang *et al*., 2013) have shown the potential of microalgae in DW treatment to biotransform pollutant into valuable biomass before discharging the cleaned water to the environment. Thereby, this study may allow the use of DW for the development of culture method in biomass production and for further purposes of phycoremediation study coupled with hydrocarbon production.

Table 5.1: Characteristics of domestic wastewater

Parameter	[a] Concentration, [*]mg/L	Effluent standard, mg/L Environmental Quality Act, (2009)	
		Standard A	Standard B
Physiochemical			
Chemical oxygen demand, COD	129.00±2.35	120	200
Biochemical oxygen demand, BOD	71.08±2.80	20	50
Total Phosphorus, TP	8.99±0.53	5	10
Total Nitrogen, TN	15.89±6.24	-	-
Total suspended solid, TSS	2158.00±10.32	50	100
Total dissolved solid, TDS	240.00±8.86	-	-
Total organic carbon, TOC	14.14±4.36	-	-
Total carbon, TC	25.54±2.36	-	-
Inorganic carbon, IC	11.36±2.68	-	-
Nitrate	8.39±0.43	-	-
Phosphate	27.50±1.61	-	-
Dissolved oxygen, DO	4.73±0.83	-	-
Chloride	79.11±4.73	-	-
pH	6.96±0.31	6.0 – 9.0	5.5 – 9.0
Turbidity (NTU)	37.51±2.43	-	-
Salinity (%)	0.25±0.03	-	-
Heavy metals			
Zinc, Zn	0.3346±0.03	1.0	1.0
Ferum, Fe	0.8915±0.03	1.0	5.0
Cadmium, Cd	0.0007±0.00	0.01	0.02
Copper, Cu	0.0250±0.00	0.20	1.0
Aluminium, Al	0.2763±0.08	-	-
Lead, Pb	0.0574±0.02	0.10	0.50
Manganese, Mn	0.1593±0.02	0.20	1.0
Arsenic, As	0.0065±0.00	0.05	0.10

* All unit in mg/L except for pH, turbidity & salinity
[a] All experiments conducted in replicates (n=9)

5.2.2 Nutrients reduction

The variations in TP (total phosphorus), TN (total nitrogen), and TOC (total organic carbon) reduction with time in different initial cell concentrations of *Botryococcus* sp. for the 18 days of phycoremediation in outdoor and indoor cultures are presented in Figure 5.1, 5.4, and 5.7, respectively.

The substantial reduction in TP concentration over the phycoremediation period for both outdoor and indoor cultures was plotted in Figures 5.1a and Figure 5.1b, respectively. After plotting the experimental data, the first-order kinetic coefficients of TP for both the outdoor and indoor cultures in different initial cell concentrations were obtained by linear regression. The highest coefficient is for the indoor culture of up to 0.1479 at 10^6 cell/mL of initial cell concentration (refer to **APPENDIX E**). The mathematical model patterns of TP for outdoor and indoor

cultures showed a constant decrease along with increasing phycoremediation time. The Figure 5.2 indicates that the TP reduction model comparison plot was uniformly distributed around the datum line, showing a good relationship between mathematical model and experimental data. In fact, the scatters present an error occurred within ±20% accuracy. Although the experimental TP reduction in domestic wastewater for both cultures over time was not statistically different (p>0.05), it is strongly opposed to the total removal of TP for the entire phycoremediation study, as stated in Figure 5.3.

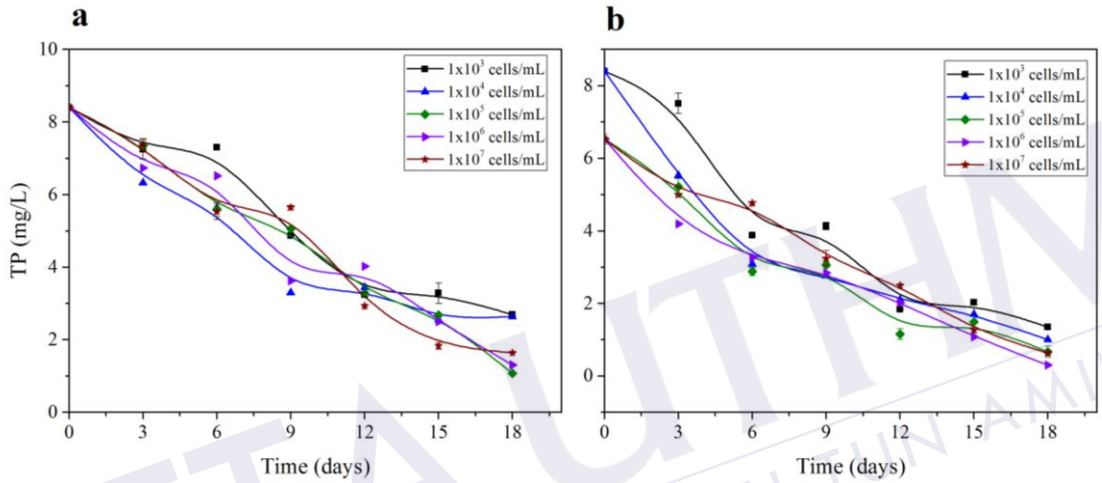


Figure 5.1: Removal of total phosphorus (TP) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in DW.

There was a significant (p<0.05) amount of different initial cell concentrations for the TP total removal efficiency for both the outdoor and indoor cultures (refer to **APPENDIX G**). Moreover, the Post hoc comparison using the Tukey HSD test indicated that the mean score for each initial cell concentration was significantly different compared to the control sample (wastewater without algae) in terms of TP total removal. Therefore, Figure 5.3 shows that the highest TP removal is at a concentration of 10^6 cell/mL with a total removal of 95.4% for the indoor culture. While for the outdoor culture, the most efficient TP removal is up to 85.5% at a concentration of 10^5 cell/mL. This result is much better than the result obtained by Gokulan *et al.* (2013) who reported that microalgae *Botryococcus braunii* removed about 77.6% of TP from greywater. Can *et al.* (2013) found that the highest TP in the form of phosphate removal was up to 99% when using cultivated *Botryococcus braunii* in municipal wastewater.

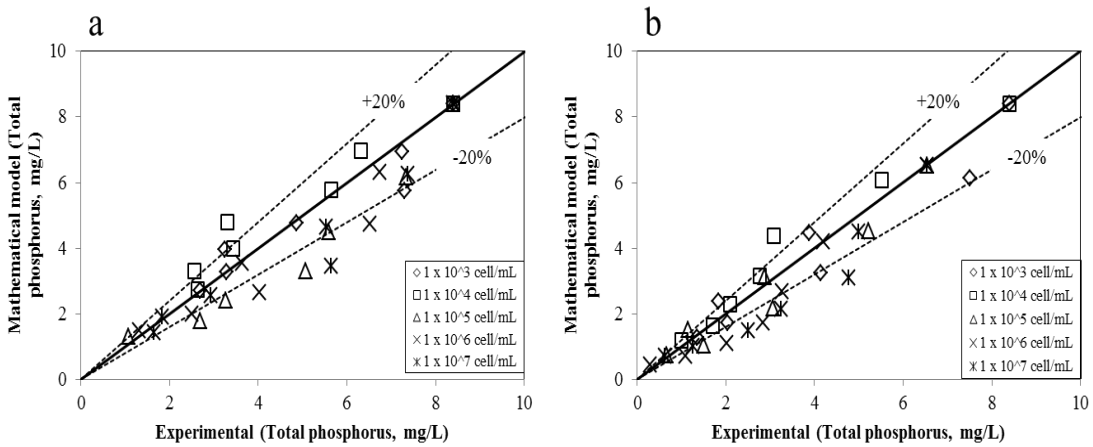


Figure 5.2: Comparison of TP phycoremediation between mathematical model and experimental for a) outdoor and b) indoor culture in DW

Since TP plays a crucial role in the algae cell growth and metabolism response of algae-causing phosphorus elements in the form of $H_2PO_4^-$ and HPO_4^{2-} is consolidated into an organic compound through the phosphorylation process. This is an active process and requires a certain amount of photon energy from a light source. Then, the generation of ATP (adenosine triphosphate) from ADP (adenosine diphosphate) during phosphorylation lets the microalgae growth mechanism assimilate and store phosphorus within the algae cells in the form of volutin granules (Cohen, 2000).

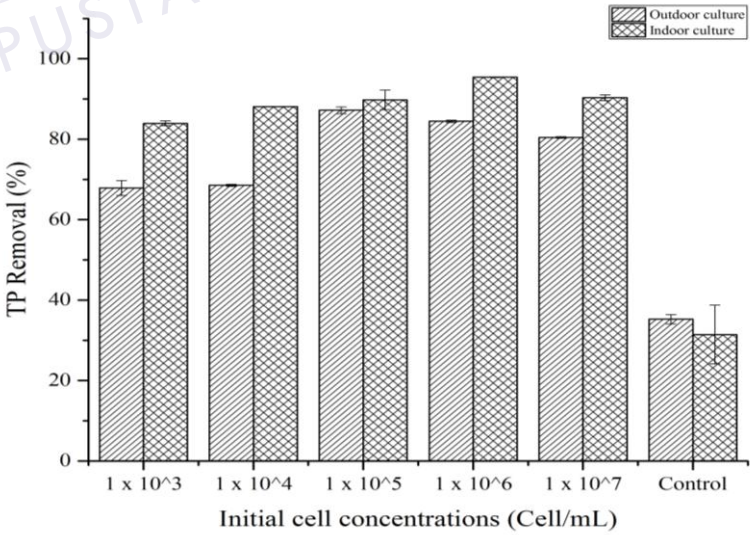


Figure 5.3: Total removal of TP after 18 days of phycoremediation.

The potential of *Botryococcus* sp. in treating domestic wastewater was also evaluated for the removal of TN. As shown in Figures 5.4a and Figures 5.4b, a considerable reduction of TN was recorded for each initial cell concentration for both outdoor and indoor cultures. The *Botryococcus* sp. took up TN efficiently from the domestic wastewater after a lag period of about 3 days, and the concentration of TN in both the outdoor and indoor cultures decreased dramatically with time (Figures 5.4a and Figures 5.4b). However, there was no statistical difference (p>0.05) among the initial cell concentrations tested in terms of TN daily reduction during phycoremediation. In addition, the significance of phycoremediation efficiency in domestic wastewater was also examined statistically for total TN removal, as illustrated in Figure 5.6. One-way ANOVA analysis proved that total removal of TN at the p<0.05 level for the six different initial cell concentration including control sample was significantly efficient (refer to **APPENDIX G**).

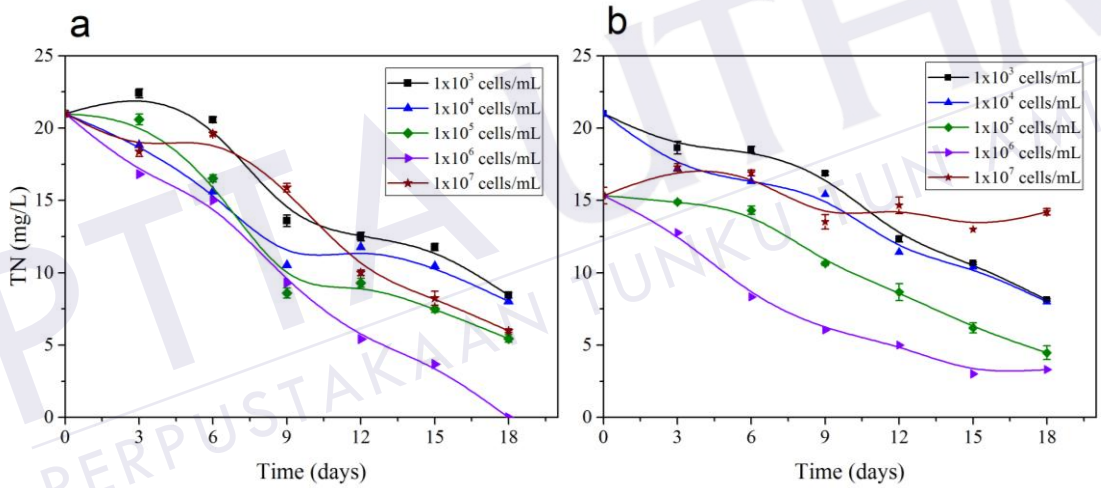


Figure 5.4: Removal of total nitrogen (TN) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in DW.

Furthermore, analysis using the Post hoc Tukey HSD test by comparing each different initial cell concentration in the form of TN total removal revealed that *Botryococcus* sp. is effective for the removal of TN when statistically compared with the control sample. Similar to TP, the mathematical model values of TN for both the outdoor and indoor culture samples were found to be more linear compared to that of the experimental. Figure 5.5 presents the comparison between mathematical model and experimental data of TN reduction during phycoremediation. It revealed that a few data at the concentration of 1×10^6 cell/mL are below underestimated -20% of

error while other concentrations showed the experimental data located within ±20% of accuracy for both culture conditions.

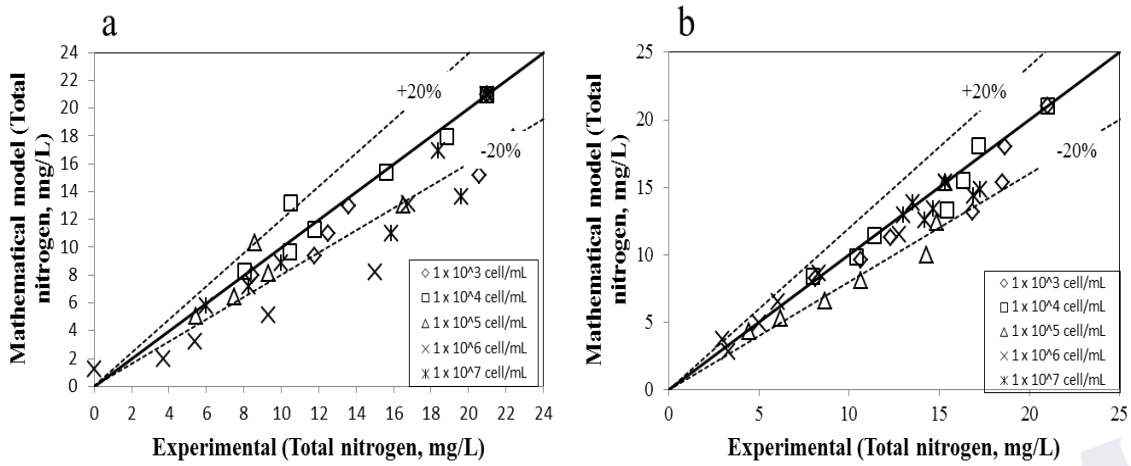


Figure 5.5: Comparison of TN phycoremediation between the mathematical model and experimental for a) outdoor and b) indoor culture in DW.

The highest TN first-order kinetic coefficient was found to be 0.157 at 10^6 cell/mL concentration in the outdoor culture. This indicated that *Botryococcus* sp. effectively removed TN in the outdoor culture by up to 100%. This result is in good agreement with previous study (Can *et al*., 2013). Can *et al*. (2013) studied TN in terms of nitrate, and found that 60.3% removal could be achieved when integrating municipal wastewater with *Botryococcus braunii.*

The success of TN removal by microalgae *Botryococcus* sp. in this study is due to the assimilation process. The assimilation of TN refers to the ability of microscopic plants to convert inorganic nitrogen in the form of nitrite, nitrate, and ammonium to organic nitrogen (Cai *et al.*, 2013). In terms of the scientific mechanism, inorganic nitrogen takes place across the plasma membrane of the algae cell where a reduction of nitrate occurs followed by the incorporation of ammonium into amino acids and glutamine (Laura and Paolo, 2006). Thus, all the inorganic elements of TN are finally reduced to organic elements (Cai *et al.*, 2013).

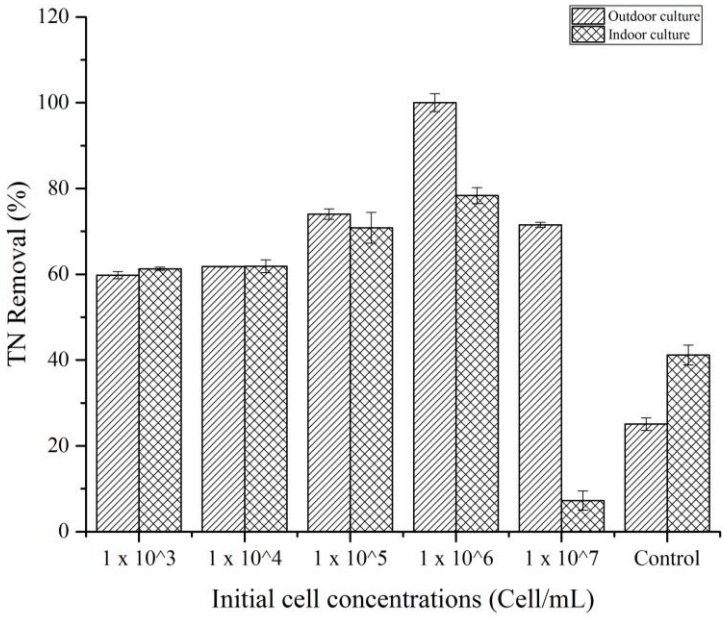


Figure 5.6: Total removal of TN after 18 days of phycoremediation.

The reduction of TOC was also determined at intervals during domestic wastewater treatment with *Botryococcus* sp. in both outdoor and indoor cultures (Figures 5.7a and Figures 5.7b). Similar to the other nutrients, TOC reduction also experienced the same pattern for both outdoor and indoor cultures. However, the TOC concentration for 10^7 cell/mL in the indoor cultures increased over time making it impossible to execute the mathematical model (Figure 5.7b). This also has been proved by Figure 5.8 in which the concentrations of 1×107 cell/mL for indoor culture indicating the error more than acceptable limit -20% of accuracy. However, other concentration presented an acceptable difference of less than ±20% accuracy.

This situation probably occurs due to the excessive algae cells inoculated in the domestic wastewater since indoor culture was applied using continues photoperiod and growth inhabitation happened to lead to the incomplete TOC removal (Mahapatra *et al*, 2014). Notably, other concentrations caused the TOC value to decrease linearly over time during phycoremediation. The highest removal of TOC occurred at a concentration of 10^7 cell/mL for the outdoor culture and 10^6 cell/mL for the indoor culture at 70.1% and 85.7%, respectively.

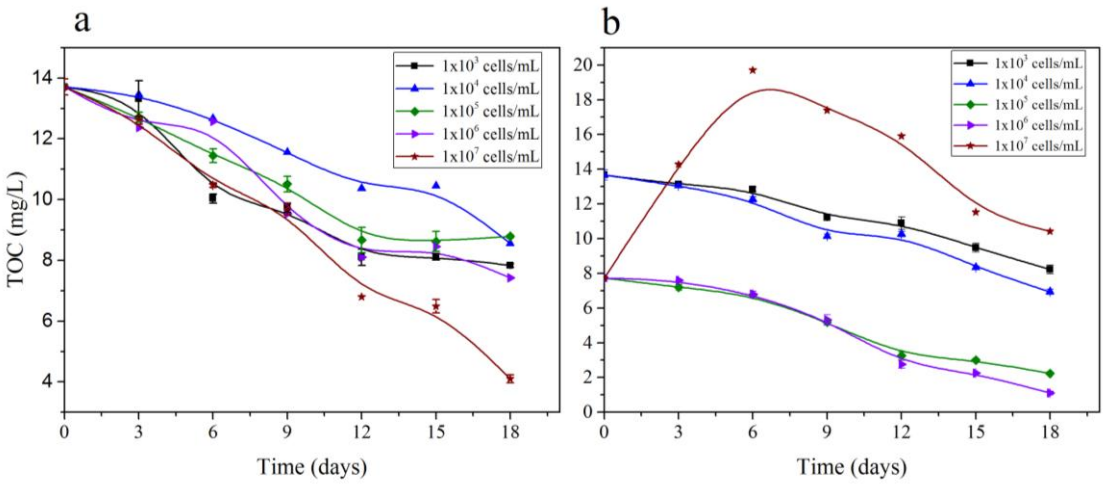


Figure 5.7: Removal of total organic carbon (TOC) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in DW.

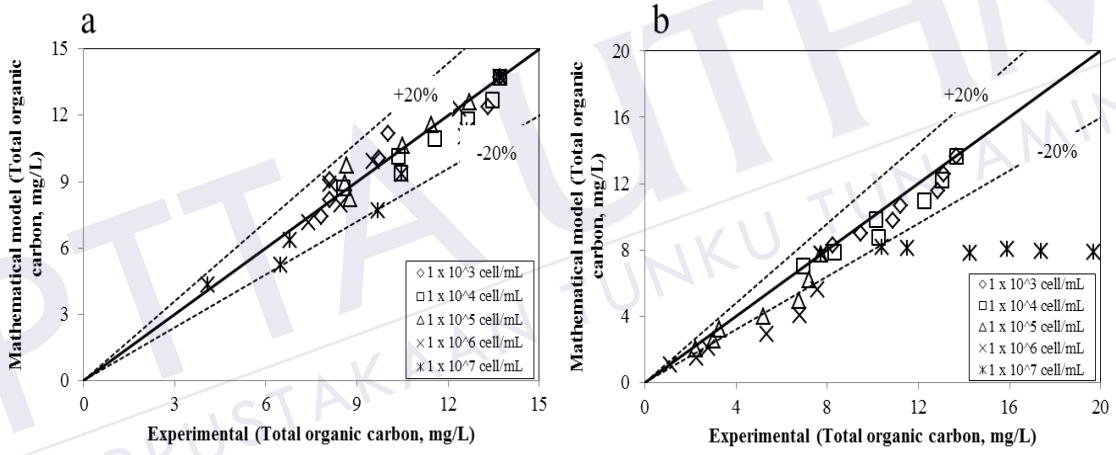


Figure 5.8: Comparison of TOC phycoremediation between mathematical model and experimental for a) outdoor and b) indoor culture in DW

Statistically different initial cell concentrations had a significant effect on TOC removal, as indicated in Figure 5.9. In fact, Post hoc comparisons using the Tukey HSD test showed that each initial cell concentration used in this phycoremediation was significantly different (p<0.05) compared to the control sample (wastewater without algae). This finding is consistent with the findings of the previous study of Mahapatra *et al.* (2014), in which the removal efficiency of TOC was 86% when they used mixotrophic algal consortia to treat municipal wastewater. In addition, the TOC removal in this study is also slightly higher than the study by Gani *et al.*

(2015a) who used the same microalgae species, *Botryococcus* sp. to bioremediate 100% concentration of dairy wastewater and obtained about 65.1% of TOC removal.

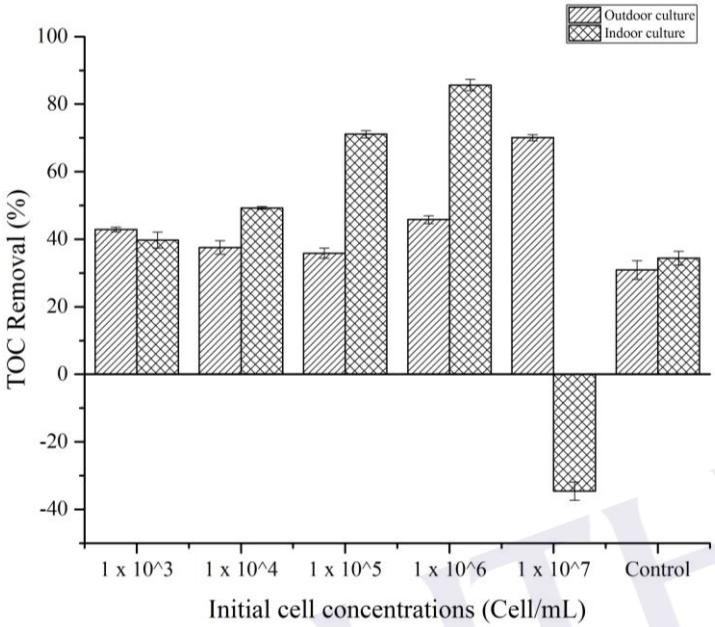


Figure 5.9: Total removal of TOC after 18 days of phycoremediation.

This finding revealed that the effectiveness of phycoremediation by *Botryococcus* sp. in domestic wastewater varies according to the culture conditions. However, both cultures (outdoor and indoor) showed a very positive sign for phycoremediation of domestic wastewater since it is able to remove nutrients (TP, TN, and TOC) concentrations by between 85 and 100%.

5.2.3 Heavy metals removal

The variations in zinc (Zn), ferum (Fe), cadmium (Cd) and manganese (Mn) bioaccumulation with time in a different initial cell concentration of *Botryococcus* sp. for 18 days of bioremediation are presented in Figure 5.10, 5.12, 5.14, and 5.16, respectively. In addition, the removal trends of these metals had been investigated by comparing the effect of different culture conditions (outdoor and indoor).

An interesting trend was shown on the Zn removal in DW (Figure 5.10a and Figure 5.10b). In the 18 days of DW phycoremediation, both culture conditions (outdoor and indoor) indicate a great removal of Zn. However, varies microalgae

concentrations exhibited different of Zn pattern removal. Statistically, one-way ANOVA proved that there was no significant difference (p>0.05) amongst the concentrations tested in term of Zn daily removal during treatment.

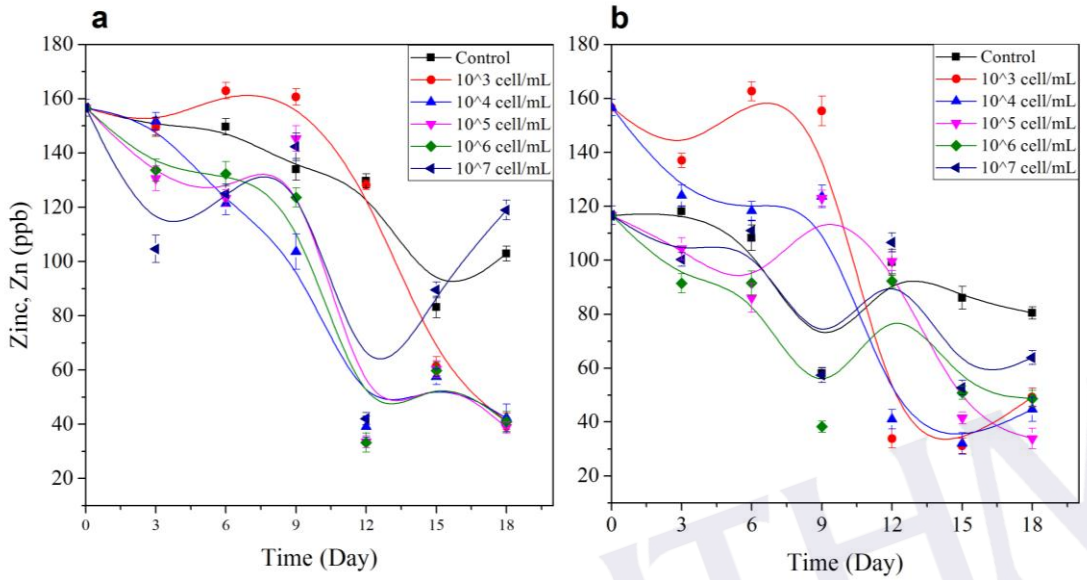


Figure 5.10: Trend removal of Zn in (a) outdoor and (b) indoor conditions

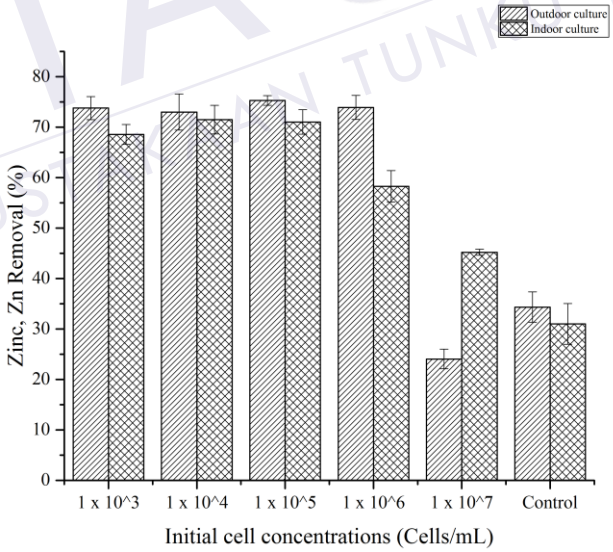


Figure 5.11: Total removal of Zn after 18 days of phycoremediation

But, the highest Zn reduction occurred at 1×10^6 cells/mL of concentration on the Day 12 which is up to 78.2% of removal. This amount of removal happened in outdoor condition while for the indoor condition, it happened at the concentration of 1×10^3 cells/mL with removal about 80% (Day 9) which is a little bit higher than the

outdoor condition. After 18 days of phycoremediation, the highest Zn removal occurred at 1×10^5 cells/mL concentration at outdoor condition while 1×10^4 cells/mL concentration for the indoor condition. Both concentrations successfully reduced Zn in DW is about 75.25% and 71.46%, respectively (Figure 5.11).

As shown in trend removal of Fe (Figure 5.12), the highest removal by *Botryococcus* sp. in DW occurred on the day 12 (outdoor) at 1×10^6 cells/mL concentration. The Fe concentration in DW has effectively reduced from 333.67 ppb to 82.59 ppb which is equivalent to 75.2% (Figure 5.12a). Meanwhile, indoor condition showed less effective removal than the outdoor condition in which the best removal up to 58.2% (day 12) at 1×10^3 cells/mL concentration (Figure 5.12b). A one-way between subjects ANOVA was conducted to compare the effect of different initial cells concentrations on Fe removal in DW at outdoor and indoor culture condition. There was not a significant effect of the amount on Fe removal at the $p>0.05$ level for the five concentrations. In contrast, total removal of Fe done by *Botryococcus* sp. after 18 days of phycoremediation (Figure 5.13) proved that the best concentration is 1×10^5 cells/mL in an outdoor condition which is up to 58% (reduced from 33.7 ppb to 10.23 ppb).

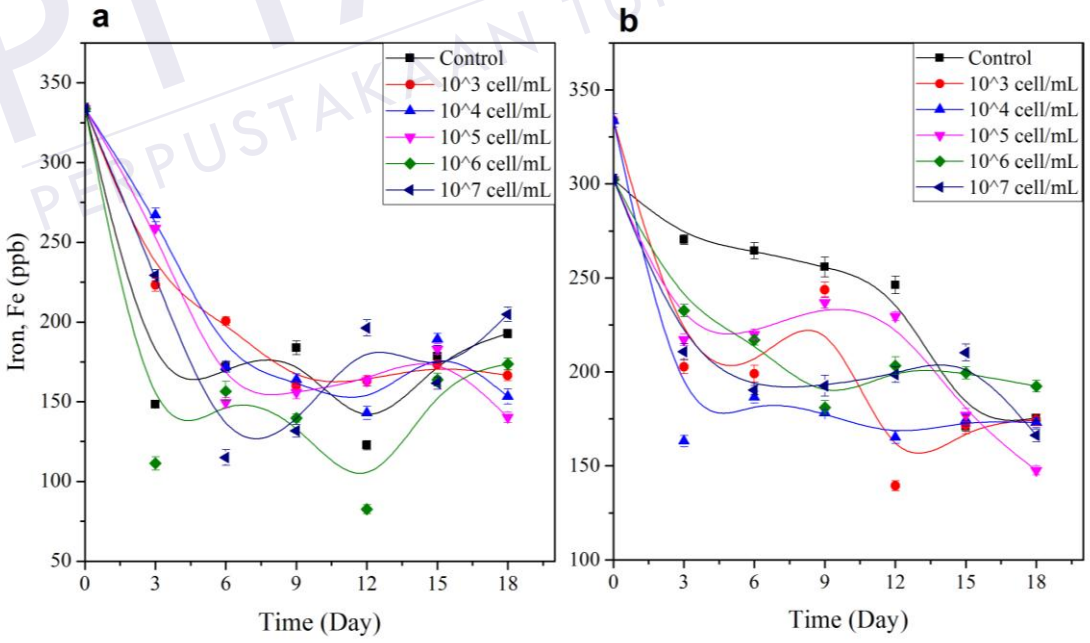


Figure 5.12: Trend removal of Fe in (a) outdoor and (b) indoor condition.

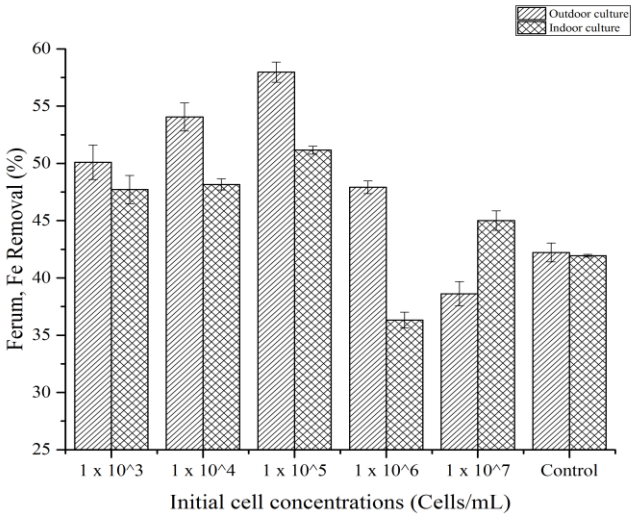


Figure 5.13: Total removal of Fe after 18 days of phycoremediation

Cultivation of *Botryococcus* sp. in DW showed a not consistent removal of Cd in outdoor condition but quite smooth reduction when exposed to indoor condition (Figure 5.14). The cause of this condition occurs may be affected by fluctuations in environmental factors (light and temperature) in outdoor condition during the study.

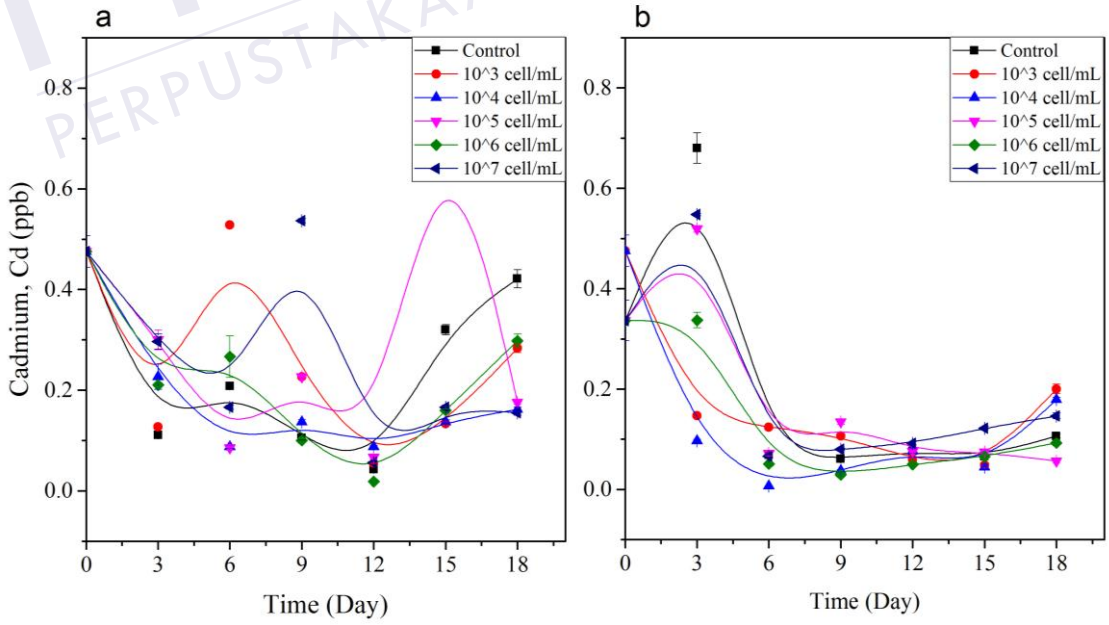


Figure 5.14: Trend removal of Cd in (a) outdoor and (b) indoor conditions

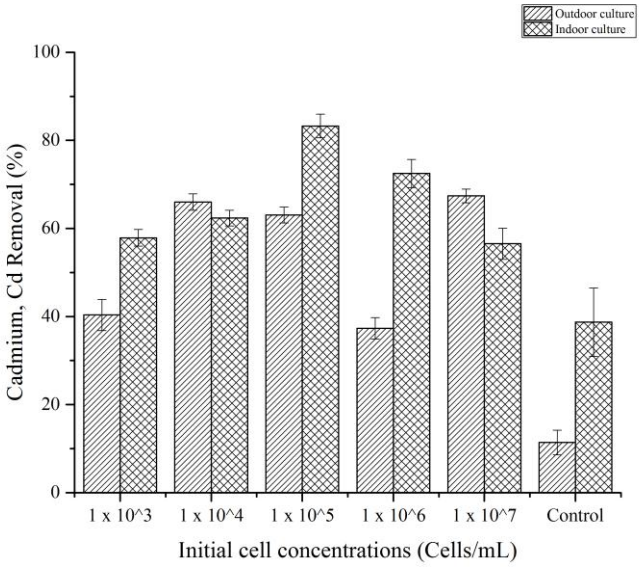


Figure 5.15: Total removal of Cd after 18 days of phycoremediation

Most of the algae concentrations show the same level removal of Cd for both culture conditions (outdoor and indoor). Therefore, statistical analysis revealed that there was no significant difference in the reduction and total removal done by *Botryococcus* sp. However, the highest removal of Cd in outdoor condition (Figure 5.14a) happened on the day 12 (96.2%) at the concentration of 1×10^6 cells/mL which is reduced from 0.4755 ppb to 0.0183 ppb. For indoor condition, 1×10^4 cells/mL depicted the best removal on the day 6 which is reduced from 0.4755 ppb to 0.006967 ppb (98.5%) (Figure 5.14b). Overall, total removal of Cd throughout of the study (18 days) presented in Figure 5.15 and found that 1×10^5 cell/mL in outdoor condition indicates the best removal of Cd up to 83.3%.

The same removal pattern (Figure 5.16) also was obtained in Mn treatment over time of phycoremediation for both culture conditions (outdoor and indoor). Most of all initial concentration applied effectively reduce Mn in DW about 89.3% to 96.4% for outdoor culture while 86.6% to 97.2% for indoor culture (Figure 5.17). Statistical analysis also revealed that there was no significant difference ($p>0.05$) for all initial concentration applied on the removal of Mn in DW. This result indicated that microalgae *Botryococcus* sp. able to remove Mn effectively in any initial concentration either in outdoor or indoor culture condition.

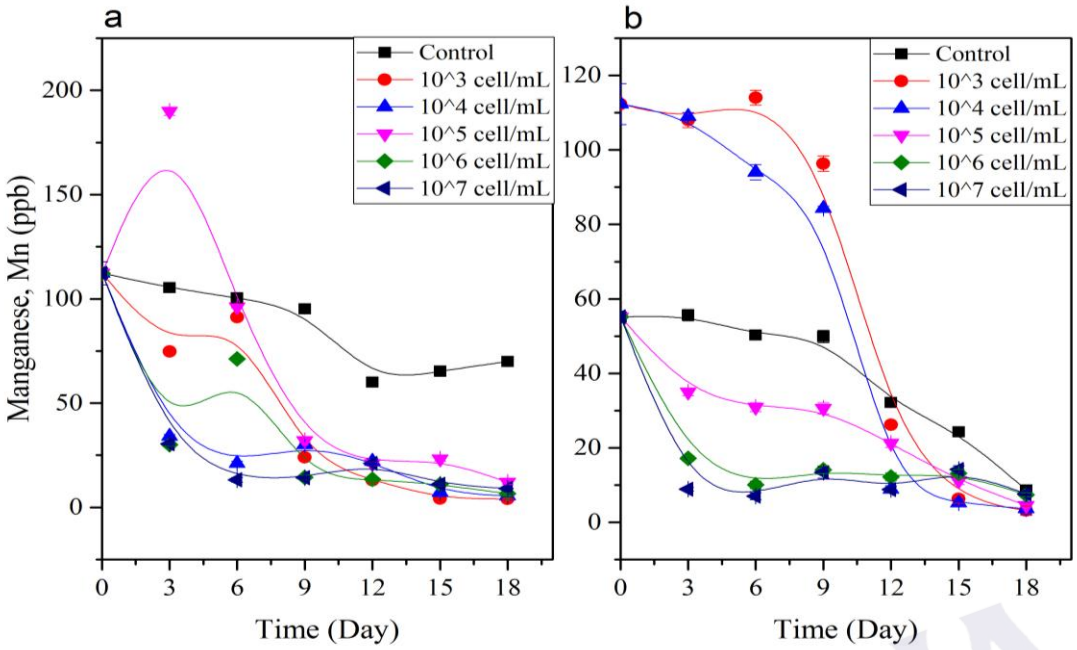


Figure 5.16: Trend removal of Mn in (a) outdoor and (b) indoor conditions

Overall, cultivation of *Botryococcus* sp. in contaminated DW for biomass production and simultaneously remove some metal elements (Zn, Fe, Cd, and Mn) was successfully performed. However, most of the initial concentration tested do not give a significant effect (p>0.05) on the removal efficiency either outdoor or indoor culture conditions. Higher microalgae concentration led to less effective in metals removal due to excessive cell was inoculated making the culture in overpopulated condition. Simultaneously, cause the food or nutrients competitive among the algae cell in the culture vessel. Previously, El-Sheekh *et al.* (2015) found that freshwater microalgae *Chlorella vulgaris* was able to remove Zn, Mn, and Fe concentration in sewage water up to 64.96%, 100%, and 100%, respectively while *Chlorella salina* successfully reduced about 15.6-28.5%, 89.94-93.71%, and 97.24%, respectively after 10 days of treatment. In another study, *Chlorella vulgaris* also successfully reduced the concentration of Zn, Fe, and Mn up to 80.1%, 100%, and 100%, respectively from domestic secondary effluent (Gao *et al.*, 2016).

In 2014, Onalo *et al.* (2014) investigated the removal of Cd in textile wastewater using *Botryococcus* sp. only up to 2%. Meanwhile, Chan *et al.* (2014) reported that *Chlorella vulgaris* and *Spirulina maxima* capable of reducing Zn concentration in DW up to 96.3% and 94.9%, respectively. Other than that, Hamouda *et al.* (2016) studied removal of heavy metals in industrial wastewater using green

microalgae and found that *Scenedesmus obliquus* able to reduce Cd about 70% in the light and dark culture condition. Therefore, heavy metals removal in wastewater mostly depending on the species of microalgae used in which has different accumulation affinities towards the tested elements. Moreover, it is also relying on the nature and charge of the cell wall polysaccharides of microalgae (El-Sheekh *et al.*, 2015). In fact, green microalgae cells cultivated in wastewater with high metal contents also reduced higher metal concentration (Priyadarshani, Sahu, & Rath, 2011). In other words, the metal accumulate was independent on the strength of external metal concentration (El-Sheekh *et al.*, 2015).

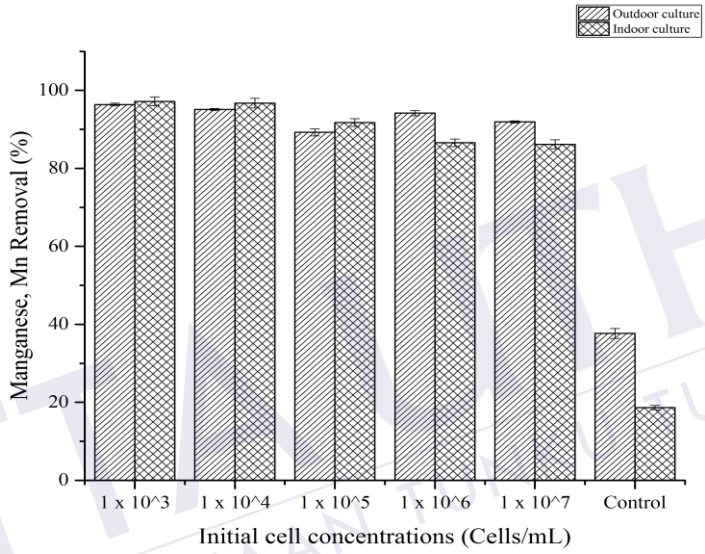


Figure 5.17: Total removal of Mn after 18 days of phycoremediation

5.2.4 Analysis of growth and biomass productivity

The growth of *Botryococcus* sp. in both outdoor and indoor cultures is shown in Figure 5.18. The growth mathematical model for each concentration was also evaluated according to Equation 3.8.

Analysis of variance (ANOVA) was conducted to compare the effect of the cell concentration on the daily growth in 10^3, 10^4, 10^5, 10^6, and 10^7 cell/mL conditions. Both the outdoor and indoor cultures showed the significant effect of the concentration at the initial algae cell on *Botryococcus* sp. growth at the $p< 0.05$ level for the five concentrations. As observed in Figure 5.18, the growing trends were different in each cell concentration, showing a lag time by about Day 3 for all

concentrations except for Day 6 for the outdoor culture at 10^6 cell/mL. These circumstances could be described by considering that, during this period, the growth was mainly because of the mixotrophic culture conditions. In fact, the growth was affected by the gradual increase in pH during phycoremediation (Alva *et al.*, 2013). Similarly, Can *et al.* (2013), and Teles *et al*. (2013) reported obtaining the same growth curve when they used domestic wastewater to grow microalgae *Botryococcus braunii* and *Chlorella vulgaris*, respectively. Obviously, interrupted growth was recorded in the concentration of 10^7 cell/mL. This is due to the limitation of nutrients to be provided to the excessive *Botryococcus* sp. in the culture. The mathematical model for cell growth was unable to be evaluated since the experimental growth line cannot be identified scientifically (Figure 5.18) for both outdoor and indoor cultures.

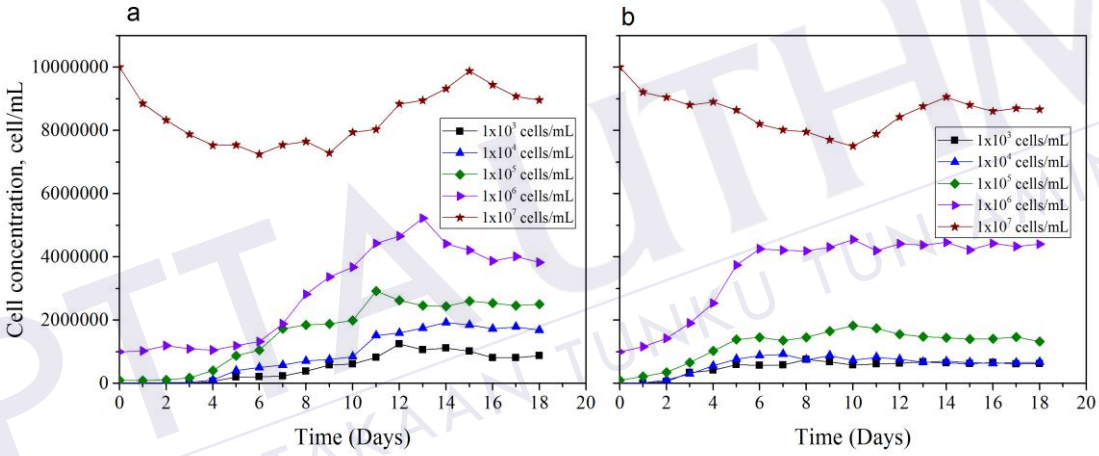


Figure 5.18: The growth of *Botryococcus* sp. in different cells concentration for (a) Outdoor culture and (b) Indoor culture.

The doubling time (day) and biomass productivity (cell/mL/day) were calculated accordingly after plotting the daily growth curve and the maximum growth rate (day^{-1}). Table 5.2 shows a summary of the growth kinetic parameters and biomass productivity for both the outdoor and indoor cultures. In terms of the significant results obtained, statistical analysis (ANOVA) reveals a significant difference (p<0.05) between the algae concentration with maximum growth rate and the biomass productivity for both the outdoor and indoor cultures (Table 5.2 and Figure 5.19). However, further analysis using the Post hoc Tukey HSD test found an insignificant difference (p>0.05) between the cell concentration of 10^3 cell/mL and 10^4 cell/mL in terms of biomass productivity for both culture conditions. In Figure

5.19, the trend for the maximum growth rate curve for the outdoor culture is similar to that of the indoor culture. Nevertheless, the greater the concentration of *Botryococcus* sp. in both cultures resulted in an impairment of the maximum growth rate (Figure 5.19). Therefore, the highest maximum growth rates for the outdoor and indoor cultures at 10^3 cell/mL were 1.35 day^{-1} and 2.00 day^{-1}, respectively. This result is quite different to the results obtained by Yoshimura *et al.* (2013) with a specific growth rate value of only up to 0.22 day^{-1} when they cultivated *Botryococcus braunii* in a modified Chu 13 medium. These findings prove that domestic wastewater as a medium to grow *Botryococcus* sp. is able to increase the productivity of cell growth compared to a synthetic medium.

Moreover, in various cell concentrations, the biomass productivity of *Botryococcus* sp. was also very different. Based on Figure 5.19, the most favourable algae concentration was 10^6 cell/mL for outdoor and indoor cultures, with a biomass production of 2.6×10^5 cell/mL/day and 2.5×10^5 cell/mL/day, respectively. These results were supported by the daily growth curve (Figure 5.18), where the optimal growth was observed at 10^6 cell/mL.

Table 5.2: Computation of growth kinetic and biomass productivity in different initial cells concentration.

Initial cell concentrations (cells/mL)	Maximum growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity (cells/mL/day)
Outdoor culture				
1×10^3	1.35	1.94	0.52	163812
1×10^4	0.69	1.00	1.00	161507
1×10^5	0.60	0.87	1.15	235521
1×10^6	0.26	0.38	2.63	258498
1×10^7	n/a	n/a	n/a	n/a
Indoor culture				
1×10^3	2.00	2.89	0.35	141422
1×10^4	1.23	1.77	0.56	138299
1×10^5	0.58	0.84	1.19	180897
1×10^6	0.27	0.39	2.56	242246
1×10^7	n/a	n/a	n/a	n/a

Data are expressed as mean (n=3)
*n/a: Not applicable

However, a concentration of 10^7 cell/mL for both cultures cannot be determined possibly because the lack of nutrients available in this domestic wastewater were unable to accommodate the high volume of algae concentration

(shown Table 5.2). In other words, this situation was due to the overpopulation of microalgae and led to the inability of microalgae cells to receive enough light intensity due to the self-shading factor by their cells in the culture (Sforza *et al.*, 2014).

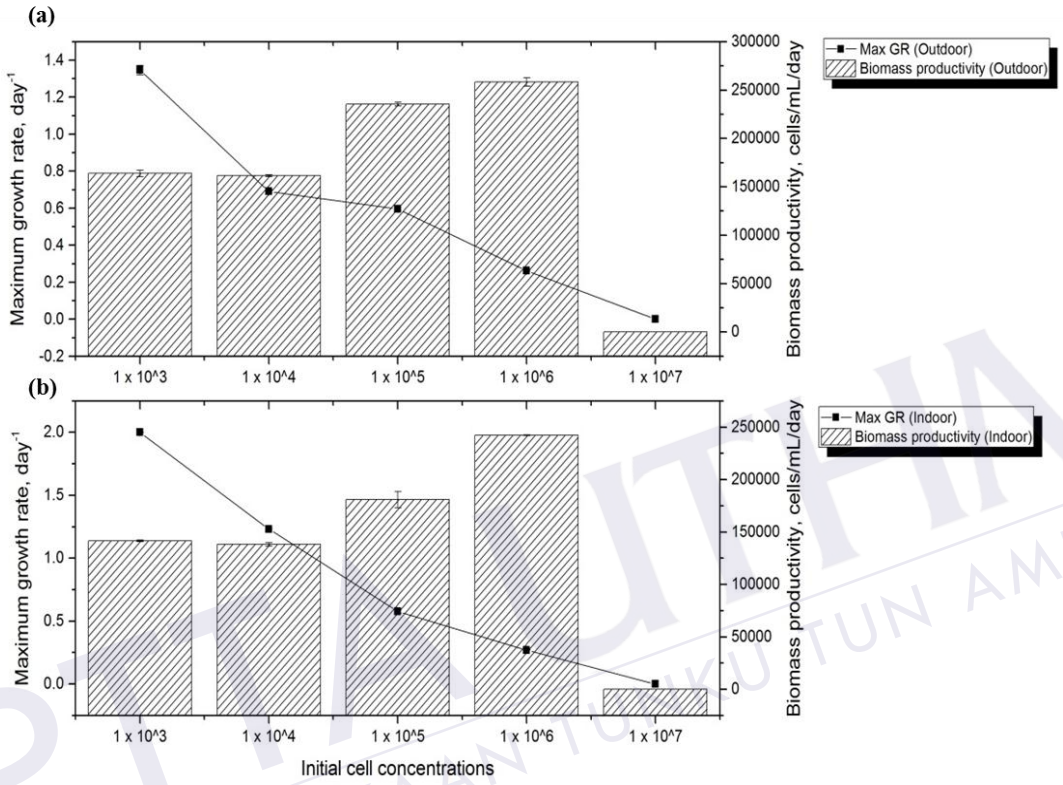


Figure 5.19: Maximum growth rate (d^{-1}) and biomass productivity (cell. mL^{-1}. d^{-1}) of *Botryococcus* sp. in different cell concentrations (cells/mL) on domestic wastewater for (a) outdoor culture and (b) indoor culture.

5.3 Phycoremediation of food processing wastewater

5.3.1 Characteristic of food processing wastewater

In the present study, raw characteristics of the food processing wastewater (FW) showed (Table 5.3) varying levels of various physiochemical and heavy metal parameters where creamish white effluent along with unpleasant odour was found. The pH of effluent was observed as acidic (5.86) while the salinity was recorded to be 0.76%. Similarly, Qasim & Mane (2013) found the pH observed in sweet-snack effluent also acidic (5.64). The turbidity of FW was higher with a value of 6720 NTU

indicating higher solids and organics contents. Therefore, FW contained 12418.67 mg/L of TSS and 765 mg/L of TDS. It clearly indicates that TSS value above the effluent standard limits set by Environmental Quality Act of Malaysia 1974 (Table 5.3).

Table 5.3: Characteristics of food processing wastewater

Parameter	[a] Concentration, [*]mg/L	Effluent standard, mg/L (Environmental Quality Act, 2009)	
		Standard A	Standard B
Physiochemical			
Chemical oxygen demand, COD	2838.62±793.46	120	200
Biochemical oxygen demand, BOD	793.89±110.20	20	50
Total Phosphorus, TP	6.81±1.93	5	10
Total Nitrogen, TN	230.11±109.40	-	-
Total suspended solid, TSS	12418.67±8989.20	50	100
Total dissolved solid, TDS	765.00±308.74	-	-
Total organic carbon, TOC	471.44±208.73	-	-
Total carbon, TC	1412.89±227.85	-	-
Inorganic carbon, IC	941.56±23.18	-	-
Nitrate	11.11±2.72	-	-
Phosphate	20.05±5.80	-	-
Dissolved oxygen, DO	4.98±2.82	-	-
Chloride	62.79±12.93	-	-
pH	5.86±0.75	6.0 – 9.0	5.5 – 9.0
Turbidity (NTU)	6720±1238.01	-	-
Salinity (%)	0.76±0.30	-	-
Heavy metals			
Zinc, Zn	0.6709±0.29	1.0	1.0
Ferum, Fe	0.6852±0.12	1.0	5.0
Cadmium, Cd	0.0013±0.00	0.01	0.02
Copper, Cu	0.0983±0.04	0.20	1.0
Aluminium, Al	0.2374±0.08	-	-
Lead, Pb	0.0432±0.03	0.10	0.50
Manganese, Mn	0.2193±0.17	0.20	1.0
Arsenic, As	0.0051±0.00	0.05	0.10

* All unit in mg/L except for pH, turbidity & salinity
[a] All experiments conducted in replicates (n=9)

Concentrations of COD and BOD were 2838.62 mg/L and 793.89 mg/L, respectively; this concentration was extremely higher than permissible limits of the effluent standard. However, a number of studies show that significant differences do exist, (Vanerkar *et al.*, 2013) findings are somewhat contradictory. They revealed that composite FW contained 11220 mg/L of COD and 6860 mg/L of BOD (Vanerkar *et al.*, 2013). But, COD concentration of current research is almost in line with findings of past studies by Shin *et al.* (2015), which digested food wastewater effluent contained about 5923 mg/L of COD. In term of metal elements, manganese shows a little bit higher of concentration compared to effluent Standard A (Table 5.3)

and the rest of elements studied were within allowable limit effluent standard as stated in Table 5.3. It clearly indicates that FW is none toxic because it contains only a few hazardous organic compounds with the exception of some toxic cleaning product (Abdalla, 2014). Nevertheless, the selected (Zn, Fe, CD and Mn) metal elements still need to be completely removed or treated before discharged to the environment. The need for this action is because the presence of heavy metal in freshwater has severe effects that threaten all the living organisms in the receiving water bodies and public health (Onalo *et al.*, 2014).

FW treatment could be conducted by using the different physical method, chemical method and biological treatment. Nowadays, the biological treatment is one of the most favourable approaches to wastewater treatment due to several advantages especially phycoremediation. Although microalgae have been widely used in the wastewater treatment, there has been limited use on FW bioremediation. Previously, Ji *et al.* (2015), Qasim & Mane (2013) and Shin *et al.* (2015) had shown the potential of microalgae in FW treatment to produce valuable biomass for lipid production. Thereby, this study may allow the use of FW for the development of culture method in biomass production and for further purposes of phycoremediation study coupled with hydrocarbon production.

5.3.2 Nutrients reduction

The trend removal of total phosphorus (TP), total nitrogen (TN) and total organic carbon (TOC) for outdoor and indoor conditions from food processing wastewater (FW) phycoremediation using *Botryococcus* sp. are presented in Figure 5.20, 5.23 and 5.26, respectively. The total removal of these nutrients after 18 days of phycoremediation is also shown in Figure 5.22, 5.24 and 5., respectively.

Botryococcus sp. demonstrated a good performance in TP reduction from FW for both outdoor and indoor culture condition (Figure 5.20). Most of the initial concentration tested showing a good reduction throughout of the study. Therefore, there was no significant effect (p>0.05) of initial cell concentration on the reduction of TP during treatment. Mathematically, the first-order kinetic coefficients of TP for both the outdoor and indoor culture in different concentrations were obtained by linear regression. The highest coefficient for outdoor and indoor culture conditions was at 1×10^6 cells/mL with the value of 0.0508 and 0.0759, respectively. In addition,

mathematical model patterns of TP for outdoor and indoor culture depicted a constant decrease along with phycoremediation time.

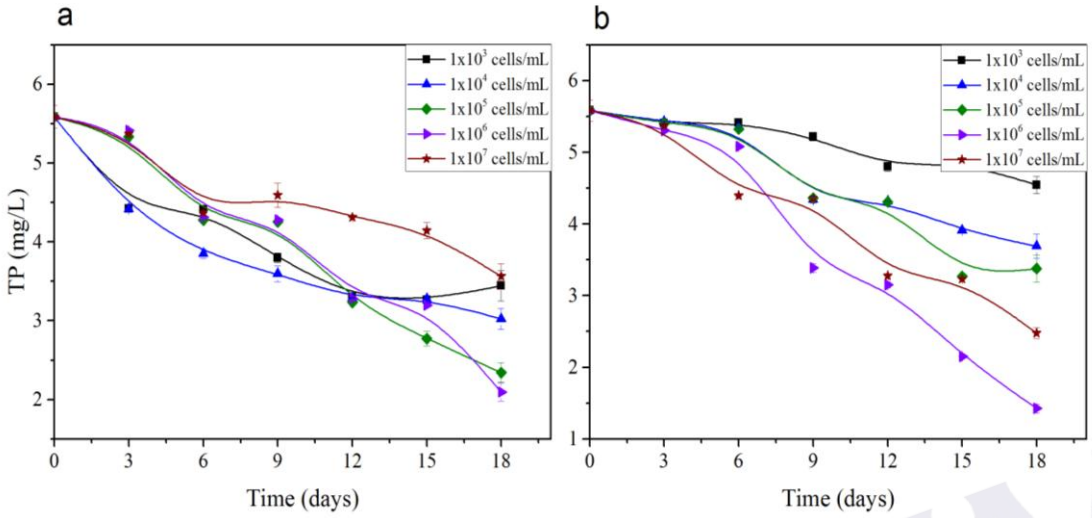


Figure 5.20: Removal of total phosphorus (TP) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in FW.

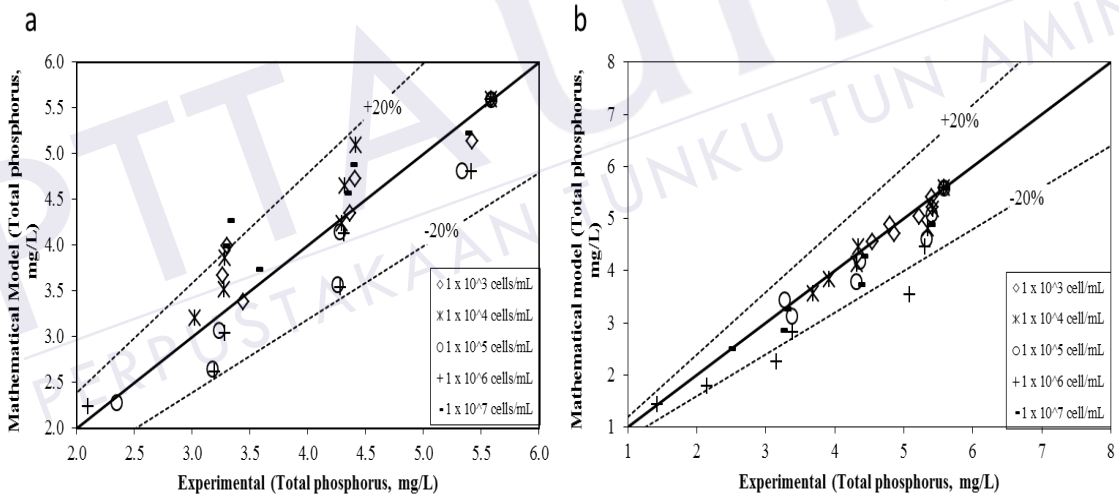


Figure 5.21: Comparison of TP phycoremediation between mathematical model and experimental for a) outdoor and b) indoor culture in FW

Thus, Figure 5.21 shows the comparison of the mathematical model with experimental data of TP removal during the phycoremediation study and found that comparison plot was uniformly distributed around the 45° straight line implying that there were no bias effects. It was apparent a good relationship between mathematical model and experimental data. As shown in Figure 5.21, compared to the experiment data, the mathematical model results of TP reduction showed an acceptable

difference within accuracy ±20%. Therefore, these model equations suitable employed to calculate the reduction of TP in the phycoremediation study of FW.

In term of total removal of TP (Figure 5.22), there was a significant (p<0.05) amount of different initial cell concentrations for the TP total removal efficiency for both outdoor and indoor conditions (refer to **APPENDIX G**). The Post hoc comparison for each initial cell concentrations using the Turkey HSD test indicated that the mean score was significantly different compared to the control sample (FW without algae). Consequently, Figure 5.22 indicates that the highest TP removal is at a concentration of 1×10^6 cell/mL with total removal of 62.5% for outdoor culture and 74.46% for indoor culture. These results are slightly lower than the result obtained by (Shin *et al.,* 2015) who reported that microalgae *Scenedesmus bijuga* remove about 90.5% of TP from 1/20 diluted FW effluent. Meanwhile, *Chlamydomonas Mexicana* was able to reduce TP only up to 28% from piggery wastewater as studied by (Abou-Shanab *et al*., 2013).

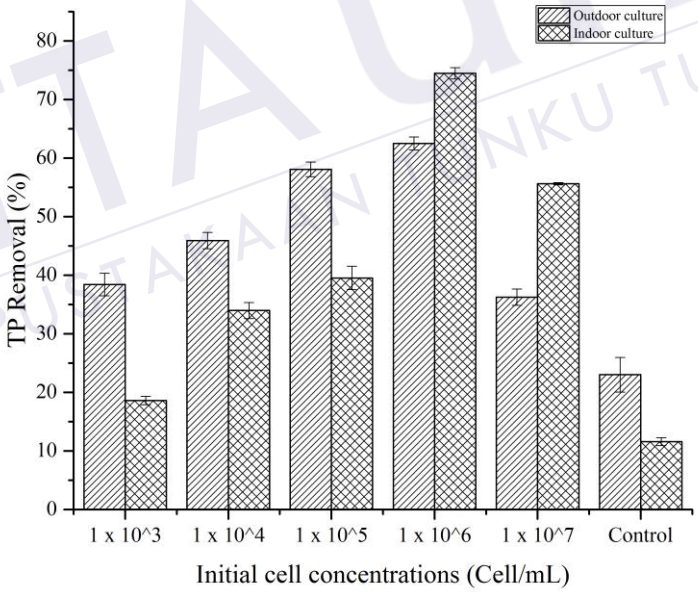


Figure 5.22: Total removal of TP after 18 days of FW phycoremediation

However, Latiffi *et al.* (2016) reported that the highest removal of TP in the form of orthophosphate successfully removed up to 94% when they cultivate *Botryococcus* sp. in meat food processing wastewater. As can be seen, the efficiency of removal mostly depending on the microalgae species and also the different wastewaters employed. According to Rawat *et al.* (2016), microalgae have the ability

to reduce phosphorus, especially orthophosphate and utilise it as a macronutrient for the synthesis of some compound such as nucleic acids, phospholipids and protein through phosphorylation process. Other than that, indirect phosphorus removal also happens because of the precipitation of phosphate at high pH since the growth of algae itself in the culture gradually increase the pH value (Rawat *et al*., 2016).

The Figure 5.23 presents the daily changes in TN of FW during cultivation of *Botryococcus* sp. for 18 days. Both culture conditions show a considerable reduction of TN for each initial cell concentration tested (Figure 5.23). Moreover, a mathematical model was plotted together with experimental data to prove the TN removal should be done by *Botryococcus* sp. during phycoremediation. The highest first-order kinetic coefficient for outdoor and indoor culture condition was at 1×10^6 cells/mL with the value of 0.1349 and 0.0799, respectively. As can be observed in Figure 5.23, there was no significant effect (p>0.05) of each concentration on the reduction of TN throughout of the study.

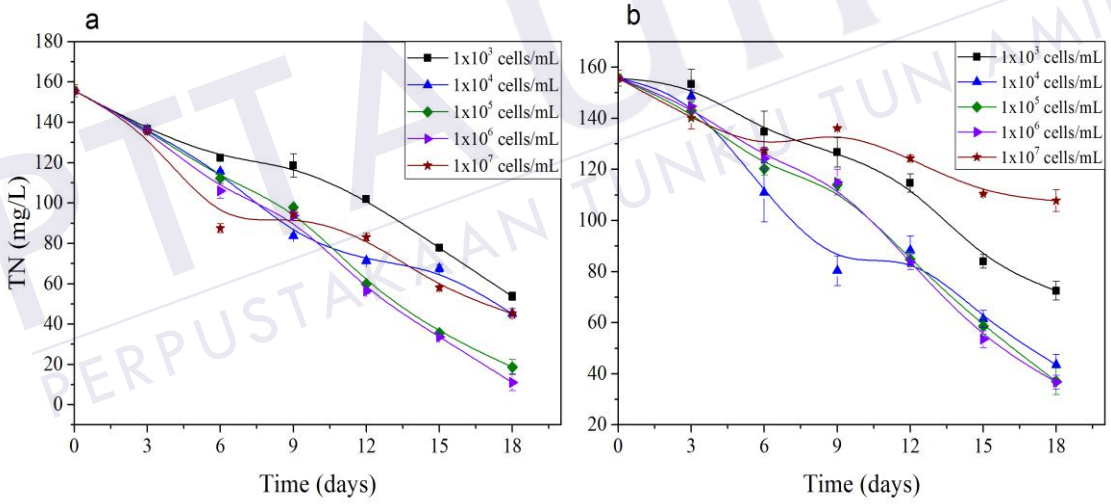


Figure 5.23: Removal of total nitrogen (TN) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in FW.

However, the highest total removal of TN was at 1×10^6 cell/mL for outdoor culture and 1×10^5 cell/mL for indoor culture. The percentage error was obtained by comparing the mathematical model with experimental data as scattered in the Figure 5.24. In outdoor culture, some data of the concentration 1×10^5 cell/mL is below underestimated -20% of error while other concentrations showed that experimental data distributed within ±20% of accuracy. Meanwhile, Figure 5.24b illustrated that

the indoor culture experimental data was estimated within the range of ±20% for all concentration tested and parallel to the datum line (45°).

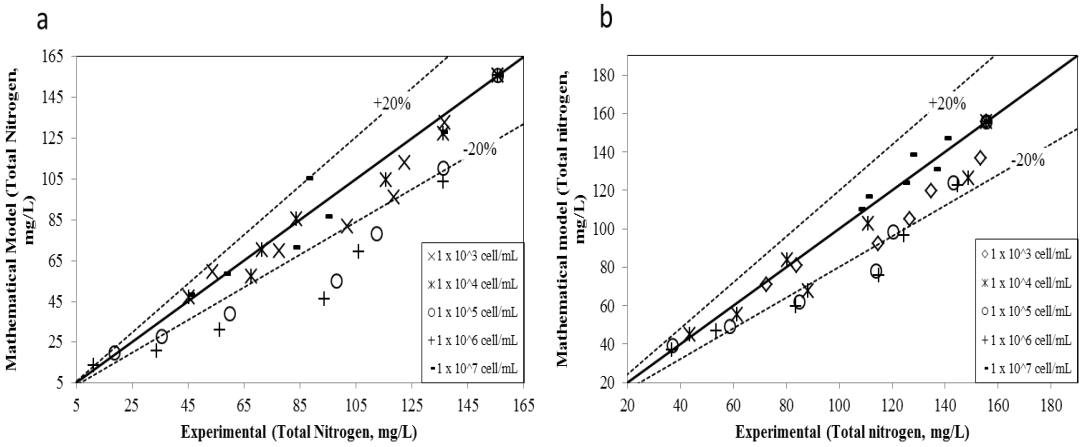


Figure 5.24: Comparison of TN phycoremediation between mathematical model and experimental for a) outdoor and b) indoor culture in FW

Data in Figure 5.25 indicates that the highest removal of TN after 18 days of FW phycoremediation was 92.9% (1×10^6 cell/mL) for outdoor culture and 76.9% (1×10^5 cell/mL) for indoor culture. This result was in line with the daily changes of TN value in FW as illustrated in Figure 5.23. These findings were reinforced by a statistical analysis where there was exist a significant difference ($p<0.05$) between concentration tested over total TN removal (refer to **APPENDIX G**). In addition, Figure 5.25 showed that higher initial cell concentration reduces more of TN concentration in FW but too much of algae cell in the culture causing the removal efficiency become less effective. The comparison of the culture condition found that the removal of TN was higher and effective in outdoor culture compared to indoor culture. The present finding of TN removal in FW also supported by Yang *et al.* (2016) study which found that *Chlorella vulgaris* and *Scenedesmus obliquus* cultivated in small scale photobioreactor successfully remove TN concentration in municipal wastewater up to 99%. Different removal of TN in wastewater as influenced by culture conditions due to the different adaptation of environmental factor used. In fact, statistical analysis also had been proved that both condition was highly significant ($p<0.05$) in term of total removal efficiency. These situations due to the different growth pattern when subjected to different environmental factors such as light intensity, photoperiod and temperature. Higher or better removal occurred in outdoor condition because of the light exposure factor where natural

sunlight provide better light wavelength for the photosynthesis process of microalgae compared to artificial light. Therefore,

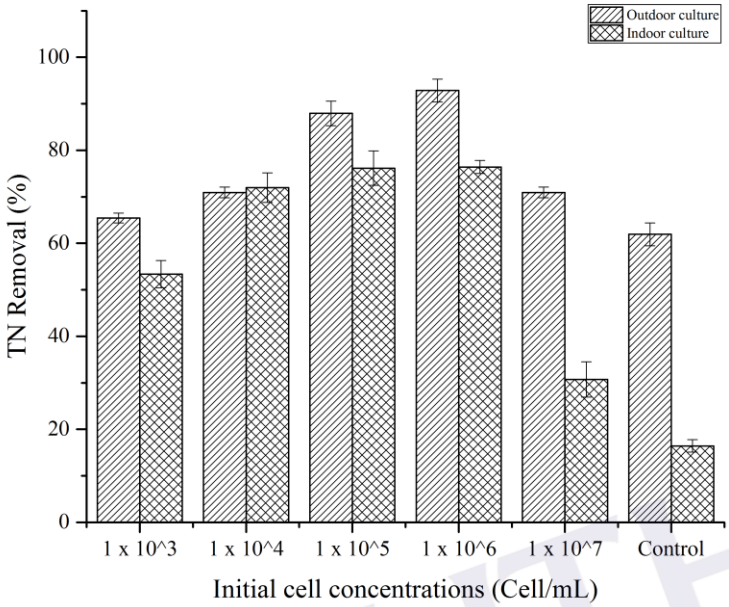


Figure 5.25: Total removal of TN after 18 days of FW phycoremediation

Other than that, present result also in good agreement with our previous study where reported that *Botryococcus* sp. was able to remove TN in DW about 60.8% in outdoor culture (Gani *et al*., 2016b). Since nitrogen is a crucial nutrient for the growth of microalgae, so, this *Botryococcus* sp. presents a great potential to effectively remove nitrogen in FW. Generally, green algae assimilate the inorganic nitrogen in the form of ammonium, nitrate, and nitrite; and transform them to the organic nitrogen which required for cell accumulation or synthesis (Rawat *et al*., 2016). In fact, assimilation of nitrogen in the microalgae cell causing the reduction of ammonia with the increase of pH during photosynthesis process.

The effectiveness of *Botryococcus* sp. to remove TOC from FW in five different initial concentrations is presented in Figure 5.26, 5.27 and 5.28 for both outdoor and indoor culture. This study indicates that experimental value of TOC decreases linearly over a period of phycoremediation (Figure 5.26). Similarly, a mathematical model of TOC removal showed a constant decrease proportionate to the increase of phycoremediation time. The percentage error obtained from experimental was compared to mathematical model and presented in the Figure 5.27

for both cultures. Figure 5.27a shows that comparison plot of the data was uniformly distributed along the datum line for outdoor culture except a few data located over underestimated -20% in the range of 3.67 mg/L – 14.27 mg/L (1×10^6 cell/mL) of the experimental result. Meanwhile, indoor culture depicts that the comparison data between the mathematical model with experimental scattered evenly and shows acceptable difference within an accuracy of ±20% (Figure 5.27b).

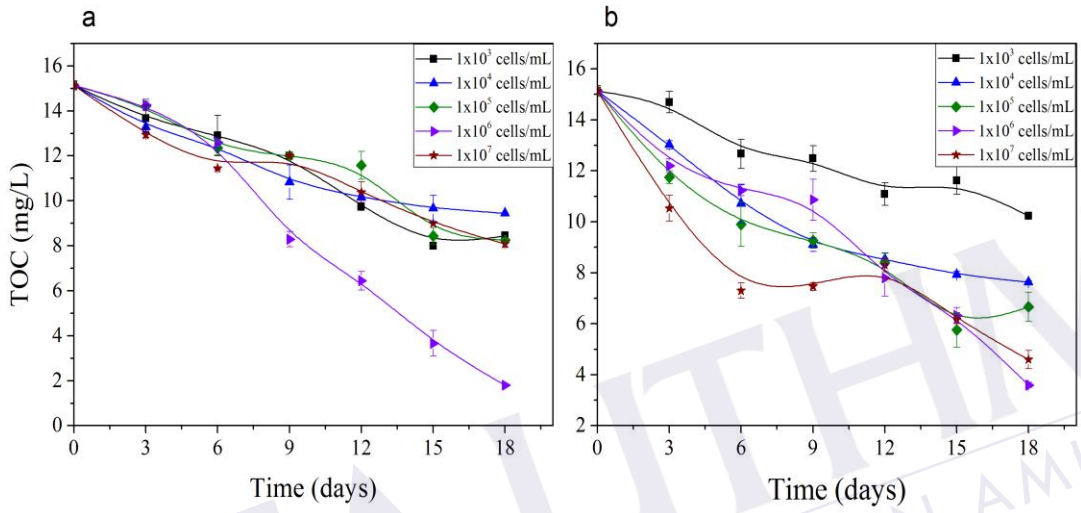


Figure 5.26: Removal of total organic carbon (TOC) from (a) outdoor and (b) indoor culture condition with different microalgae concentration in FW.

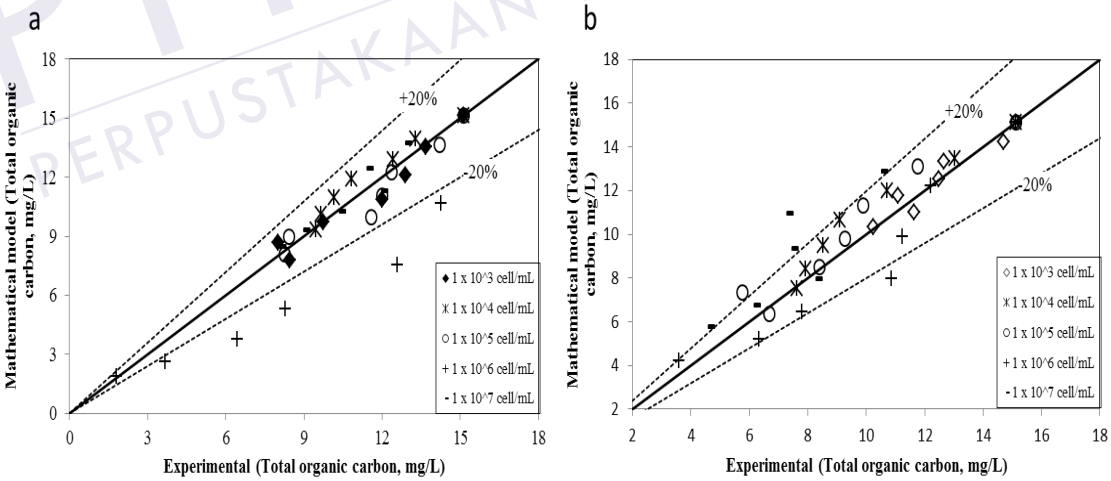


Figure 5.27: Comparison of TOC phycoremediation between mathematical model and experimental for a) outdoor and b) indoor culture in FW

Experimentally, the highest TOC first-order kinetic coefficient obtained from linear regression was found t be 0.1164 and 0.0712 at 1×10^6 cell/mL for outdoor and

indoor cultures, respectively. Thus, 1×10^6 cell/mL concentration has resulted in the highest TOC removal in FW up to 88.1% for outdoor and 76.2% for indoor culture (Figure 5.28). The above finding is consistent with the finding of past study by Shen *et al.* (2015) in which the removal efficiency of TOC was 97.8% when they employed *Scenedesmus obliquus* to treat synthetic wastewater. Similarly, Mahapatra *et al*. (2014) who used mixotrophic algae consortia to treat municipal wastewater and revealed about 86% of TOC removal was achieved. The high removal of TOC in wastewater using microalgae cultivation can be explained further where existing carbohydrate in which referring to glucose in the wastewater can be used as carbon sources for heterotrophic or mixotrophic algae growth (Lee & Lee, 2001; Shen *et al*., 2015).

The present study, however, makes several noteworthy contributions to the effectiveness of phycoremediation by *Botryococcus* sp. in FW varies according to the culture conditions and initial cell concentrations applied. Both cultures (outdoor and indoor) presents a very positive sign for FW treatment since the best concentration, 1×10^6 cell/mL was able to remove TP, TN and TOC by between 62.5 to 92.9%.

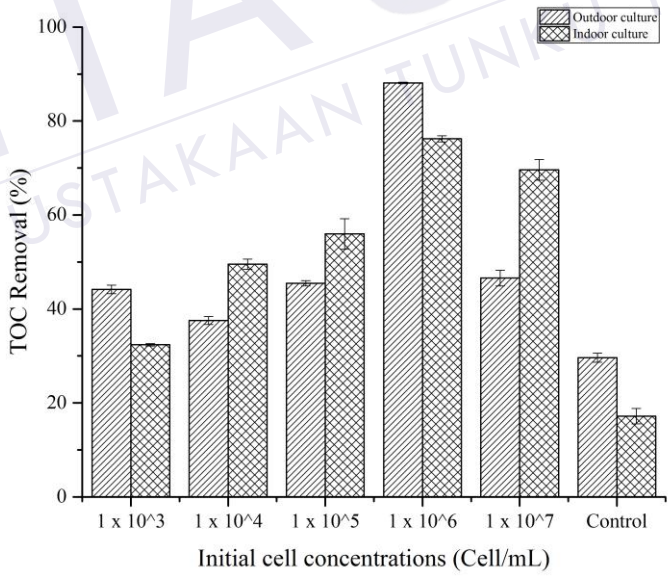


Figure 5.28: Total removal of TOC after 18 days of FW phycoremediation

5.3.3 Heavy metal removal

The potential of *Botryococcus* sp. to eliminate selected heavy metals (Zn, Fe, Cd and Mn) from FW during phycoremediation was presented in Figure 5.29, 5.30, 5.31 and

5.32 while total removal after 18 days of phycoremediation was stated in Figure 5.33 for both outdoor and indoor cultures. The data presented that the removal efficiencies differed according to the *Botryococcus* sp. concentrations, culture conditions and heavy metals studied. Interestingly, *Botryococcus* sp. has moderate ability to remove heavy metals from FW.

Therefore, analysis of variance (One-way ANOVA) shows a significant difference (p<0.05) on the amount of initial concentration for the Fe daily removal for both cultures while outdoor culture for Mn removal. However, there was no statistical difference (p>0.05) among the initial concentration tested in term of Zn and Cd daily removal during phycoremediation for both outdoor and indoor culture. But, total removal (Figure 5.33) after 18 days of treatment which is expressed as a percentage (%) showed a very significant effect (p<0.05) between initial concentrations on the selected heavy metal (Zn, Fe, Cd and Mn) tested for both outdoor and indoor culture.

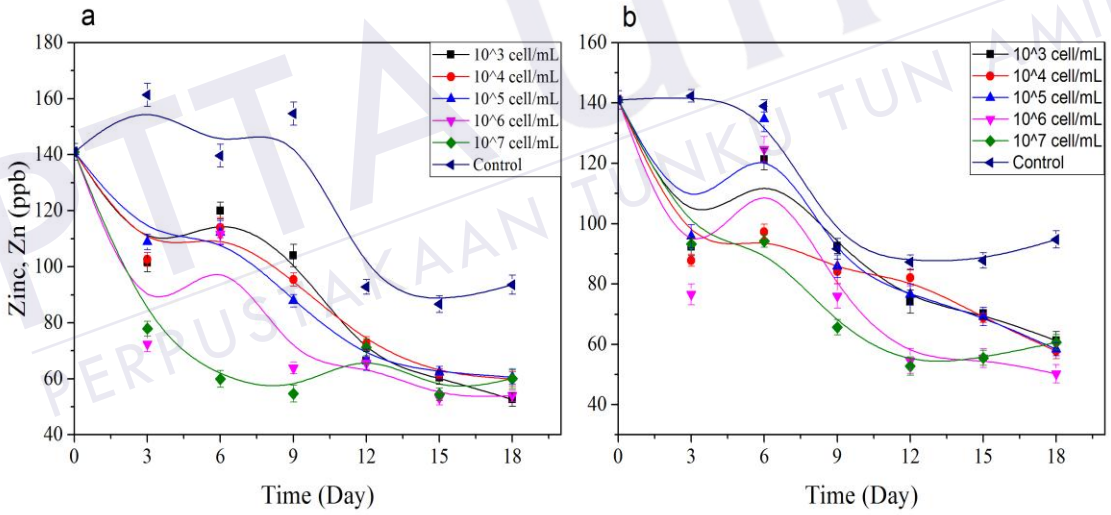


Figure 5.29: Trend removal of Zn from FW during phycoremediation; (a) outdoor culture and (b) indoor culture.

A considerable reduction in interval 3 days of Zn (Figure 5.29), Fe (Figure 5.30), Cd (Figure 5.31) and Mn (Figure 5.32) was observed in each concentration for both cultures except control sample. The *Botryococcus* sp. accumulated Zn, Fe, Cd and Mn quite effectively from FW after a lag period of about 3 to 6 days and continuously decreased with time. Nevertheless, there was some of fluctuates reading was recorded during 18 days of treatment. This may due to the several factors such

as environmental factor, pH changes and shaking time (Kirrolia *et al.*, 2012). Overall, patterns removal of Zn, Fe, Cd and Mn depicted a constant reduction proportionate to the increase of treatment time.

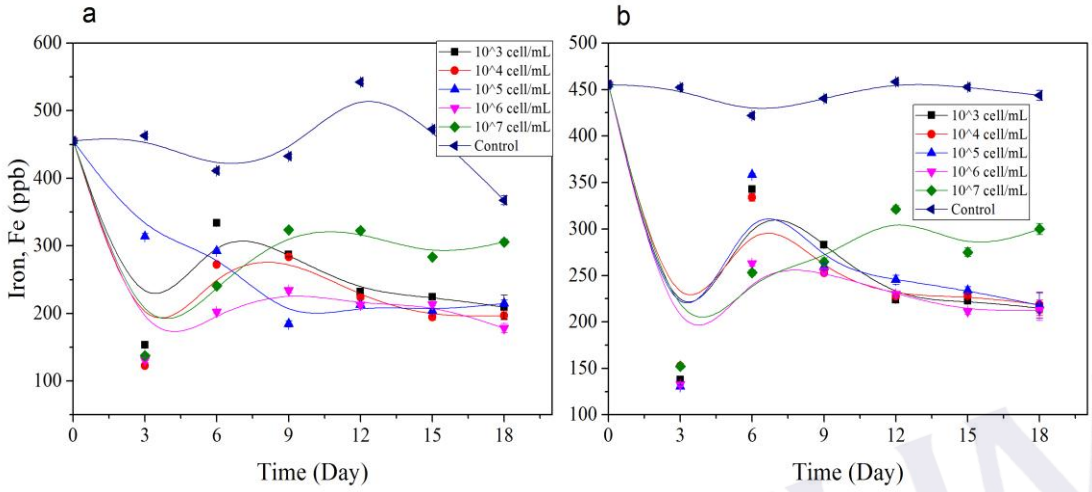


Figure 5.30: Trend removal of Fe from FW during phycoremediation; (a) outdoor culture and (b) indoor culture.

After 18 days, *Botryococcus* sp. successfully assimilated Zn in FW about 57% - 62.5% for outdoor culture and 56.6% - 64.4% for indoor culture (Figure 5.33a). In the concentration of 1×10^3 cells/mL to 1×10^6 cells/mL (Figure 5.33b), this microalga was able to accumulate Fe between 52.8% and 60.8% for outdoor culture while 52% and 53.2% for indoor culture. However, 1×10^7 cell/mL demonstrated the lowest removal of Fe (32.9% - 34.1%) for outdoor and indoor culture. The total removal of Cd from FW was ranged between 28.3% and 50.4% for outdoor culture and ranged between 36.1% and 52.9% for indoor culture for all initial concentration tested (Figure 5.33c). Therefore, indoor culture shows a better removal efficiency of Mn compared to outdoor culture from FW using microalgae (*Botryococcus* sp.). In this investigation, *Botryococcus* sp. was only able to absorb Mn up to 12-26.7% for indoor culture while 7.6-20.7% for outdoor culture (Figure 5.33d). Luckily, removal efficiencies obtained for Mn are still higher than control sample (FW without algae) and at once indicates that *Botryococcus* sp. successful reduce Mn concentration in FW compared to control sample.

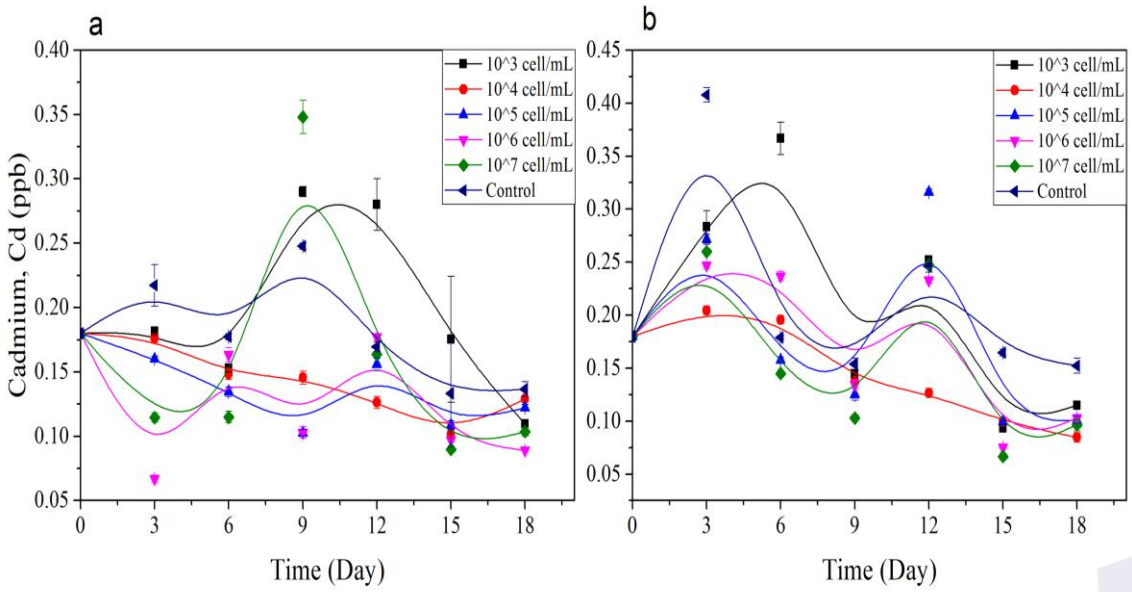


Figure 5.31: Trend removal of Cd from FW during phycoremediation; (a) outdoor culture and (b) indoor culture.

Differences use of wastewater, microalgae species and strain, concentration and culture conditions openly showed a very significant difference in term of heavy metal removal efficiency. Recently, Mar *et al.* (2016) used *Desmodesmus* sp. to bioremediate oil refinery factory wastewater obtained from Hong Run DA Chemical Ltd. Cangzhou, Hebei in China. They reported that Zn, Fe and Mn were successfully removed up to 59.8%, 34.6% and 97.29%, respectively via *Desmodesmus* sp. cultivation. Ajayan & Selvaraju (2012) have demonstrated the Zn removal of undiluted tannery effluent using two microalgae species differently in indoor condition. They found that *Chlorella pyrenoidosa* and *Scenedesmus* sp. were able to assimilate Zn up to 68.9% and 66%, respectively after 12 days of treatment (Ajayan & Selvaraju, 2012).

Meanwhile, Chan *et al.* (2014) reported that Zn removal in the autoclave secondary effluent from the city of waterloo WWTP was very high with removal efficiency up to 96.3% and 94.9% for *Chlorella vulgaris* and *Spirulina maxima*, respectively. In another study, Hamouda *et al.* (2016) examined green microalgae (*Scenedesmus obliquus*) for the removal of Cd from Wastewater Company collected in Quesna-Egypt in indoor culture. They reported that *Scenedesmus obliquus* reduces Cd in the light and dark condition about 70% (Hamouda *et al.*, 2016). Worku & Sahu (2014) and Hammouda *et. al.* (2015) studied the removal of Fe from wastewater

using *Synechocystis salina* and *Chlorella* sp., respectively. The *Synechocystis salina* removed Fe in synthetic wastewater about 66% while *Chlorella* sp. was able to reduce Fe up to 92.2% from mixed industrial wastewater. This indicates that the removal efficiency of Fe using green microalgae is much higher in real wastewater compared to artificial wastewater. Other than that, Hammouda *et al.* (2015) also tested the removal of Mn and revealed that *Chlorella* sp. was successfully remove 73.2% of Mn concentration in that wastewater while Gao *et al.* (2016) used the same microalgae species to treat domestic secondary effluent with 100% removal of Mn when integrate the cultivation using lab-scale membrane photobioreactor.

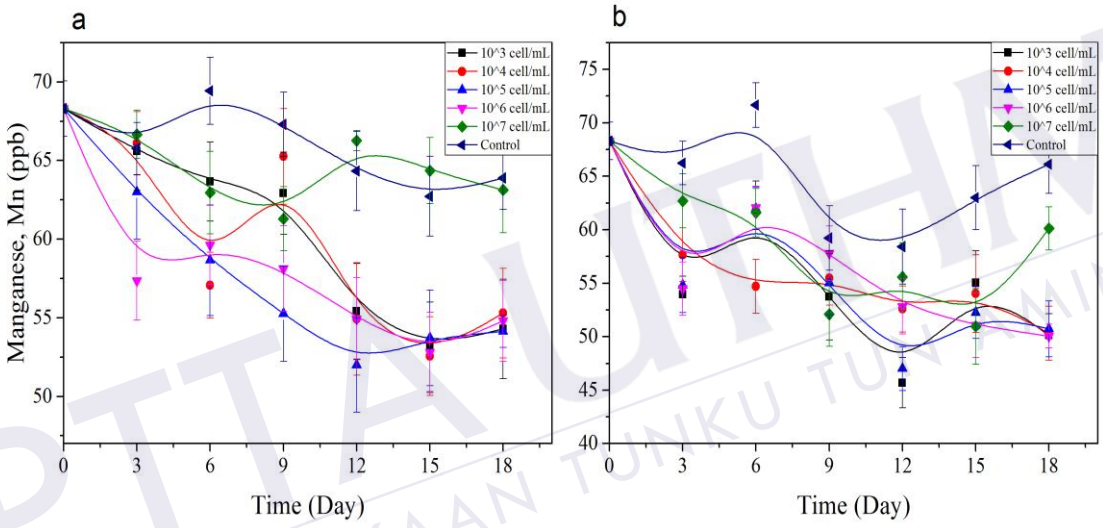


Figure 5.32: Trend removal of Mn from FW during phycoremediation; (a) outdoor culture and (b) indoor culture.

The results of our experiments are in good agreement with most of the previous study who found that the different microalgae species accumulate difference of metal assimilation. In addition, some of the metal removal via microalgae cultivation is dependent on various physiochemical factor, mainly pH, salinity and hardness of the water (Rawat *et al*., 2016). In fact, an ionic charge of metal ions itself and chemical composition of the media also plays essential roles. More recently, Kumar *et. al.* (2015) gave a comprehensive review of factors affecting heavy metal remediation using microalgae. They summarised that heavy metal removal using aquatic organisms like microalgae can be extremely affected by biotic and abiotic factors. The most significant biotic factors such as microalgae species and biomass concentration while abiotic factors such as pH level, salinity and

hardness, and temperature (Kumar *et al.,* 2015). These factors could be the possible factors that involved as heavy metal removal mechanisms in this study.

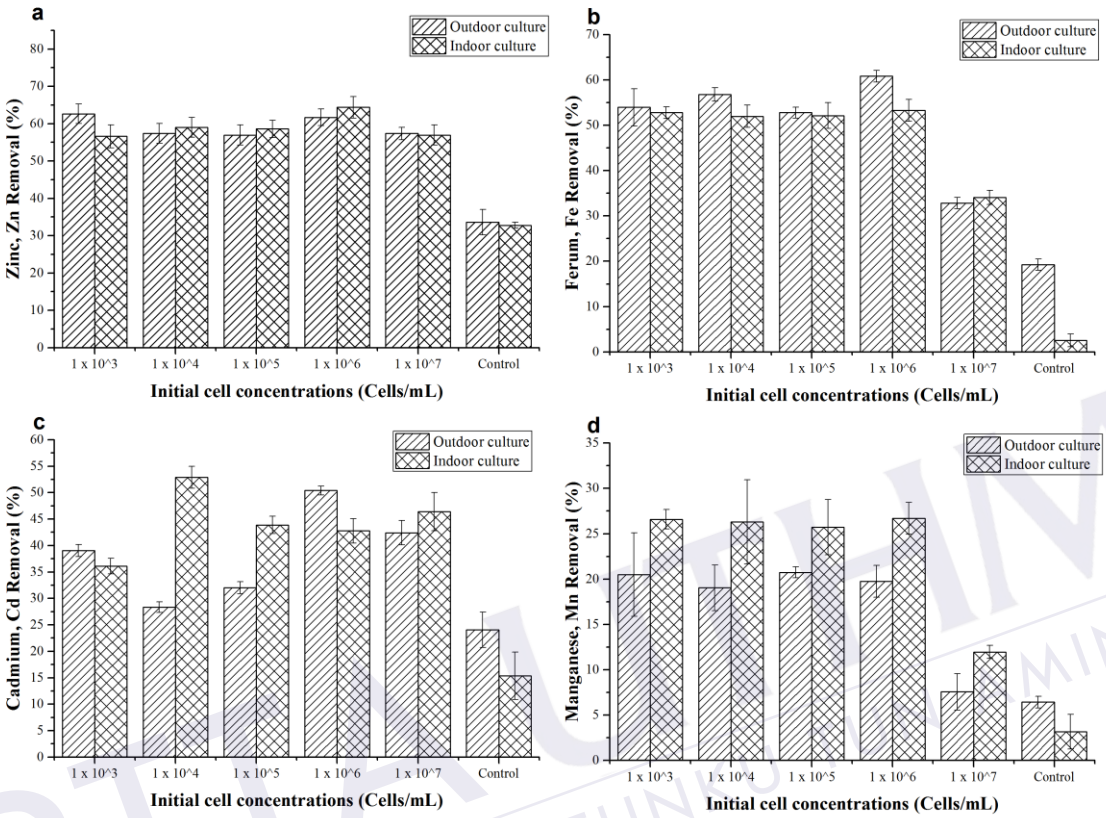


Figure 5.33: Total removal of (a) Zn, (b) Fe, (c) Cd and (d) Mn from FW after 18 days of phycoremediation

5.3.4 Analysis of growth and biomass productivity

The growth characteristics of *Botryococcus* sp. in FW were observed at different initial cell concentrations within 18 days for both outdoor and indoor culture (Figure 5.34). The experimental growth curve was plotted together with the mathematical model curve. Most of the mathematical model data for some concentrations and culture conditions are in line with that of the experimental. It indicated a good correlation and relationship between mathematical model and experimental values.

Statistical analysis (One-way ANOVA) was performed to compare the influence of the concentrations tested on the daily growth of microalgae. Both the outdoor and indoor cultures showed the significant difference (p<0.05) amount of concentrations tested on the daily growth of *Botryococcus* sp. cultivated in FW.

Experimentally, the growth patterns were different in each concentration and culture conditions (Figure 5.34). There was no lag phase was recorded during cultivation of *Botryococcus* sp. in FW for both cultures as typically reported in ordinary aquatic plant growth (Mar *et al*., 2016). This situation occurred probably due to the exponentially growing cells of precultures were used as an inoculum (Hempel *et al.*, 2012). The present finding is consistent with the study by Boonma *et al*. (2014) and Guo *et. al.* (2013). They reported obtaining the same pattern of growth curve when using synthetic medium and aquaculture wastewater as a growth medium for the cultivation of *Botryococcus braunii* and *Platymonas subcordiformis,* respectively (Boonma *et al.*, 2014; Guo *et al.*, 2013). Most of the concentrations tested already experience a period of exponential phase from the first day up to the Day 5 of cultivation. After that, *Botryococcus* sp. fluctuate grew in stationary phase until the Day 18 of treatment. From the Figure 5.34, the majority of mathematical model follows the curve of experimental values except for the concentration of 1×10^5 cell/mL (Figure 5.34c) and 1×10^7 cell/mL (Figure 5.34e). The mathematical model for 1×10^5 cell/mL concentration under indoor culture was uniformly scattered below the experimental data. Similarly, 1×10^7 cell/mL concentration also depicted the same trends for both outdoor and indoor cultures. This phenomenon possibly may because of the several factors such as the effect of environmental factors, shaking time, the availability of nutrients provided in medium and culture condition itself (Santos-Ballardo *et al*., 2016).

After identifying the exponential phase of each concentration for both outdoor and indoor culture, the maximum growth rate (day^{-1}), doubling time (day) and biomass productivity (cell/mL/day) were calculated scientifically as stated in Table 5.4. Analysis of variance (One-way ANOVA) proved that a significant difference ($p<0.05$) between the *Botryococcus* sp. concentration with maximum growth rate and the biomass productivity for both outdoor and indoor cultures. But, Post hoc Tukey test found an insignificant difference ($p>0.05$) between the concentration of 1×10^3 cell/mL and 1×10^4 cell/mL in term of maximum growth rate for outdoor culture while for indoor culture, all concentration applied showing a very significant effect on the amount of maximum growth rate.

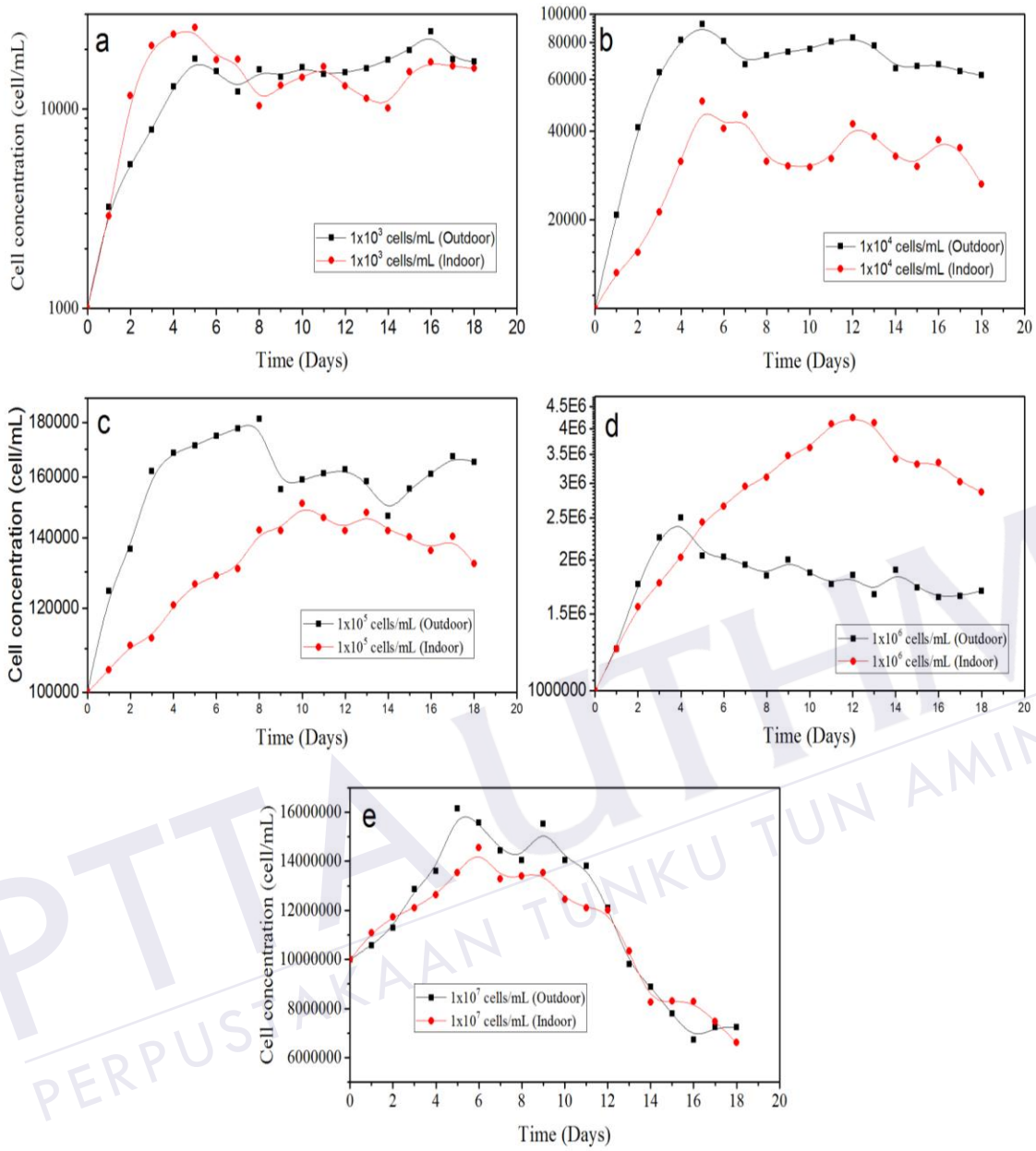


Figure 5.34: Growth of *Botryococcus* sp. during phycoremediation of FW in different cell concentrations.

Likewise, biomass productivity also showed a significant result (p<0.05) as affected by difference algae concentrations for both culture conditions. Obviously, the trend for the maximum growth rate curve for outdoor culture is almost similar to that of the indoor culture (Figure 5.35). What is interesting in this data is that the more concentration of *Botryococcus* sp. in FW for both culture conditions showed a decrement of the growth rate (Figure 5.35). Thereby, the highest maximum growth rate can be achieved by *Botryococcus* sp. in FW for outdoor and indoor cultures at

1×10^3 cell/mL were 0.543 day^{-1} and 1.051 day^{-1}, respectively. However, interestingly, this is contrary to a study conducted by (Arbib *et al.*, 2014) and (Santos-Ballardo *et al*., 2016). The *Chlorella vulgaris* was found be able to reach growth rate about 0.48 day^{-1} when cultivated in urban wastewater (Arbib *et al.,* 2014). Meanwhile, *Phaeodactylum tricornutum* was able to survive in synthetic wastewater using bubble column photobioreactor with the growth rate up to 0.4058 day^{-1}.

Hereby, the present findings would add substantially to our understanding since the application of various microalgae concentrations, the biomass productivity of *Botryococcus* sp. was also very different. From the data in Figure 5.35, it is apparent that the best concentration to be inoculated in FW was between 1×10^6 cell/mL and 1×10^7 cell/mL. Therefore, the highest biomass productivity was 2.41×10^5 cell/mL/day for outdoor culture at the concentration of 1×10^7 cell/mL while 1.45×10^5 cell/mL/day under indoor culture with the concentration of 1×10^6 cell/mL. The current findings also add to a growing body of literature on the cultivation of *Botryococcus* sp. in FW did influence by the culture conditions and microalgae concentrations applied. Thus, high growth rate normally depends on the cells proliferation and it does not reflect the microalgae capacity used in the initial culture. Moreover, biomass production is commonly obtained during the stationary phase, when the cells have most of the biosynthesis capacity to the production of hydrocarbon (Nascimento *et al.*, 2012).

Although the results show a significant impact on the culture condition differences, application of outdoor still remains an option for the cultivation of microalgae in large scale to enhance the efficiency and the energetic balance of the microalgae biomass production from microalgae. Since tropical country receives high sunlight irradiation, the application of outdoor culture in microalgae cultivation is much recommended as a source of energy in the photosynthetic process. However, another country, especially during winter season, may be faced some difficulty to implement this condition.

Table 5.4 Computation of growth kinetic and biomass productivity in different initial cells concentration

Initial cell concentrations (cells/mL)	Maximum growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity (cells/mL/day)
Outdoor culture				
1×10^3	0.543	0.783	1.277	2170
1×10^4	0.531	0.766	1.305	9129
1×10^5	0.154	0.222	4.505	4648
1×10^6	0.278	0.402	2.488	131267
1×10^7	0.094	0.136	7.352	230777
Indoor culture				
1×10^3	1.051	1.516	0.660	4354
1×10^4	0.315	0.454	2.203	3125
1×10^5	0.040	0.058	17.24	857
1×10^6	0.173	0.250	4.000	145009
1×10^7	0.057	0.082	12.195	112445

Data are expressed as mean (n=3)
*n/a: Not applicable

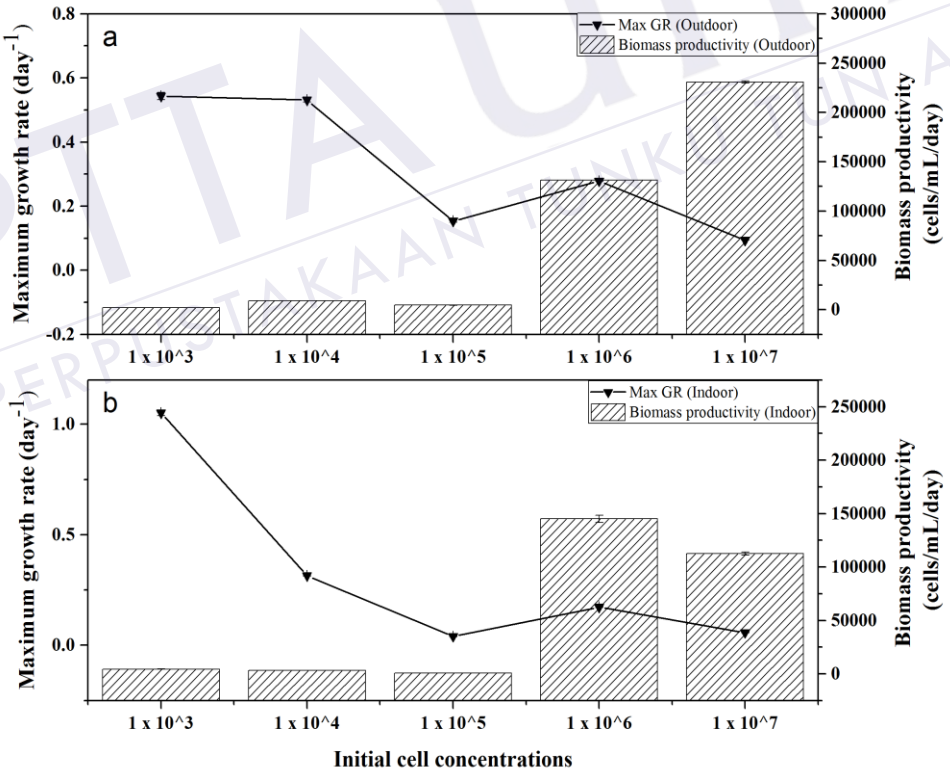


Figure 5.35 Growth rate (day^{-1}) and biomass productivity (cell/mL/day) of *Botryococcus* sp. in different cell concentration (cell/mL) on food processing wastewater; a) Outdoor culture and b) Indoor culture

5.4 Summary

The relevance of microalgae, *Botryococcus* sp. to be used for phycoremediation and potential biomass productivity is clearly supported by the currents finding in this chapter. In fact, phycoremediation efficiencies and heavy metal uptake are highly depending on the culture conditions and microalgae concentrations applied. Similarly, this chapter has shown that the maximum growth rate and biomass productivity extremely rely on these two crucial factors. According to current laboratory experimentation, the most promising culture condition for the cultivation of microalgae is under outdoor culture condition. This has been proved by the biomass productivity assessment and pollutants removal efficiencies that can be achieved using this microalgae species. The best microalgae concentration was selected in enhancing this finding for both phycoremediation and biomass production is 1×10^6 cell/mL. The highest removal of TP, TN and TOC based on the best microalgae concentration for DW is 84.4%, 100% and 45.8%, respectively while for FW is 62.5%, 92.9% and 88.1%, respectively. Further investigation and experimentation into huge biomass productivity for the potential of hydrocarbon production is strongly recommended. Further analysis is to evaluate the effect of outdoor culture condition on the cultivation of *Botryococcus* sp. for biomass productivity using vertical closed photobioreactor in mini-pilot scale; as discussed in next chapter.

CHAPTER 6

MICROALGAE BIOMASS PRODUCTION

6.1 Introduction

In this chapter, we discussed the results of the experiments that conducted for the growth of *Botrycoccus* sp. using vertical closed photobioreactor under outdoor culture condition (refer to **APPENDIX D**). The selected of outdoor condition was made according to work that been discussed previously in Chapter 5. Mainly, it is divided into two sections. The first section explains the details of the microalgae biomass production using domestic wastewater (DW) as growth media. The effect of DW on the growth and biomass productivity are described in section 6.2.1 and harvesting efficiency experiments using flocculation technique are discussed in section 6.2.2. Meanwhile, the second section explains the growth and harvesting efficiency result obtained using FW culture media. The details of the analysis of growth and biomass productivity using FW are explained in section 6.3.1 while recovering microalgae biomass from FW is described in section 6.3.2 using response surface methodology (RSM) analysis. There are two types of coagulant agents was tested namely alum and chitosan. The quadratic model had been obtained according to RSM suggestion and fitted well with the experimental data. Finally, the results and discussion obtained in this chapter are concluded as stated in section 6.4. End of this chapter, the best coagulants agent for further separating of microalgae biomass from culture media is suggested accordingly.

6.2 Biomass production using domestic wastewater

6.2.1 Analysis of growth and biomass productivity

The growth of *Botryococcus* sp. cultivated in photobioreactors using domestic wastewater as a medium is shown in Figure 6.1. The experimental curve was compared to the mathematical model prediction and evaluated according to Equation 3.8. It was found that the mathematical model curve was in line with that of the experimental data on the initial day of cultivation up to the day four only. After that, the predicted model trend shows that slightly higher growth occurred from Day 5 until 8. The growth of *Botryococcus* sp. was begun to grow on the Day 1 and linearly increase to the maximum growth on Day 9. However, on the day ten and onwards, *Botryococcus* sp. growth started to decrease steadily until Day 12. Likewise, Can *et al*. (2013) and Teles *et al.* (2013) were obtained the same growth trend when they apply domestic wastewater to grow *Botryococcus braunii* and *Chlorella vulgaris*, respectively.

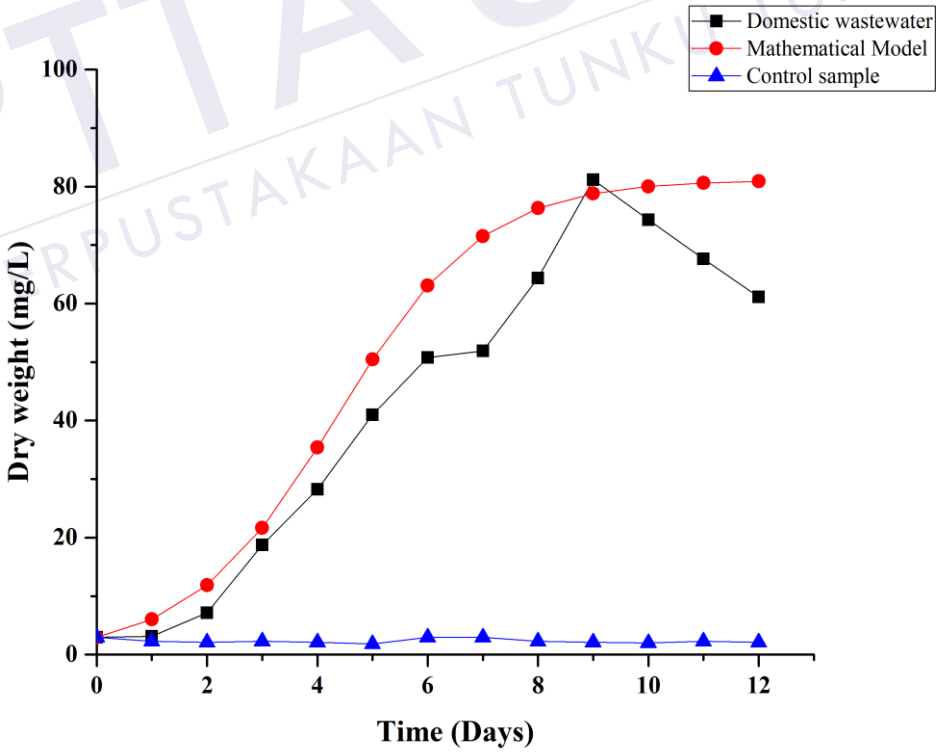


Figure 6.1: Daily growth measurement of *Botryococcus* sp. in domestic wastewater.

The maximum growth rate was determined from the slope of the exponential phase, followed by the calculations of division per day, doubling time, and biomass productivity as stated in Table 6.1. There was no growth found (Figure 6.1) in the control sample, meaning that the growth kinetic parameters were unable to calculate.

Table 6.1: The maximum growth rate, division per day, doubling time, and biomass productivity of *Botryococcus* sp. grown in domestic wastewater.

Maximum growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity ($mg.L^{-1}.Day^{-1}$)
0.7551 ± 0.035	1.089 ± 0.05	0.919 ± 0.041	9.81 ± 0.58

This may be due to the no nutrients inside the autoclave distilled water compared to domestic wastewater. Obviously, *Botryococcus* sp. grew well in domestic wastewater without any inhibition, with a maximum growth rate of 0.7551 day^{-1}. This growth rate is capable of producing doubling time up to 0.9192 days or simultaneously with 22.1 hours. Therefore, this *Botryococcus* sp. can double their cells within 22.1 hours when integrated into domestic wastewater. The daily biomass production of *Botryococcus* sp. was about 9.81 $mg.L^{-1}.day^{-1}$ during the cultivation period.

6.2.2 Harvesting efficiency

A face-centered central composite design (FCCCD) via response surface methodology (RSM) was employed to optimise the harvesting of *Botryococcus* sp. biomass through comparison of flocculation using alum and chitosan. The response in terms of harvesting efficiency (%) at different experimental runs under two selected factors, namely pH and coagulant dosage (mg/L), is shown in Table 3.8. There were a total 21 runs of the FCCCD experimental for each coagulant agent; alum and chitosan.

The ANOVA of alum coagulant model indicated an *F*-value of 5014.61 which implies that the model obtained is statistically significant ($p<0.05$) at 95% confidence level (Table 6.3). The R^2 value of 0.9994 and adjusted R^2 of 0.9992 indicated that the obtained alum coagulant was close to 1, showing the good relation between the calculated and observed values. In fact, the predicted R^2 of 0.9988 also correlated

with adjusted R^2. Moreover, the adequate precision (200.507) of alum coagulant is greater than four, which demonstrated an adequate signal of the model. Therefore, this model can be applied to navigate the design space of harvesting efficiency using alum coagulant. The model for alum coagulant also presents a statistically insignificant lack of fit (p>0.05) with a 20.9% possibility that a lack of fit value occurred due to noise. Thereby, non-significant lack of fit is good because it indicates that the harvesting efficiency responses are adequate for employing this model (Razack *et al.*, 2015).

Meanwhile, to ensure that the selected model is well fitted to the experimental design data, the normal plot of residuals was retrieved from the Design Expert 7.0.0 software. According to normal probability plot of alum coagulant (Figure 6.2a), the points follow a straight line for each case, indicating a normal distribution. Accordingly, the data is considered normally distributed in response to particular models using alum as a coagulant. To obtain the interaction between the independent variable (pH and alum dosage) and the response such as harvesting efficiency, the 3D surface, and contour plot was executed using RSM software (Figure 6.3). The surface plot for alum coagulant (Figure 6.3a) demonstrated that the optimum point is located inside the experimental region (Bezerra *et al.*, 2008). It was also demonstrated in this study that harvesting efficiency increased with the increment of alum dosage which is similar in effect as that of pH. As illustrated in Figure 6.3b, the optimum value of pH and alum dosage was 8.24 and 177.74 mg/L, respectively. The highest harvesting efficiency was 99.3%, with 1.000 of desirability. To validate the statistical model obtained, additional verification experiments in triplicate were conducted at optimum conditions. The average value obtained was 98.1%, which was in good agreement with the predicted response as in Figure 6.5a.

Table 6.3 also presents the ANOVA for the quadratic model response to harvesting efficiency for chitosan coagulant. The p-value of the coefficient was analysed, and C, D, CD, C^2, and D^2 show a very high confidence level. Based on ANOVA output, the p-value for the model obtained was less than 0.0001, showing that the model using chitosan coagulant is highly significant. By comparing the actual values with predicted values for harvesting efficiency using chitosan coagulant (Table 6.2), the R^2value was found to be 0.9928. Moreover, adjusted R^2 of 0.9905 is in reasonable agreement with predicted R^2 of 0.9855.

The adequate precision ratio of the model was about 48.862, which is an adequate signal for the model of chitosan coagulant. The desirability and confirmation of the predicted model can be used to navigate the design space since the adequate precision was greater than 4.

Another way to evaluate the goodness of model obtained is via lack of fit value (Bezerra *et al.*, 2008). According to Table 6.3, the lack of fit value of 1.69 implies the lack of fit is not significant relative to the pure error. There is a 22.18% possibility that lack of fit value can occur due to numerical discrepancy or noise and show that the model is good. Numerical noise is a result of incomplete convergence at iterative process in statistical analysis. The coefficient of each variable (pH and chitosan dosage) was calculated from the results in Table 3, and the final quadratic equation for harvesting efficiency using chitosan coagulant in terms of coded and actual value is stated in Table 6.4. Harvesting efficiency for the chitosan coagulant, the 3D response surface, and contour plot was obtained accordingly (Figure 6.4).

Table 6.2: Actual factor and response values of *Botryococcus* sp. harvesting in domestic wastewater.

Standard order	Actual factor values		Response values (Harvesting Efficiency, %)			
			Alum		Chitosan	
	pH(Coded)	Coagulant dosage, mg/L (Coded)	Actual (Experimental)	Predicted (RSM Model)	Actual (Experimental)	Predicted (RSM Model)
1	5 (-1)	30 (-1)	33.85	33.72	78.14	78.31
2	5 (-1)	30 (-1)	34.01	33.72	79.05	78.31
3	5 (-1)	30 (-1)	33.20	33.72	77.84	78.31
4	12 (-1)	30 (1)	91.81	90.83	70.23	71.83
5	12 (-1)	30 (1)	90.00	90.83	73.22	71.83
6	12 (-1)	30 (1)	91.01	90.83	71.74	71.83
7	5 (1)	180 (-1)	89.20	88.90	94.24	93.96
8	5 (1)	180 (-1)	88.79	88.90	94.58	93.96
9	5 (1)	180 (-1)	88.84	88.90	93.94	93.96
10	12 (1)	180 (1)	85.66	85.36	95.23	95.03
11	12 (1)	180 (1)	85.99	85.36	95.15	95.03
12	12 (1)	180 (1)	84.99	85.36	95.19	95.03
13	5 (0)	105 (-1)	72.30	72.32	80.04	81.01
14	12 (0)	105 (1)	98.22	99.11	78.11	78.30
15	8.5 (-1)	30 (0)	70.26	70.51	72.16	71.97
16	8.5 (1)	180 (0)	94.70	95.37	90.04	91.40
17	8.5 (0)	105 (0)	94.99	93.95	77.01	76.56
18	8.5 (0)	105 (0)	93.67	93.95	75.98	76.56
19	8.5 (0)	105 (0)	94.01	93.95	77.99	76.56
20	8.5 (0)	105 (0)	93.88	93.95	75.98	76.56
21	8.5 (0)	105 (0)	94.11	93.95	77.01	76.56

Decreasing the pH value from 12 to 5 led to a quadratic decrement in the harvesting efficiencies. However, harvesting efficiency is less influenced by the dosage of chitosan but more of the pH level; the significant effect was induced with high pH value. It can be concluded from this result (Figure 6.4) that pH plays an important role in determining the harvesting efficiencies when using chitosan as a coagulant.

Based on the regression equation, optimal conditions for harvesting efficiency were as follows: pH = 12 and chitosan dosage = 169.95 mg/L (Figure 6.4). Therefore, the highest harvesting efficiency was 94.2% with a desirability of 0.958 (Figure 6.5b). Triplicate additional experiments were performed to confirm and validate the predicted optimal value obtain previously. The mean of the extra experimental results was 93.3%, which was similar to the predicted response and at once indicates that the obtained model can be used.

Table 6.3: ANOVA for the response surface quadratic model harvesting efficiency of *Botryococcus* sp. in domestic wastewater for alum and chitosan coagulant.

	Source	Sum of Squares	df	Mean square	*F* value	*p*-value
Alum coagulant	Model	8798.49	5	1759.70	5014.61	< 0.0001
	A - Coagulant dosage	2510.99	1	2510.99	7155.56	< 0.0001
	B - pH	2163.34	1	2163.34	6164.88	< 0.0001
	AB	2758.45	1	2758.45	7860.77	< 0.0001
	A^2	213.06	1	213.06	607.15	< 0.0001
	B^2	381.07	1	381.07	1085.92	< 0.0001
	Residual	5.26	15	0.35		
	Lack of fit	1.60	3	0.53	1.75	0.2094
	Pure error	3.66	12	0.30		
	Cor total	8803.75	20			
	R^2 = 0.9994, Adj. R^2 = 0.9992, Pred. R^2 = 0.9988, Adeq precision = 200.507					
Chitosan coagulant	Model	1641.27	5	328.25	415.96	< 0.0001
	C - Coagulant dosage	25.72	1	25.72	32.60	< 0.0001
	D - pH	1320.85	1	1320.85	1673.78	< 0.0001
	CD	42.76	1	42.76	54.18	< 0.0001
	C^2	30.15	1	30.15	38.21	< 0.0001
	D^2	82.52	1	82.52	104.56	< 0.0001
	Residual	11.84	15	0.79		
	Lack of fit	3.52	3	1.17	1.69	0.2218
	Pure error	8.32	12	0.69		
	Cor total	1653.11	20			
	R^2 = 0.9928, Adj. R^2 = 0.9905, Pred. R^2 = 0.9855, Adeq precision = 48.862					

Table 6.4: The quadratic equation developed for harvesting efficiency of *Botryococcus* sp. in domestic wastewater in term of coded and actual factor.

Type of coagulant	Quadratic equation (Coded factors)	Quadratic equation (Actual factors)
Alum	$+93.95 + 13.39A + 12.43B - 15.16AB - 8.23A^2 - 11.01B^2$	$-87.62210 + 0.97689A + 24.89721B - 0.057758AB - 1.46373E\text{-}003A^2 - .89888B^2$
Chitosan	$+76.56 - 1.36C + 9.71D + 1.89CD + 3.10C^2 + 5.12D^2$	$+97.57930 - 0.19483C - 5.09066D + 7.19096E\text{-}003CD + 5.50658E\text{-}004C^2 + 0.41828D^2$

Notes: A and C: Coagulant dosage; B and D: pH

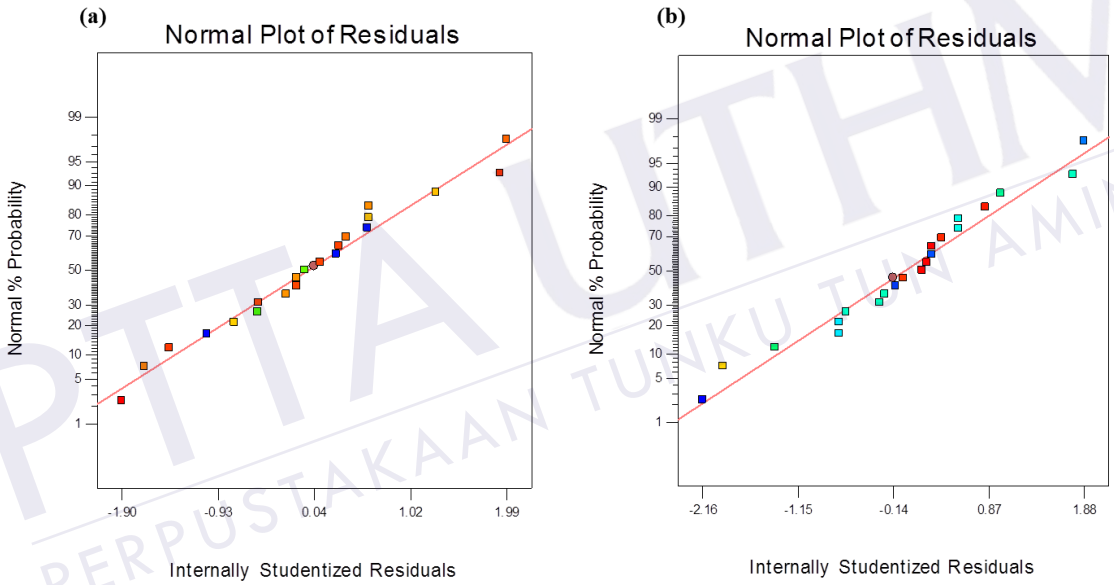


Figure 6.2: Design expert plot (domestic wastewater); normal probability plot of the internally standardised residual for harvesting efficiency using (a) alum and (b) chitosan.

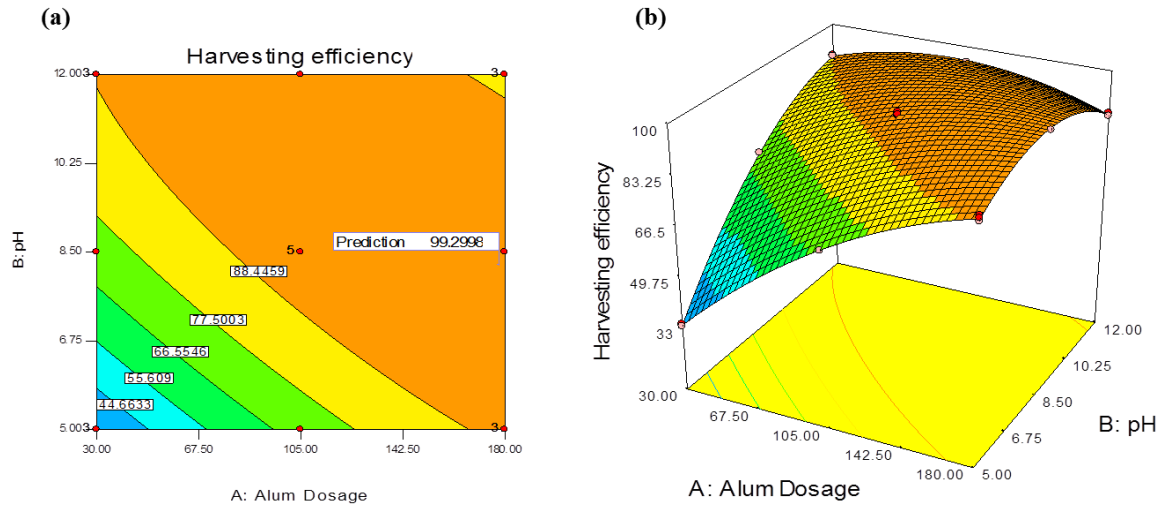


Figure 6.3: Design expert plot (domestic wastewater); (a) contour plot and (b) 3D response surface for harvesting efficiency using alum coagulant.

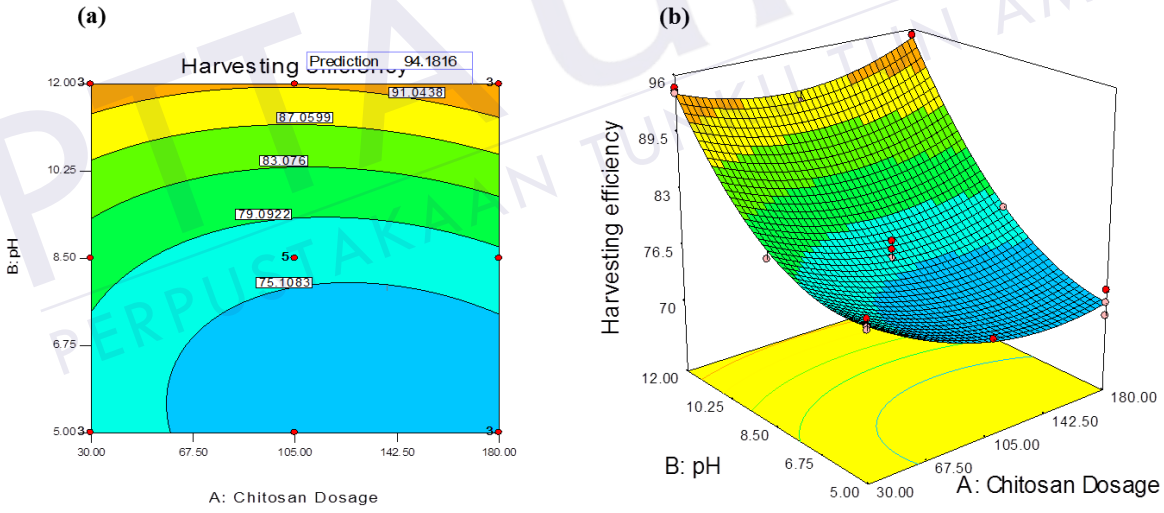


Figure 6.4: Design expert plot; (a) contour plot and (b) 3D response surface for harvesting efficiency using chitosan coagulant.

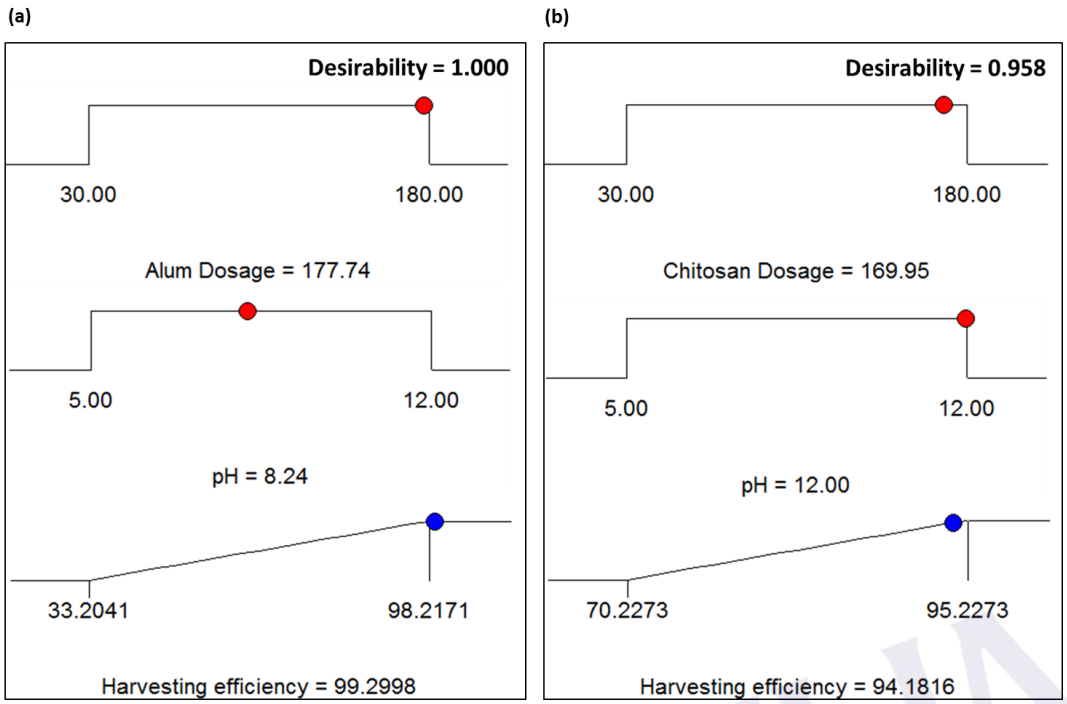


Figure 6.5: Ramps of the harvesting optimisation using (a) alum and (b) chitosan.

Table 6.5: Summary of the previous application study of RSM in microalgae harvesting.

Microalgae	Coagulant	pH	Dosage	Harvesting efficiency, %	References
Botryococcus braunii	Ferric chloride	n.a	0.79 mM	90.6	Kim *et al.* (2013)
Chlorella vulgaris	Bioflocculant (*Strychnos potatorum*)	n.a	100 mg/L	99.68	Razack *et al.* (2015)
Scenedesmus quadricauda	Sodium hydroxide	11.6	n.a	94.7	Huo *et al.* (2014)
Chaetoceros muelleri	Sodium hydroxide	11.5	n.a	100	Huo *et al.* (2014)
Microalgal- bacterial	Chitosan	n.a	214 mg/L	92	Riano *et al.* (2012)
Spirulina platensis	Chitosan	5.5	75 mL/L	98	El-mashad (2014)
Botryococcus sp.	Alum	8.24	177.74 mg/L	99.3	This study
Botryococcus sp.	Chitosan	12	169.95	94.2	This study

**n.a = Not applicable*

In the present study, alum as the coagulant shows a higher harvesting efficiency of 99.3% as compared to chitosan with an efficiency of 94.2%. Moreover, the result of this study shows that the dosage of alum and the pH level were two significant factors that influence harvesting efficiencies of microalgae biomass (Table 6.5). Previously, El-mashad (2014) used chitosan to harvest *Spirulina platensis* under

optimal conditions of pH (5.5) and dosage (75 mL/L) with 98% flocculation efficiency. The harvesting efficiencies obtained in this work is similar compared to other studies where both coagulants tested were able to harvest >90% of biomass. Therefore, it is suggested that the flocculation technique has a great potential harvesting due to the low-cost and fast method of biomass recovery.

6.3 Biomass production using food processing wastewater

6.3.1 Analysis of growth and biomass productivity

Botryococcus sp. was grown in FW under outdoor conditions with aeration using enclosed photobioreactors. The growth profile of *Botryococcus* sp. is shown in Figure 6.6. The growth was simulated with mathematical model predicted via Verhulst logistic formula (Ruiz *et al.*, 2013; Yang *et al.*, 2011). It was found that mathematical data was consistent with that of the experimental value during the experiments conducted.

Basically, the *Botryococcus* sp. cells were able to survive and quickly adapt to the conditions without any lag phase in FW. The *Botryococcus* sp. grew remarkably rapidly from the starting day of cultivation in the exponential phase. Until the fourth day, earlier exhaustion of nutrients gradually led to slower growth. On the following day, growth was assumed to be in the stationary phase up to the Day 12. Similarly, previous studies obtained the same curve when they used wastewaters to cultivate *Chlorella vulgaris*, *Platymonas subcordiformis* and *Botryococcus braunii* (Guo *et al.*, 2013; Raj GP *et al.*, 2015; Teles *et al.*, 2013). The lacking of lag phase in this study is because the inoculum was carefully prepared before the experiment as described by Teles *et al.* (2013). Additionally, exponentially growing cells in pre-cultures (microalgae stock) were used as suspension inoculum (Hempel *et al.*, 2012).

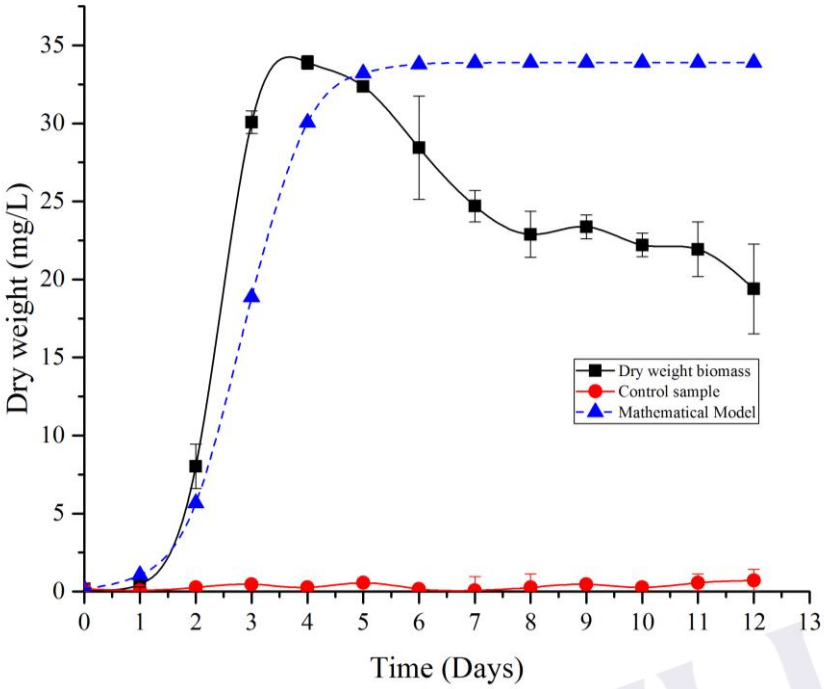


Figure 6.6: Daily growth measurement of *Botryococcus* sp. in food processing wastewater

Table 6.6: The maximum growth rate, division per day, doubling time, and biomass productivity of *Botryococcus* sp. grown in food processing wastewater

Maximum growth rate (day^{-1})	Division per day (Dd)	Doubling time (td)	Biomass productivity ($mg.L^{-1}.Day^{-1}$)
1.8304 ± 0.294	2.64 ± 0.424	0.38 ± 0.062	7.51 ± 0.276

From the exponential slope, the maximum growth rate was calculated accordingly, followed by the calculation of division per day, doubling time and biomass productivity (Table 6.6). The maximum growth rate of *Botryococcus* sp. in FW is 1.8304 day^{-1} with doubling time up to 0.38 day or 9.12 hours. This indicates that FW is a suitable source to grow *Botryococcus* sp. and simultaneously remove pollutants and nutrients effectively. This study's findings are quite difficult to compare with other studies as different wastewater was employed, in addition to variables of culture conditions and a variety of locations of study (Koreivienė *et al*., 2014; Malla *et al.,* 2015; Pathak *et al.*, 2015). Nevertheless, the present study proved that *Botryococcus* sp. was a notably useful candidate for high biomass productivity in FW. The volumetric biomass productivity of our research found that the

Botryococcus sp. was able to achieve up to 7.51 mg.L.$^{-1}$day^{-1} during the exponential phase of cultivation in FW.

6.3.2 Harvesting efficiency

Coagulant dosage and pH were selected in this study as an important variable which significantly influences the harvesting efficiency of microalgae in wastewater (Selesu *et al.,* 2016). These independent variables were categorised into three levels (+1, 0, -1). Experimental design was performed according to the face-centered central composite design (FCCCD) technique. The coded levels of actual factors, experimental design and results (actual and predicted) of microalgae harvesting in FW are stated in Table 6.7. The experiments were conducted in replicates of factorial points. Based on Table 3.8, FCCCD determines and suggests total standard runs up to 21, alpha face-centring, and five centre points.

The analysis of variance (ANOVA) for the response surface quadratic model in terms of harvesting efficiency for alum coagulant is shown in Table 6.8. The *F*-Test and ANOVA showed that the selected model was statistically significant at 95% of confidence level ($p<0.05$) with an *F*-value of 450.63 for alum coagulant. The R^2 of 0.9934 and adjusted R^2 of 0.9912 shows that the alum coagulant model could be used predicting the response and explaining 95% of the variability in this model. The most significant factors of harvesting efficiency using alum coagulant are A, B, and AB (Table 6.8). The model is significant if $p<0.05$ and not significant if $p>0.05$ (Razack *et al.,* 2015). This model also indicates the statistically non-significant lack of fit ($p>0.05$) with 28.1% chance, showing that the response is adequate for use in this alum coagulant model. The developed equation is a regression model for harvesting efficiency for microalgae cultivated in FW using alum coagulant as follows where A is alum dosage and B is pH:

$$\text{Harvesting efficiency}, \% = 11.49 + 0.20A + 5.38B - 9.92\times10^{-3}AB + 6.6\times10^{-5}A^2 + 8.19\times10^{-3}B^2 \quad (6.1)$$

Table 6.7: Actual factor and response values of *Botryococcus* sp. harvesting in food processing wastewater

Standard order	Actual factor values		Response values (Flocculation Efficiency, %)			
			Alum		Chitosan	
	pH(Coded)	Coagulant dosage, mg/L (Coded)	Actual (Experimental)	Predicted (RSM Model)	Actual (Experimental)	Predicted (RSM Model)
1	5 (-1)	30 (-1)	40.63	43.15	93.75	95.25
2	5 (-1)	30 (-1)	44.79	43.15	95.83	95.25
3	5 (-1)	30 (-1)	42.71	43.15	94.79	95.25
4	12 (-1)	30 (1)	66.67	67.67	89.58	89.63
5	12 (-1)	30 (1)	68.75	67.67	89.58	89.63
6	12 (-1)	30 (1)	67.71	67.67	89.58	89.63
7	5 (1)	180 (-1)	80.21	79.73	76.04	74.45
8	5 (1)	180 (-1)	79.17	79.73	72.92	74.45
9	5 (1)	180 (-1)	79.69	79.73	74.48	74.45
10	12 (1)	180 (1)	95.83	93.82	77.08	76.64
11	12 (1)	180 (1)	92.71	93.82	77.08	76.64
12	12 (1)	180 (1)	94.27	93.82	77.08	76.64
13	5 (0)	105 (-1)	62.79	61.34	90.63	89.32
14	12 (0)	105 (1)	79.17	80.64	86.42	87.61
15	8.5 (-1)	30 (0)	56.25	55.04	88.54	87.02
16	8.5 (1)	180 (0)	85.17	86.4	68.71	70.12
17	8.5 (0)	105 (0)	68.75	70.62	85.42	83.04
18	8.5 (0)	105 (0)	71.88	70.62	80.21	83.04
19	8.5 (0)	105 (0)	70.83	70.62	82.81	83.04
20	8.5 (0)	105 (0)	69.79	70.62	82.29	83.04
21	8.5 (0)	105 (0)	71.88	70.62	84.38	83.04

Table 6.8: ANOVA for the response surface quadratic model harvesting efficiency of *Botryococcus* sp. in food processing wastewater for alum coagulant.

Source	Sum of Squares	df	Mean square	*F* value	*p*-value
Model	4829.22	5	965.84	450.63	< 0.0001
A – Alum dosage	1304.2	1	1304.2	608.5	< 0.0001
B - pH	3442.75	1	3442.75	1606.29	< 0.0001
AB	81.38	1	81.38	37.97	< 0.0001
A^2	0.43	1	0.43	0.2	0.6595
B^2	0.032	1	0.032	0.015	0.9049
Residual	32.15	15	2.14		
Lack of fit	8.5	3	2.83	1.44	0.2808
Pure error	23.65	12	1.97		
Cor total	4861.37	20			
R^2 = 0.9934, Adj. R^2 = 0.9912, Pred. R^2 = 0.9895, Adeq precision = 64.746					

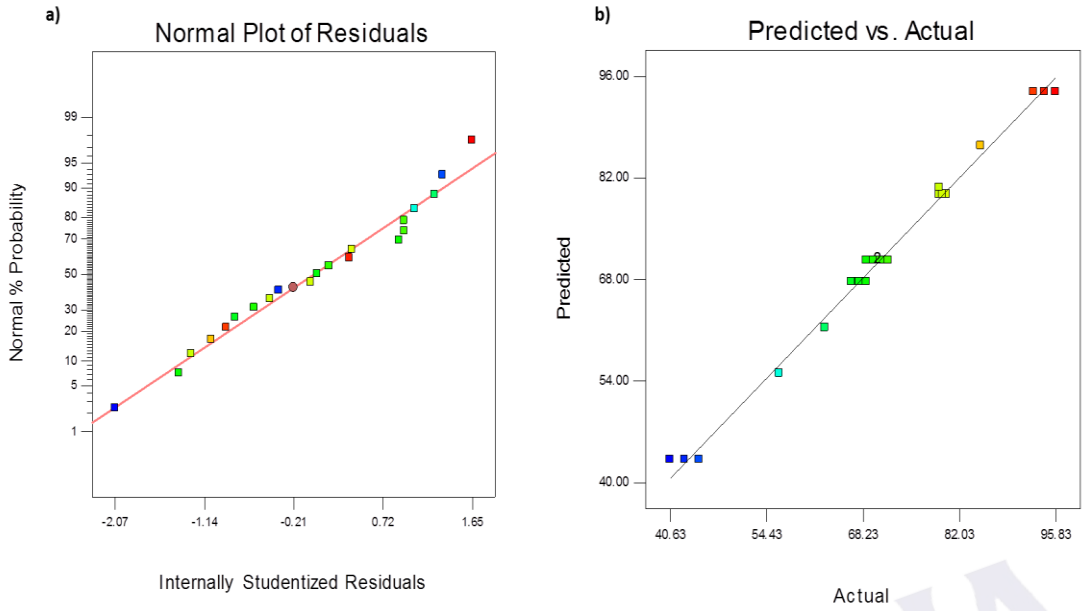


Figure 6.7: Design expert plot (food processing wastewater); a) normal probability plot and b) predicted vs actual value for flocculation efficiency using alum.

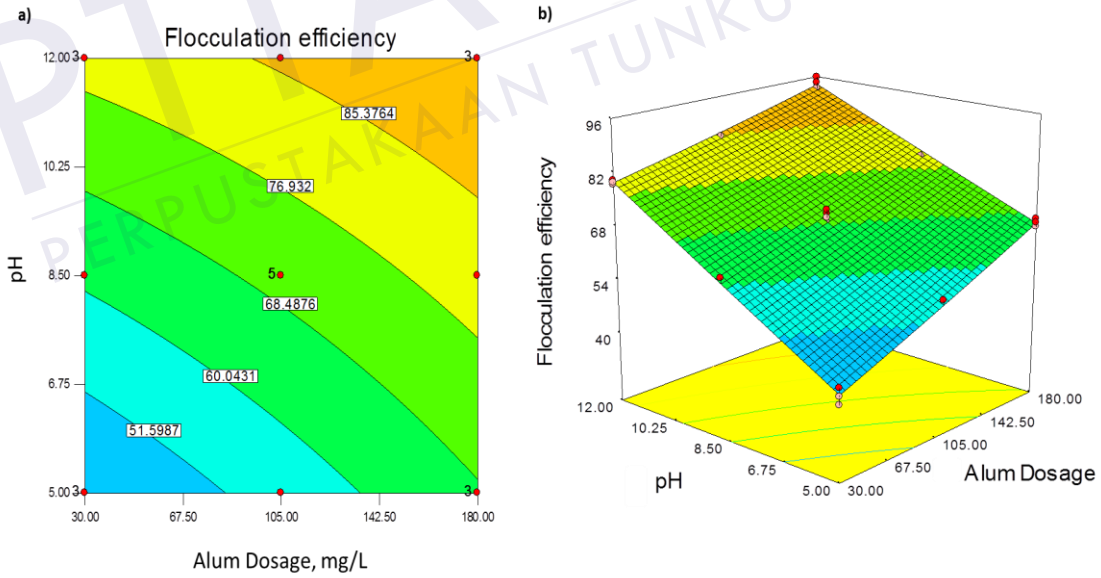


Figure 6.8: Design expert plot (food processing wastewater); a) contour plot and b) and 3D response surface for flocculation efficiency using alum.

Table 6.9: Comparison of FCCCD result with validation experiments in food processing wastewater.

Flocculant	Face centered central composite design (FCCCD) result			Validation experiments, %
	Dosage, mg/L	pH	Predicted flocculation efficiency, %	
Alum (Inorganic)	166	12	92.4	89.04
Chitosan (Organic)	30	5.54	94.9	92.17

The normal probability plot shows that the selected model for harvesting efficiency using alum coagulant is well fitted to the experimental data (Figure 6.7a). In fact, predicted vs actual plots show that the data is distributed uniformly along the straight line indicating a good agreement between actual and predicted values (Figure 6.7b). The contour plot and three-dimensional (3D) response surfaces were depicted for a better understanding of harvesting efficiency using alum coagulant (Figure 6.8). The 3D surface for alum demonstrated that the optimum point is located at the edge of the experimental region (Figure 6.8b). This figure reveals that by increasing the pH level and alum dosage, the harvesting efficiency was increasing accordingly. The optimum values of pH and alum dosage were 12 and 166 mg/L, respectively (Table 6.9). The maximum harvesting efficiency was 92.4%, with 0.945 desirabilities (Figure 6.11a). To confirm the optimum values obtained from statistical experimental strategies, three runs of confirmation experiments were conducted. As shown in Table 6.9, validation experiments (89.04%) were close to those estimated using RSM indicated that optimised conditions were reproducible.

The results of a quadratic model of harvesting efficiency using chitosan in the form of analysis of variance (ANOVA) are shown in Table 6.10. The developed second order polynomial equation in terms of actual factors was obtained where C is chitosan dosage and D is pH value:

$$\text{Harvesting efficiency}, \% = 95.65 - 0.28C + 7.44\times10^{-3}CD + 9.64\times10^{-4}C^2 - 0.37D^2 \quad (6.2)$$

Table 6.10: ANOVA for the response surface quadratic model harvesting efficiency of *Botryococcus* sp. in food processing wastewater for chitosan coagulant.

Source	Sum of Squares	df	Mean square	*F* value	*p*-value
Model	1156.48	5	231.3	109.17	< 0.0001
C – Chitosan dosage	10.32	1	10.32	4.87	0.0433
D - pH	999.14	1	999.14	471.58	< 0.0001
CD	45.78	1	45.78	21.61	0.0003
C^2	92.44	1	92.44	43.63	< 0.0001
D^2	62.87	1	62.87	29.67	< 0.0001
Residual	31.78	15	2.12		
Lack of fit	8.67	3	2.89	1.5	0.2645
Pure error	23.11	12	1.93		
Cor total	1188.26	20			
R^2 = 0.9733, Adj. R^2 = 0.9643, Pred. R^2 = 0.9499, Adeq precision = 32.300					

The function of this equation was to adequately present the interaction of factors influencing the harvesting efficiency of *Botryococcus* sp. in FW. The normal probability plot is provided by the Design Expert 7.0.0 software to ensure that the selected model provides an adequate approximation. It was found that the residual points follow a straight line, as stated in Figure 6.9a, meaning that the data obtained is considered normally distributed in the response of the model. Meanwhile, the observed harvesting efficiency values also vary between 68.71% and 95.83% which are in good agreement with the predicted values (Figure 6.9b). According to Table 6.10, the second order polynomial function indicates that the model is highly significant, as the *F*-Test is 109.17 with probability values of less than 0.0001. In this case, *C*, *D*, *CD*, C^2 and D^2 are presenting a significant model (Table 6.10). The goodness of the fit of the model obtained was observed in terms of R^2 value. Consequently, the value of R^2 for chitosan was 0.9733, which implies that this coagulant model is statistically significant and in reasonable agreement with the adjusted R^2 (0.9643). In addition, the adequate precision of this model was 32.3 which is greater than 4 and indicates an adequate signal to navigate the design space. The model also depicted the statistically non-significant lack of fit (p>0.05), indicating that the responses are adequate for use in this model.

A graphical interpretation such as 3D surface and contour plot is highly recommended and may be used to determine the interaction effect between the independent variables (pH, chitosan dosage) and harvesting efficiency of *Botryococcus* sp. (Figure 6.10). These plots allow us to establish the optimum value

of each variable studied for the maximum harvesting could be achieved. From this optimisation study, the optimal values of chitosan dosage and pH were found as 30 mg/L and 5.54, respectively (Figure 6.11b). The maximum harvesting efficiency of *Botryococcus* sp. using chitosan was estimated to be 94.4%. Validation of the model obtained was conducted by conducting another additional experiment in the optimised conditions. The mean observed value obtained was 92.17%, which was in good agreement with the predicted response (Table 6.9).

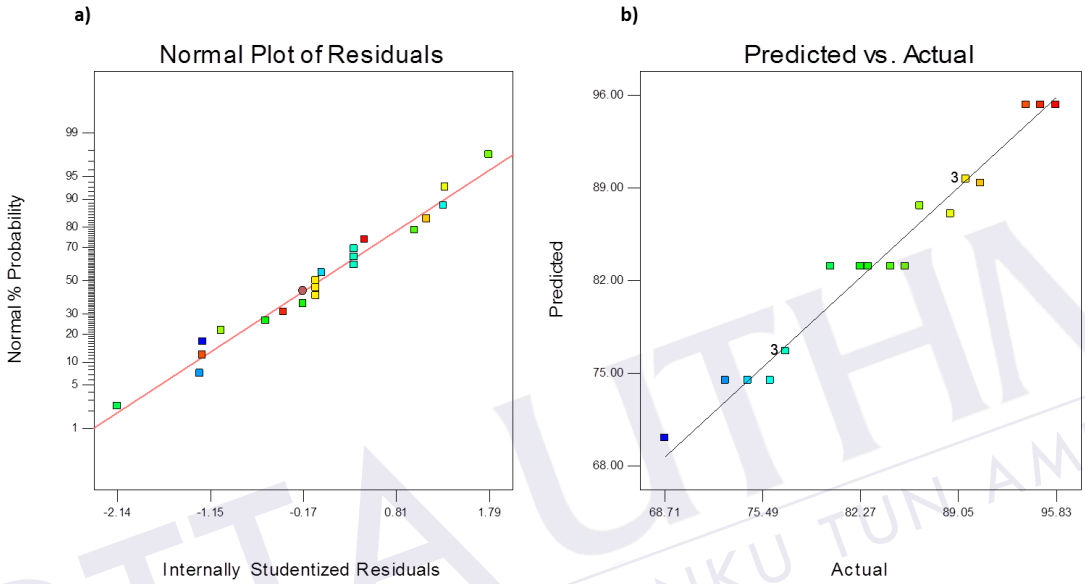


Figure 6.9 Design expert plot; a) normal probability plot and b) predicted vs actual value for flocculation efficiency using chitosan.

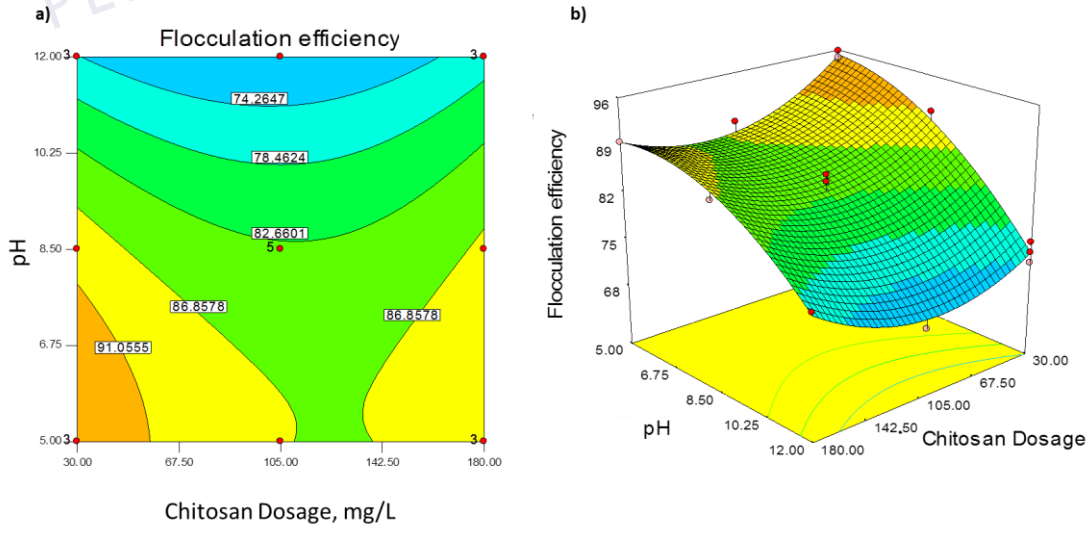
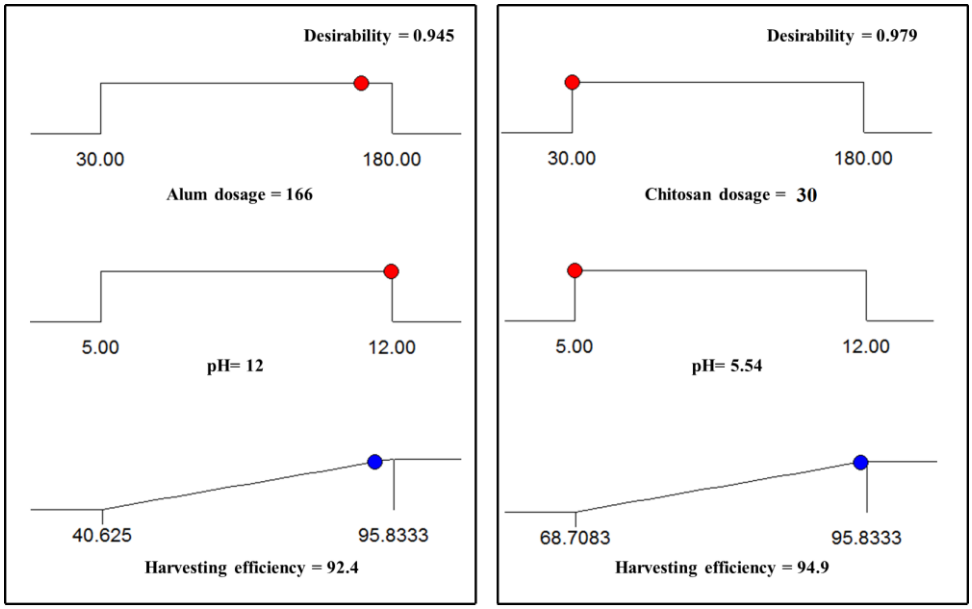


Figure 6.10 Design expert plot; a) contour plot and b) and 3D response surface for flocculation efficiency using chitosan.



(a) (b)

Figure 6.11: Ramps of the harvesting optimisation using (a) alum and (b) chitosan for FW.

Table 6.11: Comparison of present study findings with previous studies

Microalgae	Growth media	Coagulant	Dosage	pH	Harvesting efficiency, %	References
Chlorella vulgaris	Rudic medium	Chitosan	216 mg/L	7.23	94	Barekati-Goudarzi *et al.* (2016)
Spirulina platensis	Zarrouk medium	Chitosan	75 mL/L	5.5	98.7	El-mashad (2014)
Scenedesmus sp.	Swine wastewater	Tanfloc SG	210 mg/L	7.8	96.7	Selesu *et al.* (2016)
Chlorella sp.	Synthetic medium	Ferric ions ($FeCl_3$)	560 mg/L	-	>99	Kim *et al.* (2015)
Botryococcus sp.	Food processing wastewater	Alum	166 mg/L	12	92.4	Current study
Botryococcus sp.	Food processing wastewater	Chitosan	30 mg/L	5.54	94.9	Current study

The present study significantly enhances our understanding of the harvesting efficiency of microalgae based on their strain, culture media, pH level, dosage and type of coagulant. Therefore, Table 6.11 presents the harvesting efficiencies of microalgae from the culture medium using different types of coagulant. In this study, chitosan coagulant prefers to harvest *Botryococcus* sp. grown in FW with acidic pH

(5.54) compared to alum coagulant with alkaline pH (12) condition. In terms of dosage, chitosan also indicates a lower concentration compared to alum coagulant but both of them still lower than ferric ions (560 mg/L) as studied by Kim *et al*. (2015) when they recovered *Chlorella* sp. from synthetic medium and harvested the biomass up to 99%. The most obvious finding to emerge from this study is the type of coagulant used and pH sensitivity of the culture in microalgae harvesting. However, the selection of appropriate coagulant mostly depends on the desired product to be produced from microalgae. For instance, if the purpose of coagulation is the production of food feedstock, then an inorganic coagulant (e.g. chitosan) is appropriate. If the purpose is the production of biofuel (e.g. hydrocarbon), then selection of the fastest and cheapest coagulant is appropriate, namely alum coagulant (Papazi, Makridis, & Divanach, 2010; Selesu *et al.,* 2016).

6.4 Summary

The most obvious finding to emerge from this study is that the application of both DW and FW for microalgae cultivation was successful. It was shown that photobioreactor used in this study effectively to produce valuable biomass in massive quantity under outdoor culture condition. In fact, both inorganic (alum) and organic (chitosan) coagulants was able to harvest microalgae, *Botryococcus* sp. from both wastewater significantly more than 90% of efficiency. The inorganic (alum) coagulant was an option for harvesting the biomass cultivated in both wastewaters due the selection of the appropriate coagulant is directly correlated to the desired product to be produced. For instance, if the purpose is the production of biodiesel and hydrocarbon, then efficiency and economy are important, which means that selection of the fastest and cheapest coagulant is appropriate, that is an alum. Meanwhile, the usage of chitosan is more important in bio-fertilizer, agro feedstocks and medical industry. Next chapter would discuss the hydrocarbon content in microalgae oil obtained from *Botryococcus* sp. biomass cultivated in DW and FW.

CHAPTER 7

HYDROCARBON PRODUCTION FROM MICROALGAE OIL

7.1 Introduction

This chapter discusses the results analysis of microalgae oil extraction and hydrocarbon composition presence in *Botryococcus* sp. cultivated in different culture media (domestic wastewater – DW) and (food processing wastewater – FW). The microalgae oil extracted was conducted using soxhlet apparatus via solvent extraction and the result obtained was discussed as in section 7.2. Meanwhile, the result of hydrocarbon composition from algae oil extraction is analysed using FT-IR and GC-MS machine as discussed in section 7.3.1 and 7.3.2, respectively. Each hydrocarbon compound obtained from the GC-MS analysis was discussed in term of advantages and potentially applied as high and chemical value added in a related industry.

7.2 Microalgae oil extraction

The productions of oil from *Botryococcus* sp. in two different culture media were calculated as shown in Figure 7.1. Each culture media was extracted in triplicate and written as means of data provided in **APPENDIX F**. The calculation of oil content recovery based on dry weight biomass and expressed as a percentage. The results indicated that the oil extraction from DW culture media was higher than FW culture media (Figure 7.1). Accordingly, oil content could be obtained from *Botryococcus* sp. biomass cultivated in DW and FW culture media was up to 70.7% and 53.1%,

respectively (refer to **APPENDIX F**). These results show that the percentage of bio-oil recovery is in line with the statement as reported by Chisti (2007).

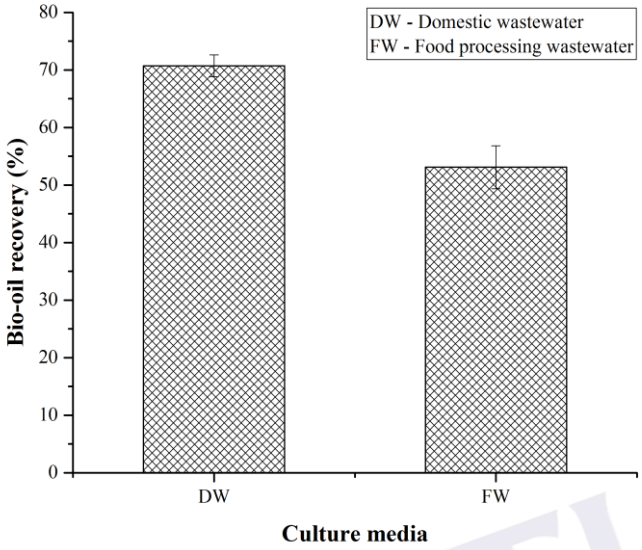


Figure 7.1: The percentage of bio-oil recovery of *Botryococcus* sp. growth in two difference culture media

The derivation of oil from plants especially microalgae is a potential renewable energy to the conventional source to overcome the standard biodiesel from a crop which cannot realistically satisfy for global transportation demand (Chisti, 2007). In this study, the differences in oil content of *Botryococcus* sp. between different culture media as treatment mainly resulted from the difference in their biomass, since the deviations in the bio-oil content of *Botryococcus* sp. within different culture media were not large in contrast. These results are supported by Zhu *et al*. (2013) studies where they examined green microalgae (*Chlorella zofingiensis*) integrated with piggery wastewater for the nutrient removal and biodiesel production. Thus, the result proved that the solvent extraction using soxhlet apparatus was successful and can be applied to this experimental. Lastly, analysis of algal oil in term of hydrocarbon composition would be discussed in the next section.

7.3 Hydrocarbon oil analysis

There are two type of hydrocarbon analysis namely Fourier transform infrared spectroscopy (FT-IR) and Gas chromatography–mass spectrometry (GC-MS). Both

analyses are a common test to analyse the presence of hydrocarbon compound in microalgae bio-oil.

7.3.1 FT-IR analysis

The crude oil extracted from microalgae biomass potentially used as a feedstock for biodiesel production (Maity *et al.*, 2014). The hydrocarbons are the main elements of petrodiesel and similar to the biodiesel properties obtained from microalgae oil (Banerjee *et al.*, 2002). The FT-IR spectra of bio-oil extracted from *Botryococcus* sp. biomass cultivated in different wastewaters (DW and FW) compared to palm oil-based biodiesel (as a guideline) are shown in Figure 7.2 and 7.3. The Figure 7.2 presents the FT-IR spectra of a triplicate sample (Sample A, Sample B and Sample C) of each culture medium and indicating that the bands of each sample almost similar in their pattern then producing similar wavenumber (cm^{-1}). Therefore, the bands obtained from crude oil in each culture medium were averaged and compared with the standard as illustrated in Figure 7.3. The possible functional group composition assignments are listed in Table 7.1.

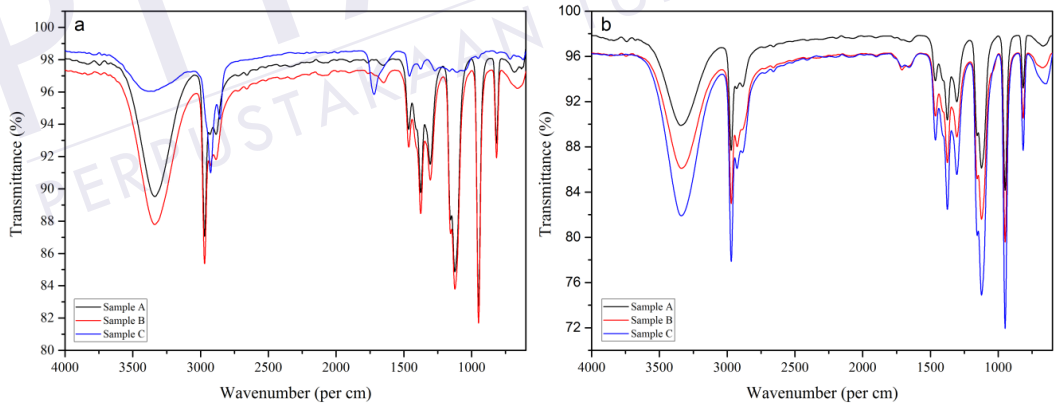


Figure 7.2: The IR-Spectra of microalgae oil cultivated in (a) domestic wastewater and (b) food processing wastewater.

The strong and broad absorbance peak of O-H stretching was obtained in culture media of DW and FW at 3241.71 cm^{-1} and 3339.45 cm^{-1}, respectively. The presence of this peak normally caused by water or alcohol in the bio-oil (Zou *et al.*, 2010). The absorption peaks are almost the same for all three oil sample (Figure 7.2) where the C-H stretching absorption occurs at the wavenumber between 2850 cm^{-1}

and 3000 cm^{-1}. These peaks appear strong in all oil sample tested due to the presence of alkanes and alkyls (Table 7.1).

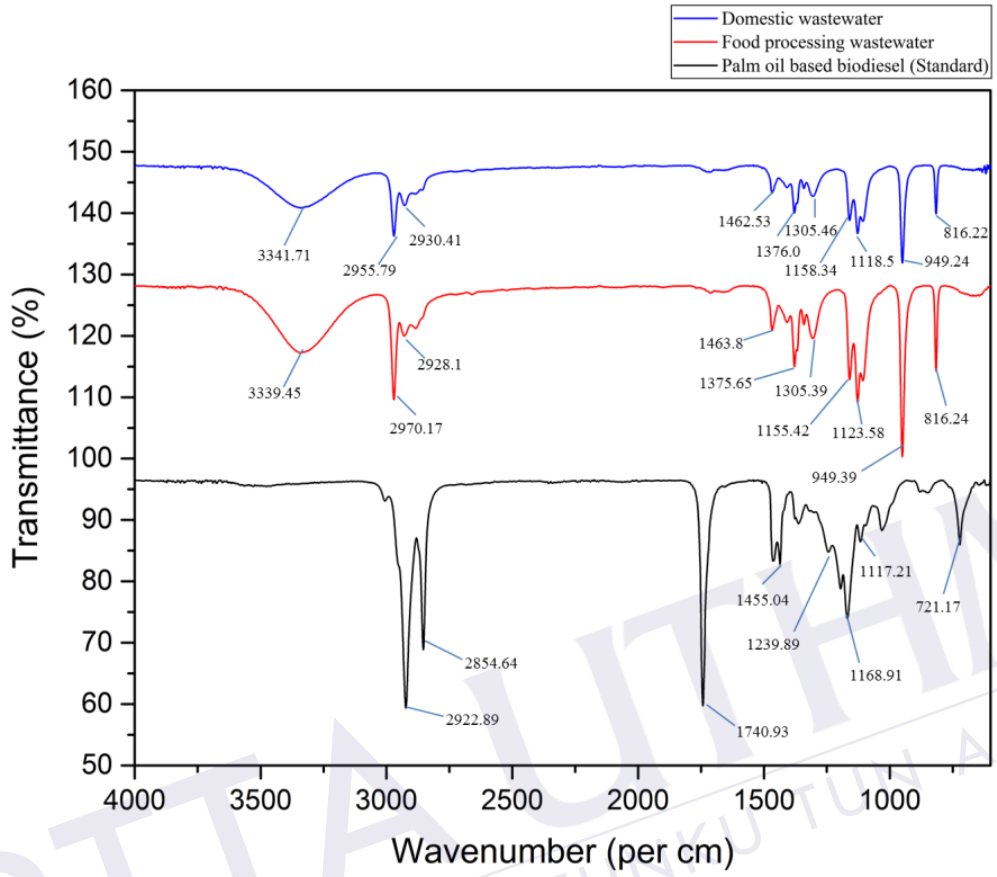


Figure 7.3: The IR-Spectra of microalgae oil compared to palm oil-based biodiesel.

The bands at 1462.53 cm^{-1} for DW, 1463.8 cm^{-1} and 1455.04 cm^{-1} for the standard are due to the strong CH_2 bending vibration in the algae oil and classified as alkanes in the form of methylene compound. However, alkanes in the form of methyl only exist in algae oil cultivated in both wastewaters because of the medium bending of CH_3. The ethers compound only presence in standard oil with medium strong stretching =C-O-C symmetric and asymmetric at the band of 1239.89 cm^{-1}. In the range of 1085 cm^{-1} to 1150 cm^{-1}, all oil sample (Figure 7.3) tested contain ether, epoxides, acetals and ketals due to the strong C-O-C stretching vibration. The FT-IR analysis falls mainly in the very related to the hydrocarbon categories such as fatty acid, fatty acid methyl esters, ketones and aldehydes (Table 7.1 and Figure 7.3).

Similarly, Shuping *et al.* (2010) reported the same outcome when they examined bio-oil obtained from *Dunaliella tertiolecta*. This may because of the microalgae biomass composed of proteins, fat and raw cellulose with the total crude

protein and crude fate extracted using an organic solvent (Shuping *et al*., 2010). Interestingly, it clearly indicates that the spectrum of DW and FW almost in line with palm oil based biodiesel spectrum (Figure 7.3).

Table 7.1: The summary of IR-Spectra of FT-IR analysis.

Absorption range (cm^{-1})	Absorption (cm^{-1})			Group	Intensity	Class of compound
	DW	FW	Standard			
2500-3500	3341.71	3339.45	N/A	O-H Stretching	Strong, broad	Alcohols, phenols, water impurities
2850-3000	2955.79, 2930.41	2970.17, 2928.1	2922.89, 2854.64	C-H Stretching	Strong	Alkanes and alkyls
1735-1750	N/A	N/A	1740.93	C=O Stretching	Very strong	Esters (Aliphatic)
1450-1470	1462.53	1463.8	1455.04	C-H Bending	Strong	Alkanes (Methylene)
1370-1390	1376.00	1375.65	N/A	CH_3 C-H Bending	Medium	Alkanes (Methyl)
1000-1350	1305.46	1305.39	N/A	C-F Stretching	Very strong	Alkyl halides
1200-1275	N/A	N/A	1239.89	=C-O-C sym. & asym. Stretching	Medium-strong	Ethers
1125-1205	1158.34	1155.42	1168.91	C-O Stretching	Medium-strong	Alcohols, phenols
1085-1150	1118.50	1123.58	1117.21	C-O-C stretching	Strong	Ethers, epoxides, acetals, ketals
790-840	816.22	816.24	N/A	=C-H Bending	Strong	Alkenes
665-730	N/A	N/A	721.17	=C-H Bending	Medium-strong, broad	

*N/A = Not available

Therefore, triglycerides and phospholipids do exist in the spectrum of *Botryococcus* sp. which similar to the spectrum of methyl ester in petrodiesel spectra (Santhoshkumar *et al*, 2015). These results are in line with previous past studies that most of microalgae oil containing chemical compound such as alkane and alkyl, esters, ethers and alcohol (Mahapatra & Ramachandra, 2013; Maity *et al.*, 2014; Prabakaran & Ravindran, 2012; Yin *et al.*, 2011; Zou *et al.*, 2010).

7.3.2 GC-MS analysis

In this study, triplicate samples of different culture media were discussed separately. Moreover, all of them were compared to the analysis of palm oil-based biodiesel as a guideline. The identified peaks and compounds in the palm oil based biodiesel (as a guideline) are shown in Figure 7.4 and Table 7.2, respectively.

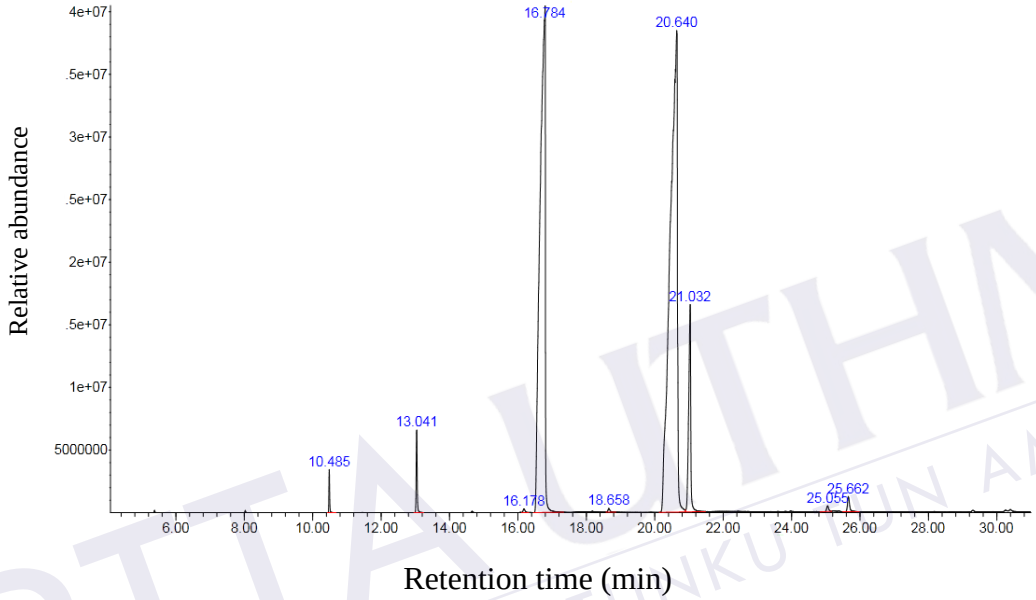


Figure 7.4: GC-MS analysis for palm oil-based biodiesel

Table 7.2: Major compounds in the palm oil based biodiesel

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	10.487	Undecanoic acid, 10-methyl-, methyl ester	$C_{13}H_{26}O_2$	214.3443	0.4654
2	13.040	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242.3975	1.2221
3	16.180	11-Hexadecenoic acid, methyl ester	$C_{17}H_{32}O_2$	268.4348	0.1182
4	16.783	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	39.6823
5	18.660	Hexadecanoic acid, 15-methyl-, methyl ester	$C_{18}H_{36}O_2$	284.4772	0.1109
6	20.641	9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	296.4879	51.3649
7	21.034	Octadecanoic acid, methyl ester	$C_{19}H_{34}O_2$	294.4721	6.3903
8	25.055	11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	324.5411	0.1609
9	25.663	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326.5570	0.485
Total					100

Roughly, the peaks obtained clearly indicate the characteristic of palm oil biodiesel since it contains hydrocarbon compound classified as fatty acid methyl ester (FAME). The carbon numbers of each compound detected were ranged from C12 to C20 which is similar to important properties of conventional biodiesel (C16 – C18). This was confirmed with FT-IR result as discussed in past study (Mumtaz *et al.*, 2014).

7.3.2.1 Domestic wastewater media

The identified peaks and compounds in the sample A of domestic wastewater (DW) culture media are shown in Figure 7.5 and Table 7.3, respectively. The pure hydrocarbons were observed at the peaks no. 1, 2, 4 and 14 with a peak area of 1.98%, 2.94%, 2.52% and 22.14%, respectively. The name of the hydrocarbon compound as follow: 2-Dodecene, (Z)-, 1-Octadecene, 1-Hexadecene and 2, 3-Dihydroxypropyl elaidate. The remain peaks and compound identified as hydrocarbon derivative since they attached with another atom such as oxygen (O), silicon (Si), bromine (Br) and nitrogen (N). Most of them classified as fatty acid methyl ester (FAME) which almost similar to the biodiesel (standard). The peak at no. 16 showed the highest peak area of 31.5%. This largest peak is due to the presence of tetracosanoic acid, methyl ester ($C_{25}H_{50}O_2$) with 382.66 g/mol of molecular weight. Next, the peak at no. 14 had a peak area about 22.14%, indicating the presence of 2, 3-Dihydroxypropyl elaidate ($C_6H_{12}N_2$).

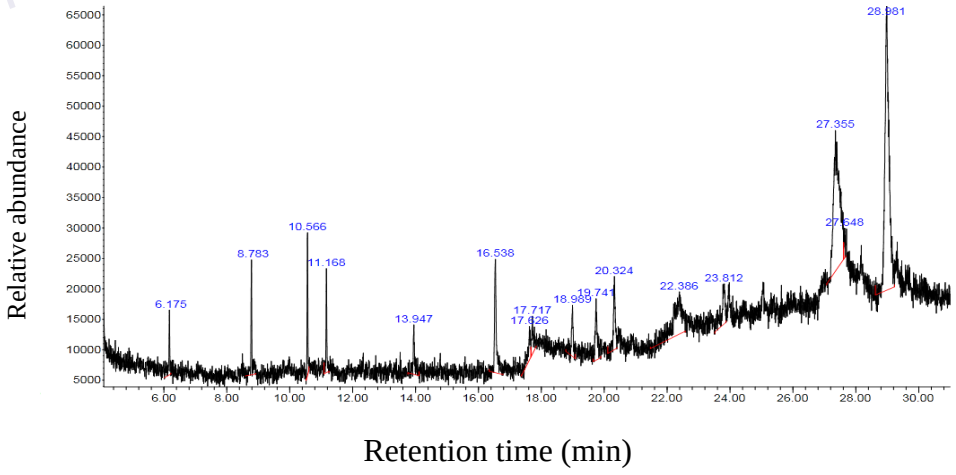


Figure 7.5: GC-MS analysis for microalgae oil biomass cultivated in domestic wastewater (Sample A)

Meanwhile, the third less significant peak at no. 12 with a peak area up to 9.4% showed the characteristic of Tetradecanoic acid, 12-methyl-, methyl ester ($C_{16}H_{32}O_2$). According to the three largest peak identified in this sample A of DW culture media may be potentially significant to be used as a chemical value added to biofuel industry and other biotechnology application.

Table 7.3: Major compounds in microalgae oil biomass cultivated in domestic wastewater (Sample A)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	6.1733	2-Dodecene, (Z)-	$C_{12}H_{24}$	168.3190	1.9782
2	8.7838	1-Octadecene	$C_{18}H_{36}$	252.4784	2.9386
3	10.5661	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	278.5050	2.9419
4	11.1689	1-Hexadecene	$C_{16}H_{32}$	224.4253	2.5225
5	13.9472	Bromoacetic acid, hexadecyl ester	$C_{18}H_{35}BrO_2$	363.373	2.2105
6	16.5367	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.4507	5.999
7	17.627	Benzene, 1-isothiocyanato-2-methyl-	C_8H_7NS	149.213	2.223
8	17.7161	Carbonic acid, hexadecyl 2,2,2-trichloroethyl ester	$C_{18}H_{36}O_3$	300.4766	1.7118
9	18.99	Benzimidazol-2(3H)-one, 1-(1-methylethenyl)-4-nitro-	$C_{10}H_9N_3O_3$	219.1968	2.5585
10	19.7396	3,4-Dimethyl-isoxazol-5(4H)-one	$C_5H_7NO_2$	113.1146	3.6741
11	20.3267	8-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	296.4879	3.5004
12	22.3867	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	256.4241	9.4023
13	23.8126	2(1H)-Naphthalenone, octahydro-4a,7,7-trimethyl-, trans-	$C_{13}H_{22}O$	194.3132	3.8146
14	27.3561	2,3-Dihydroxypropyl elaidate	$C_{21}H_{40}$	356.540	22.1364
15	27.6497	1H-Imidazole, 2-ethyl-4,5-dihydro-	$C_6H_{12}N_2$	112.17	0.8883
16	28.9812	Tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	382.6633	31.4999
Total					100

The identified peaks and hydrocarbon compounds in sample B of DW culture media are stated in Figure 7.6 and Table 7.4, respectively. The bio-oil of *Botryococcus* sp. in this sample contains several of chemical compounds in which dominated by pure hydrocarbon. The only peak at the retention time of 10.56 observed as hydrocarbon derivative since containing oxygen and silicon with 28.38% of peak area. This peak was named as phenol, 2,4-bis(1,1-dimethylethyl)-

($C_{17}H_{30}OSi$) contributing to the largest peak area amongst other peak detected in this sample (sample B).

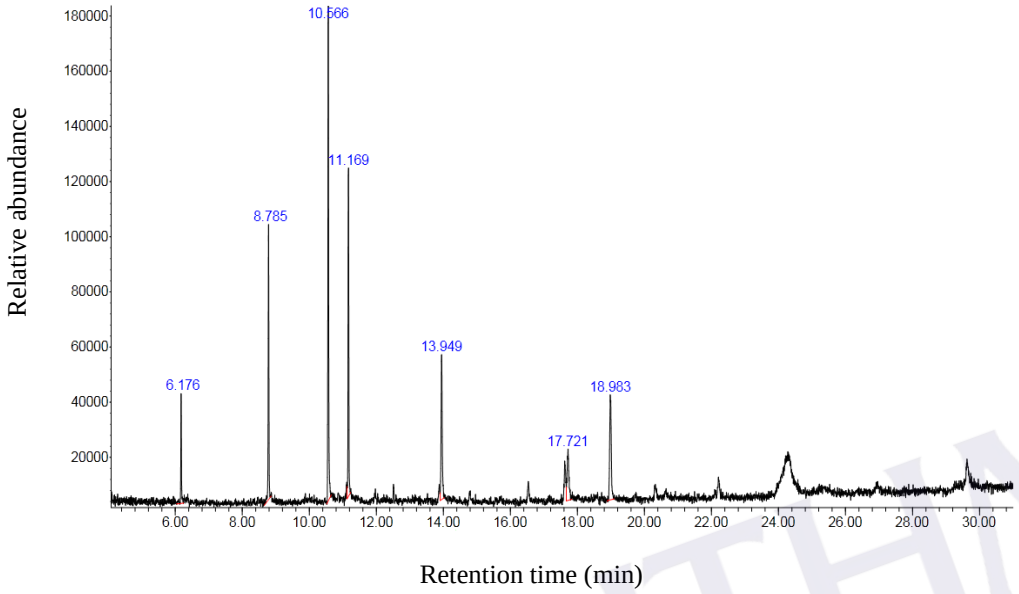


Figure 7.6: GC-MS analysis for microalgae oil biomass cultivated in domestic wastewater (Sample B)

Table 7.4: Major compounds in microalgae oil biomass cultivated in domestic wastewater (Sample B)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	6.1733	Cyclododecane	$C_{12}H_{24}$	168.319	5.6264
2	8.7838	1-Tetradecene	$C_{14}H_{28}$	196.3721	14.861
3	10.5661	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	278.5050	28.3817
4	11.1689	7-Hexadecene, (Z)-	$C_{16}H_{32}$	224.4253	18.7345
5	13.9472	1-Octadecene	$C_{18}H_{36}$	252.4784	12.8296
6	17.7214	1-Nonadecene	$C_{19}H_{38}$	266.5050	7.0743
7	18.9847	1,4-Naphthoquinone, 3-methyl-2-[(4-isopropenyl-1-cyclohexenyl)formyl]-	$C_{21}H_{20}$	320.382	12.4925
Total					100

The second largest peak is due to the existence of 7-Hexadecene, (Z)- ($C_{16}H_{32}$) with 18.73% of peak area and listed as peak no. 4 (Table 7.4). The presence of 1-Tetradecene ($C_{14}H_{28}$) at the peak no. 2 was the third less significant peak area of 14.86% by carrying about 196.37 g/mol of molecular weight. Meanwhile, the

retention time at 6.17, 13.95, 17.72 and 18.98 showed the compounds of Cyclododecane ($C_{12}H_{24}$), 1-Octadecene ($C_{18}H_{36}$), 1-Nonadecene ($C_{19}H_{38}$), and 1,4-Naphthoquinone, 3-methyl-2-[(4-isopropenyl-1-cyclohexenyl)formyl]- ($C_{21}H_{20}$), respectively (Table 7.4).

The active hydrocarbon compound identified in the *Botryococcus* sp. oil of sample C for DW culture media by GC-MS analysis was presented and tabulated in Figure 7.7 and Table 7.5, respectively. Totally, eight compounds have been detected through GC-MS analysis based on retention time, chemical formula, molecular weight and peak area. The result revealed that the presence of 3-Dodecene, (E)- ($C_{12}H_{24}$), 3-Tetradecene, (E)- ($C_{14}H_{28}$), Phenol, 2,5-bis(1,1-dimethylethyl)- ($C_{14}H_{22}O$), Cyclohexadecane ($C_{16}H_{32}$), 9-Octadecene, (E)- ($C_{18}H_{36}$), 7-Pentadecyne ($C_{15}H_{28}$), Curan, 1-acetyl-16,17,19,20-tetradehydro- ($C_{21}H_{24}$) and Phytol ($C_{20}H_{40}O$). The total ion chromatogram profile of GC-MS confirmed the existence of these compounds with retention time 6.17 (4.01%), 8.78 (10.02%), 10.57 (29.56%), 11.17 (14.03%), 13.95 (11.45%), 14.79 (7.87%), 18.99 (18.51%) and 20.64 (4.54%), respectively.

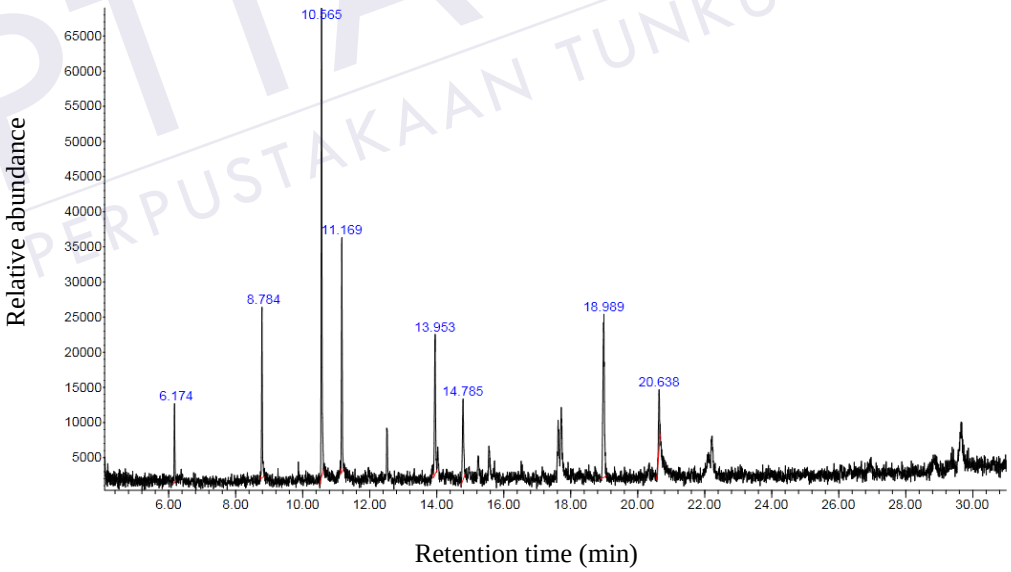


Figure 7.7: GC-MS analysis for microalgae oil biomass cultivated in domestic wastewater (Sample C)

The full individual fraction of the hydrocarbon compound is illustrated in Figure 7.6. This finding highlights where the major hydrocarbon compound was

Phenol, 2,5-bis(1,1-dimethylethyl)- with 29.57% of peak area followed by Curan, 1-acetyl-16,17,19,20-tetradehydro- with 18.51% of peak area and Cyclohexadecane with 14.03% of peak area. Basically, sample C of DW culture media was containing six of pure hydrocarbon indicated as peak no. 1, 2, 4, 5, 6 and 7 while at the peak of 3 and 8 noted as hydrocarbon derivative. However, most of them classified as fatty acid methyl ester since having carbon number ranged from C16 to C18 which is common fatty acids presence in biodiesel (Mahapatra & Ramachandra, 2013).

Table 7.5: Major compounds in microalgae oil biomass cultivated in domestic wastewater (Sample C)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	6.1732	3-Dodecene, (E)-	$C_{12}H_{24}$	168.3190	4.0108
2	8.7837	3-Tetradecene, (E)-	$C_{14}H_{28}$	196.3721	10.0237
3	10.566	Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.3239	29.5653
4	11.1688	Cyclohexadecane	$C_{16}H_{32}$	224.4253	14.028
5	13.9523	9-Octadecene, (E)-	$C_{18}H_{36}$	252.4784	11.4537
6	14.7858	7-Pentadecyne	$C_{15}H_{28}$	208.383	7.8726
7	18.9898	Curan, 1-acetyl-16,17,19,20-tetradehydro-	$C_{21}H_{24}$	320.428	18.5059
8	20.6358	Phytol	$C_{20}H_{40}O$	296.5310	4.54
Total					100

Obviously, this study has shown that different replicate produce significant different of hydrocarbon compound even they are from the same culture media sample (DW). This phenomenon may be due to the amount of chlorophyll since the growth observed of each replicate was different. A lot of hydrocarbon compound was detected in sample A compared to sample B and C probably because of *Botryococcus* sp. biomass in DW culture media was higher in sample A. Nevertheless, all of replicate sample in DW culture media was still providing the compound of hydrocarbon classified as fatty acid methyl ester which is suitable for biofuel production. Not only that, this compound may be potentially used as chemical value added in other industry such as plastic manufacturing, medical field, pharmaceutical, cosmetic and many more. For instance, major compound in sample A (tetracosanoic acid, methyl ester) is a fatty acid (C24:0) commonly identify in other oil plant extraction for biofuel production (Tomaz *et al.*, 2012). Other hydrocarbon compound

in sample A DW culture media namely tetradecanoic acid is suitable for the activity such as antioxidant, cancer preventive, cosmetic, hyperchloresterolemic and nematicide (Devi & Muthu, 2014). Meanwhile, Phenol, 2,4-bis(1,1-dimethylethyl)- in sample B of DW culture media usually subjected for evaluation of antioxidant activity (Kumar *et al.,* 2015; Nishaa *et al.,* 2013). In another study, Younis *et al.* (2014) reported that phenol was a contaminant present in petroleum refinery wastewater and need to be removed before discharging the clean water to the environment. This is because phenol is highly irritating to the skin, eyes, and mucous membranes in humans after acute inhalation or dermal exposures. Normally, excessive exposure to phenol may occur from the use of some medicinal products such as throat lozenges and ointments (U.S. EPA). The sample C of DW culture media was consisting the same compound as sample B as major compound (Phenol, 2,4-bis(1,1-dimethylethyl)-). Although hydrocarbon compound found at the peak no. 8 in sample C of DW culture media called as phytol less prominent in peak area about 4.54%, but the presence of it is very useful in the medical field such as for antimicrobial, anticancer, diuretic and anti-inflammatory (Sermakkani & Thangapandian, 2012). Other compounds in sample C of DW culture media are undetectable of the possible usage. However, most of them consist of pure hydrocarbon except hydrocarbon compound at the peak no. 3 and 8.

Overall, the result of this outcome shows that microalgae *Botryococcus* sp. oil cultivated in DW culture media potentially applied for biofuel production besides useful for other activities particularly in medical works and pharmaceutical industry.

7.3.2.2 Food processing wastewater media

The qualitative analysis of hydrocarbon compound identified in sample A of FW culture media by GC-MS was illustrated in Figure 7.8. The detected compound with their retention time, chemical formula, molecular weight and peak area are presented in Table 7.6. The major hydrocarbon compounds presence in sample A were Hexadecanoic acid, methyl ester (40.99%), 14-Octadecenoic acid, methyl ester (29.09%) and Phenol, 2,4-bis(1,1-dimethylethyl)- (5.81%). The Table 7.6 profile of GC-MS proved the presence of these three compounds with retention time of 16.54, 20.33 and 10.56, respectively. The sample A individual fragmentation of the hydrocarbon component is stated in Table 7.6. Accordingly, the sample was

dominated by the pure hydrocarbon with six out of eleven compounds are consisting only carbon and hydrogen atom. Interestingly, hydrocarbon derivative in this sample known as fatty acid methyl ester with lipid number between C16 and C18 in which in line with previous study by Mahapatra & Ramachandra (2013).

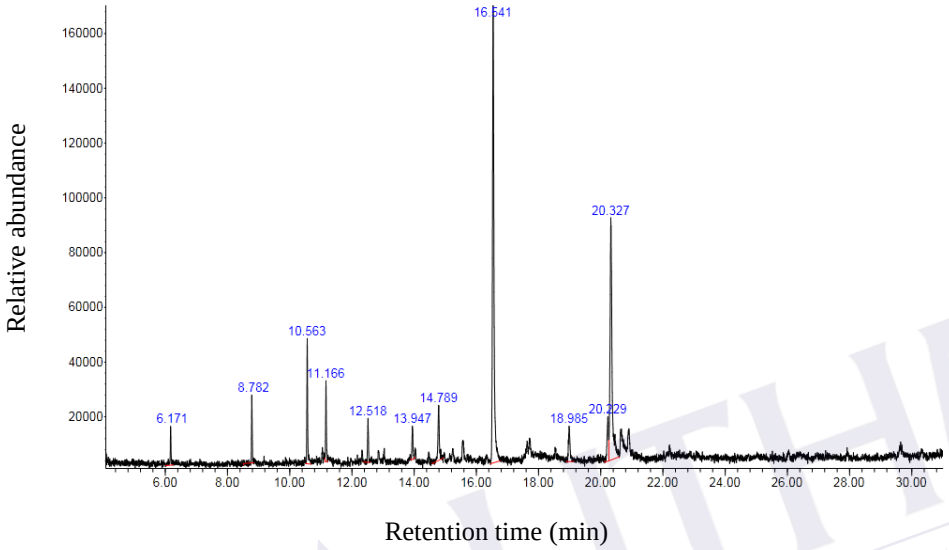


Figure 7.8: GC-MS analysis for microalgae oil biomass cultivated in food processing wastewater (Sample A)

Table 7.6: Major compounds in microalgae oil biomass cultivated in food processing wastewater (Sample A)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	6.1732	Cyclopropane, 1-ethyl-2-heptyl-	$C_{12}H_{24}$	168.319	2.1443
2	8.7837	1-Tridecene	$C_{13}H_{26}$	182.3455	2.5045
3	10.5607	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	278.5050	5.8078
4	11.1688	Cyclotetradecane	$C_{14}H_{28}$	196.3721	3.4537
5	12.516	Heneicosane	$C_{21}H_{44}$	296.5741	2.2619
6	13.9471	1-Octadecanethiol	$C_{18}H_{38}S$	286.559	2.024
7	14.791	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	$C_{10}H_{18}$	138.2499	4.6374
8	16.5418	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.4507	40.9914
9	18.9846	Curan, 1-acetyl-16,17,19,20-tetradehydro-	$C_{21}H_{24}$	320.428	3.3955
10	20.2269	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.4721	3.6852
11	20.3265	14-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.4879	29.0944
Total					100

Meanwhile, for sample B of FW culture media, Figure 7.9 presents the total ion chromatogram of the analyzed sample. In this figure, hundreds of peaks were displayed in the GC-MS analysis implying the complex hydrocarbon composition of algae oil. However, only those separated major component that were in considerable amount were semi-quantitavely evaluated based on peak area of selected ion chromatogram (Zou *et al.*, 2010).

Table 7.7: Major compounds in microalgae oil biomass cultivated in food processing wastewater (Sample B)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	6.1734	1-Dodecene	$C_{12}H_{24}$	168.3190	2.1644
2	8.7838	5-Tetradecene, (E)-	$C_{14}H_{28}$	196.3721	6.8294
3	10.5661	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	278.5050	19.674
4	11.0589	Tridecanal	$C_{13}H_{26}O$	198.3449	0.8722
5	11.1218	Z-8-Hexadecene	$C_{16}H_{32}$	224.4253	0.7634
6	11.169	1-Hexadecene	$C_{16}H_{32}$	224.4253	10.1462
7	12.3274	1-Tetracosanol	$C_{24}H_{50}O$	354.65	1.3387
8	12.5161	Heptadecane	$C_{17}H_{36}$	240.4677	2.6386
9	12.8569	1,2,3,6-Tetrahydropyridine	C_5H_9N	83.1317	1.4617
10	13.9472	1-Octadecene	$C_{18}H_{36}$	252.4784	9.4599
11	14.0363	Octadecane	$C_{18}H_{38}$	254.494	3.1718
12	14.7912	R(-)3,7-Dimethyl-1,6-octadiene	$C_{10}H_{18}$	138.2499	2.0711
13	16.5367	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.4507	3.1875
14	17.6323	Phthalic acid, isobutyl octadecyl ester	$C_{30}H_{50}O_4$	474.7156	4.4294
15	17.7214	2- Chloropropionic acid, hexadecyl ester	$C_{19}H_{37}ClO_2$	332.949	5.417
16	18.9847	2-(4-Methylphenoxy)-4a,9b-dihydro-8,9b-dimethyl-3(4H)-dibenzofuranone	$C_{21}H_{20}$	320.382	16.7012
17	20.3214	Pyridine, 1,2,3,6-tetrahydro-1-methyl-	$C_{11}H_{15}NS$	193.3085	1.8294
18	20.6307	Phytol	$C_{20}H_{40}O$	296.5310	3.8543
19	22.2033	Dichloroacetic acid, heptadecyl ester	$C_{19}H_{36}CI_2O_2$	367.3939	0.3779
20	22.219	Acetic acid, chloro-, octadecyl ester	$C_{20}H_{39}ClO_2$	346.9755	0.3326
21	29.6102	Diethylene glycol dibenzoate	$C_{18}H_{18}O_5$	314.3325	1.7493
22	29.6364	Diethylene glycol dibenzoate	$C_{18}H_{18}O_5$	314.3325	1.5302
Total					100

The Table 7.7 lists down the hydrocarbon compound in *Botyococcus* sp. oil of sample B for FW culture media under the most suitable conditions. However, each detected compound containing peak area less than 20%. The majority of compounds

were carrying below 10% of peak area except compound at the retention time of 10.57, 11.17 and 18.98. The retention time of 10.57 (peak no. 3) contained peak area about 19.67% due to the presence of Phenol, 2,4-bis(1,1-dimethylethyl)- while the second less prominent of peak area about 16.70% at the retention time of 18.98 because of the 2-(4-Methylphenoxy)-4a,9b-dihydro-8,9b-dimethyl-3(4H)-dibenzofuranone characteristic.

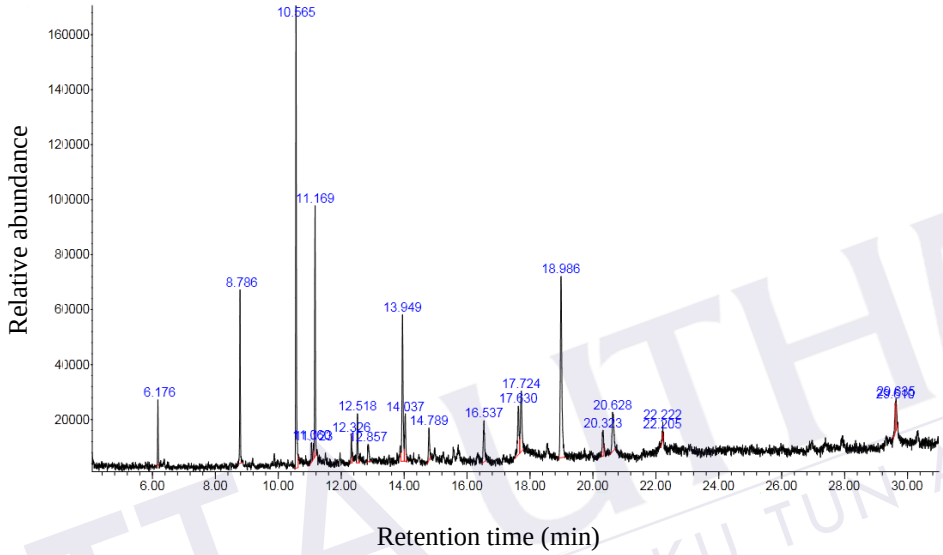


Figure 7.9: GC-MS analysis for microalgae oil biomass cultivated in food processing wastewater (Sample B)

Next, the hydrocarbon compound identified in the sample C of FW culture media from algae oil by GC-MS are provided in Figure 7.10 and Table 7.8. A total of ten compounds have been screened through GC-MS analysis based on retention time, chemical formula, molecular weight and peak area as stated in Table 7.8. the major compound presence in sample C of FW culture media were Phenol, 2,5-bis(1,1-dimethylethyl)- (22.99%), 5-Octadecene, (E)- (19.19%) and 1-Hexadecene (16.17%) etc; other major and minor compounds were also present. The Table 7.8 also showed that the peak no. 1, 3, 5, 7, 8 and 10 are hydrocarbon derivative in which attached to another atom such as oxygen, nitrogen and fluorine while other peak (no. 2, 3, 6 and 9) identified as pure hydrocarbon since contained only carbon and hydrogen element.

As same as algae oil produced from DW culture media, triplicate of oil extracted from *Botryococcus* sp. biomass cultivated in FW culture media also present

the different hydrocarbon composition. For example, compound detected in sample B much more than compound presence in sample A and C. In the sample A, two major compounds which is Hexadecanoic acid, methyl ester and 14-Octadecenoic acid, methyl ester are similar to the palm oil based biodiesel (Table 7.2). Both compound are classified as fatty acid methyl ester (C16-C18) and commonly identified in properties of biodiesel. In fact, the advantages of Hexadecanoic acid also can be used as anti-inflammatory of pathogens, damages cells or irritants (Thomas *et al.*, 2013). Meanwhile, 14-Octadecenoic acid, methyl ester also potentially be used as anti-androgenic, cancer preventive and anemiagenic insectifuge which very important in the medical field (Thomas *et al.*, 2013).

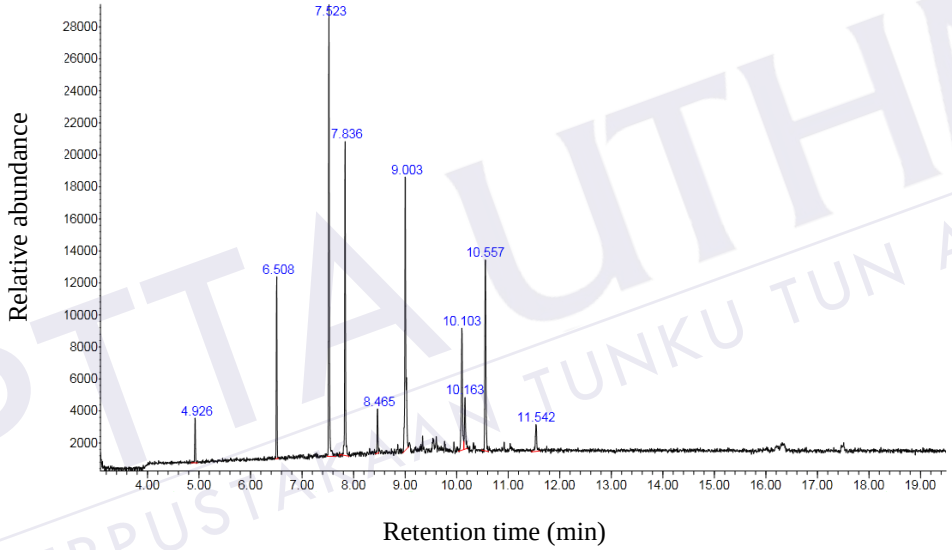


Figure 7.10: GC-MS analysis for microalgae oil biomass cultivated in food processing wastewater (Sample C)

The average hydrocarbon compound in sample B of FW culture media are less than 20% of peak area but interestingly, most of the compound representative to the FAME which is suitable to be further as biofuel feedstock in the future. Meanwhile, sample C of FW culture media presents the major hydrocarbon compound of Phenol, 2,5-bis(1,1-dimethylethyl)- with 22.99% of peak area at the retention time 7.52. Component of Phenol had been adapted as an antioxidant agent in the medical field. However, other compounds either in major or minor concentration is still categorized as FAME. Generally, all replicate samples (sample

A, B and C) contained the same component of Phenol, 2,5-bis(1,1-dimethylethyl)- but it appear at different retention time. For instance, both sample A and B located at the 10.56 retention time while sample C at 7.52.

Table 7.8: Major compounds in microalgae oil biomass cultivated in food processing wastewater (Sample C)

Peak no.	Retention time, R_T (min)	Compound	Chemical formula	Molecular weight (g/mol)	Area (%)
1	4.927	1-Octanol	$C_8H_{18}O$	130.2279	2.1603
2	6.508	5-Octadecene, (E)-	$C_{18}H_{36}$	252.4784	8.2849
3	7.5216	Phenol, 2,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.3239	22.9897
4	7.8355	1-Hexadecene	$C_{16}H_{32}$	224.4253	16.1662
5	8.4635	Oxalic acid, diisohexyl ester	$C_{14}H_{26}O_4$	258.3538	2.3071
6	9.0034	5-Octadecene, (E)-	$C_{18}H_{36}$	252.4784	19.19
7	10.1051	Trifluoroacetic acid, n-heptadecyl ester	$C_{17}H_{31}F_3O_2$	324.4220	9.2761
8	10.1602	1,2-Benzenedicarboxylic acid, butyl decyl ester	$C_{22}H_{34}O_4$	362.5030	3.701
9	10.5568	1,4-Naphthoquinone, 3-methyl-2-[(4-isopropenyl-1-cyclohexenyl)formyl]-	$C_{21}H_{20}$	320.382	13.3554
10	11.5429	2-Acetyl-1-pyrroline	C_6H_9NO	111.1418	2.5693
Total					100

One of the more significant findings to emerge from this study is that the hydrocarbon compound of microalgae oil from the extraction of *Botryococcus* sp. cultivated in wastewater is different in replicate sample. Most of compound detected was a bit different but still similar to the characteristic of biodiesel (palm oil based), showing that high potential of this crude oil to be converted into biodiesel through transesterification process (Mumtaz *et al.*, 2014). The differences of compound produced from each sample (sample A, B and C) may due to the significant influence of growth each photobioreactor used during cultivation. Besides that, according to Zou *et al*. (2010), raw material microalgae itself such as the content of protein, fats and lipid also do affect the presence of hydrocarbon compound in algae oil. Therefore, composition of fatty acids in algae oil in addition to the chemical characteristic such as number of carbon atom, hydrogen and other attached element governs the quality of bio-oil that produced (Mahapatra *et al*., 2014). This had been proved by the sample B (Figure 7.8) in which the chromatogram profile showed the peak was dominated by C16:0 and C18:0. The above finding is consistent with the

study by Dayananda *et al.* (2007) and Manchanda *et al*. (2016). According to Dayananda *et al.* (2007) that examined the Botryococcus braunii extracted oil using GC-MS analysis, found that the presence of Heneicosane, Tetracosane and Octacosane which is similar to compound revealed in the present study. Similarly, Manchanda *et al*. (2016) also obtained the similar hydrocarbon compounds (e.g Heneicosane, Heptadecane, 1-Hexadecene and 5-Octadecene, (E)-). Mostly saturated, but some unsaturated compounds were identified by GC-MS semi-quantitative analysis in FW culture media mainly ranged from C11 to C23 (as stated in Table 7.6, 7.7 and 7.8).

7.4 Summary

The present chapter makes several noteworthy contributing to the latest precious findings result from hydrocarbon produced form microalgae oil cultivated in difference wastewater. But this study specifically conducted by using microalgae species, *Botryococcus* sp. that locally isolated from the area of southern region of Peninsular Malaysia. Interestingly, different culture media used in cultivation produced difference kinds of hydrocarbon compounds. The biggest contribution of this algae oil used as biofuel feedstock that contributes to the development of renewable energy technology. Besides, this study revealed that the hydrocarbon compounds obtained potentially to be used as chemical value added in many industries such as pharmaceutical, agro-feedstock, cosmetic, medical field and etc. This chapter has thrown up many questions in need of the further investigation as intensively included in the Chapter 8.

CHAPTER 8

CONCLUSION AND FURTHER WORK

8.1 Introduction

This is the last chapter that made to conclude all outcomes based on the research aim and set up objectives. The main objective of the undertaken study is to conduct a phycoremediation approach for domestic and food processing wastewater beside to explore new potential hydrocarbon extracted from microalga *Botryococcus* sp. biomass. Lastly, some recommendations had been emerged for further investigation in the future.

8.2 Conclusion

Working towards a sustainable microalgae hydrocarbon production in combination with wastewaters phycoremediation provides a niche opportunity for community-level algae biomass production that has several superiorities over other approaches. The aim of present study was designed to investigate the potential of freshwater green microalgae, *Botryococcus* sp. in treating domestic (DW) and food processing (FW) wastewater associated with biomass generation for precious hydrocarbon production. All objectives of this study were successfully achieved and concluded as follow:

1) The optimisation of environmental factors (temperature, light intensity, photoperiod and salinity) in term of growth rate and biomass productivity of *Botryococcus* sp. in synthetic media BBM found that:

i. The best concentration of temperature, light intensity, photoperiod and salinity are 33°C, 18000 Lux, 24:0 hours and 0M, respectively in term of biomass productivity.
ii. The highest biomass is 81.52×10^4 cell/mL/day with maximum growth rate, division per day and doubling time are 1.31 day^{-1}, 1.89 and 0.53.
iii. The optimum day of culture is revealed to be 18 day to achieve the maximum of growth under controlled condition.

2) The development of a new technique employing microalgae *Botryococcus sp.* in treating wastewater (DW and FW) at different culture conditions had been achieved in this study, based on the experimental results as follow:

i. The best condition is under natural outdoor culture while the most appropriate concentration to be inoculated in wastewaters is 1×10^6 cell/mL in term of nutrients removal and biomass productivity.
ii. The maximum removal of TP, TN and TOC based on the best microalgae concentration for DW is 84.4%, 100% and 45.8%, respectively while for FW is 62.5%, 92.9% and 88.1%, respectively.
iii. Cultivation of *Botryococcus* sp. in contaminated DW and FW for biomass production and simultaneously remove some metal elements (Zn, Fe, Cd, and Mn) was successfully performed for both culture condition and all concentration applied.
iv. The highest biomass productivity when integrates *Botryococcus* sp. in DW is 25.9×10 cell/mL/day with maximum growth rate, division per day and doubling time are 0.26 day^{-1}, 0.38 and 2.63, respectively while in FW is 13.1×10^4 cell/mL/day with maximum growth rate, division per day and doubling time are 0.28 day^{-1}, 0.4 and 2.49.

3) The assessment of the potential biomass production from microalgae *Botryococcus* sp. cultivated using mini pilot scale photobioreactor in DW and FW. Simultaneously, response surface methodology (RSM) was applied to optimize the effects of alum and chitosan dosage and pH sensitivity on flocculation efficiency. The intensive investigations found that:

i. The maximum growth rate and biomass production of *Botryococcus* sp. cultivated in photobioreactor using DW is 0.76 day $^{-1}$ and 9.81 mg/L/day, respectively while using FW is 1.83 day^{-1} and 7.51 mg/L/day, respectively.

ii. Response surface methodology via face centered central composite design statistically proved that second order polynomial function fit well with the experimental results. Both coagulant dosage and pH significantly (p<0.05) affect the flocculation efficiency of *Botryococcus* sp. biomass cultivated in both DW and FW.

iii. The harvesting efficiency of *Botryococcus* sp. in DW using alum was 99.3%, with optimum dosage and pH of 177.74 mg/L and 8.24, respectively. Chitosan achieved 94.2% biomass recovery at an optimal dosage of 169.95 mg/L at pH of 12.

iv. The highest flocculation efficiency (92.4%) in FW was obtained at a dosage of 166 mg/L and pH 12 for alum coagulant, while 94.9% flocculation efficiency was achieved with optimum chitosan dosage and pH of 30 mg/L and 5.54, respectively.

4) The establishment of the chemical composition profile for bio-hydrocarbon production from extracted microalgae biomass of both wastewaters using FT-IR and GC-MS analysis.

i. Obviously, this study presents the latest discovery of hydrocarbon compound in algae oil other than for biodiesel feedstock. The FT-IR analysis shows the similar functional group to standard palm oil based biodiesel. Therefore, triglycerides and phospholipids do exist in the spectrum of *Botryococcus* sp. which in line to the spectrum of methyl ester in petro-diesel spectra.

ii. Similarly, GC-MS analysis revealed that the hydrocarbon compounds obtained potentially to be used as chemical value added in many industries such as pharmaceutical, agro-feedstock, cosmetic, medical field, bioplastic and many more. With this invention, it will be expected

to contribute significantly to a product that can be produced using this local isolated microalga.

8.3 Further work

This research has thrown up many questions in need of further intensive investigation. Further works of research were identified as follows:

- The effect of pH, CO_2 concentration and shaking time of microalgae cultivation should be further investigated in order to maximize the production of microalgae biomass.
- In term of growth, intensive optimization study need be undertaken in the future by employing response surface methodology (RSM) analysis.
- Besides using synthetic medium to optimize the microalgae growth, it is recommended to use real contaminated wastewater to study the potential of biomass production.
- Intensive bioremediation of heavy metals using *Botryococcus* sp. need to be further investigated using synthetic wastewater and simultaneously, to perform the kinetic removal of heavy metal using this microalgae.
- The application of HRAP to produce massive biomass in the real wastewater treatment plant need to be carried out to validate the cultivation using photobioreactor.
- Since microalgae harvesting contribute to the high cost of biomass production, it is recommended to compare the method of harvesting microalgae such as filtration, sedimentation, flocculation, settling and flotation.
- The hydrocarbon study in this study should be further examined by converting the bio-oil produce from algal biomass into biodiesel through transesterification process and to be tested in the real engine to identify the emission rate.
- It is recommended to study the intensive cost related to the microalgae hydrocarbon production to make it reliable to be implemented in low-cost and sustainable way.

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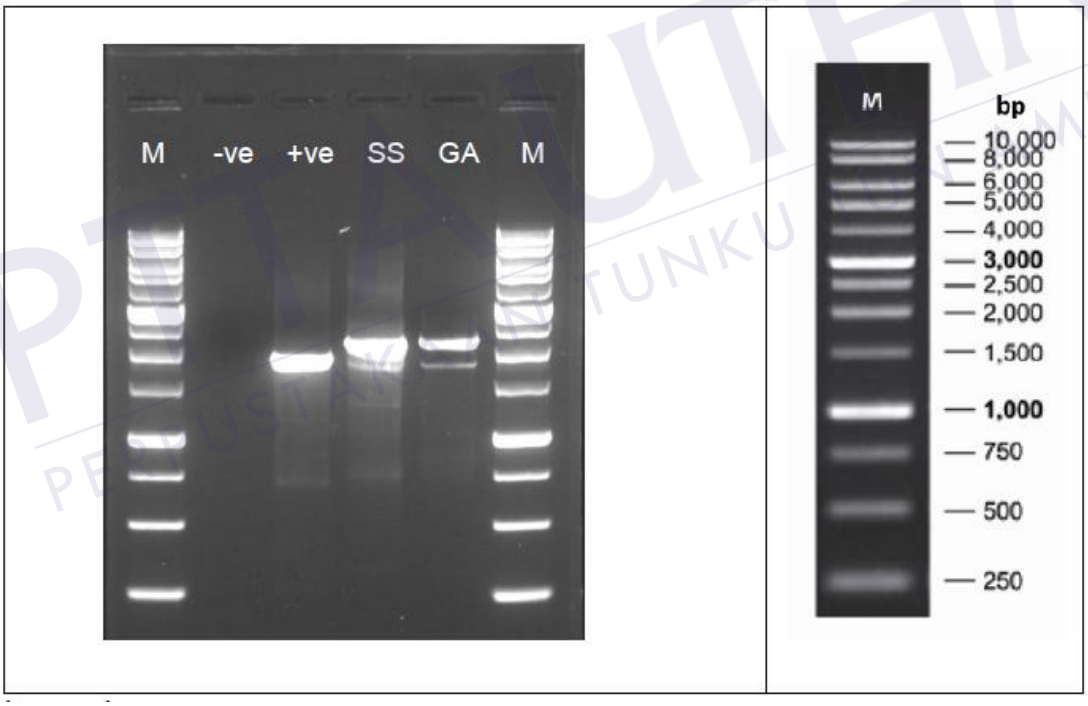
APPENDIX A

Result of Molecular Analysis

SAMPLE INFORMATION		
Sample Name	:	Green Algae (*Botryococcus* sp.)
PCR Product	:	18S rRNA, ~ 1.8 kb

RESULT

1) Gel Photo



Legend:

Lane Label	Sample
M	1kb DNA Ladder
-ve	Negative control
+ve	Positive Control
SS	*Scenedesmus* sp.
GA	Green Alga

2) PCR Product Cloning

Purified PCR product was cloned into pJET1.2/blunt. Positive clone pJET1.2-18SrRNA-Green Alga was sent for sequencing.

Sequence of PCR Product

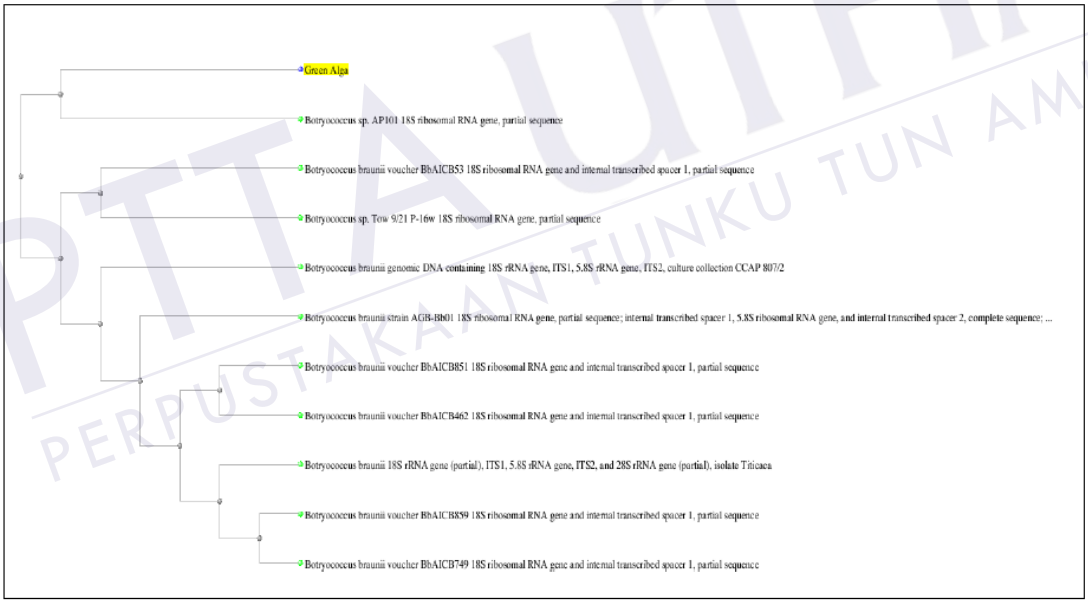
>Green Alga

GATCCCGGTTGATTCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAA
CTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTGGTACCTTCTTACT
CGGATAACCGTAAGAAATTTAGAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATATATTA
GATAAAAGGCCGACCGGACTTTGTCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCACAGCCTCG
CGCTGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTG
GTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG
AAGGCAGCAGGCGCGCAAATTACCTAATCCTGATACGGGGAGGTAGTGACAATAAATAACAATACCGGGC
ATTTCATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCT
GGTGAACACAACAACGCATTTTGCTGTTGACGCCAGAGATAGTAGGGCAGTTTCACGACTGTTATGCCTG
CTAGTCGAGCAGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAGCGGCAGGTTCACCTACGGAAACCTTGTTACGACT
TCTCCTTCCTCTAGGTGGGAGGGTTTAATGAACTTCTCGGCAATCGAGGGTGTTGCCACCTTCAACTGCC
AATCCGAACACTTCACCAGCACACCCAATCGGTAGGAGCGACGGGCGGTGTGTACAAAGGGCAGGGACGT
AATCAACGCAATCTGATGAATTGCGCTTACTAGGCATTCCTCGTTGAAGACTAATAATTGCAATAATCTA
TCCCCATCACGATGCAGTTTCAAAGATTACCCGTACCTTTCGGCAAGGGATAGGCTTGTTGAATGCATCA
GTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCACAGACCTGTTATTGCCTCATACTTCCGTTGA
CTAAACGCCAACTGTCCCTCTAAGAAGTCAAGCCAGCCGCAAAAAGCGACAGTGACTATTTAAAGGCTGA
GGTCTCGTTCGTTACCGGAATCAACCTGACAAGACAACCCACCAACTAAGAACGGCCATGCACCACCACC
CAAATAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTCATTTGTCTGGACCTGGTAAGTTTTCCCGTGT
TGAGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCCTT
GCGACCATACTCCCCCCGGAACCCAAAAACTTTGATTTCTCTCAAGGTGCTGACGGAGTCATGCAAA

3) Top 10 Hits Blast Result against NCBI Nucleotide Collection (nr/nt) Database excluding Bacteria

Description	Max score	Total score	Query cover	E value	Ident	Accession
Botryococcus sp. AP101 18S ribosomal RNA gene, partial sequence	989	989	45%	0.0	92%	JQ585723.1
Botryococcus braunii strain KMRR 18S ribosomal RNA gene, partial sequence	984	984	45%	0.0	92%	KU678200.1
Botryococcus braunii 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2, and 28S rRNA gene (partial), isolate Titicaca	971	1694	82%	0.0	90%	AJ581912.1
Botryococcus braunii strain AICB 53 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	969	1645	81%	0.0	90%	JF261250.2
Botryococcus braunii genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, culture collection CCAP 807/2	969	1679	82%	0.0	90%	FR865761.1
Botryococcus sp. Tow 9/21 P-16w 18S ribosomal RNA gene, partial sequence	969	1703	83%	0.0	90%	AY197640.1
Botryococcus braunii strain AICB 859 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	967	1638	80%	0.0	90%	JF261269.2
Botryococcus braunii strain AICB 851 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	967	1643	81%	0.0	90%	JF261264.2
Botryococcus braunii strain AICB 749 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed space	967	1629	81%	0.0	90%	JF261263.2

4) Phylogenetic Tree – Neighbour Joining (Unrooted Tree) by NCBI Blast Tree Method



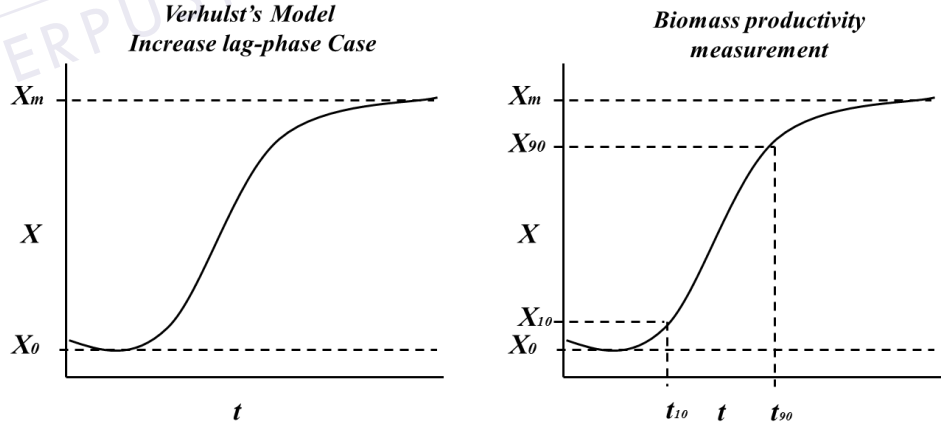
APPENDIX B

Biomass productivity measurement

The volumetric biomass productivity of *Botryococcus* sp. from batch culture experiments is calculated based on the formula below.

$$P = \frac{X_m - X_0}{t_m - t_0}$$

where t_0 is time at the beginning of the experiment, X_0 is the initial biomass concentration and t_m is the time spent in reach X_m. In this case, we consider that the time spent in the lag phase and in the late stationary phase of the cultures must not be included in calculations, in order to reduce sources of variation that can hide productivities (initial biomass concentration of the inoculum or its preservation conditions), as can be seen in Figure below. Then, we arrive at the following expression considering only the biomass generated once initial biomass has increased in a 10% and until 90% of the maximum biomass is reached (Álvarez-Díaz *et al.*, 2014).



$$Biomass\ productivity = \frac{\mu_{max}\ (0.9X_m\text{-}1.1X_0)}{\ln \frac{9(X_m - 1.1X_0)}{1.1X_0}}$$

APPENDIX C

Growth mathematical model derivation

Therefore, Verhulst logistic formula was employed for the microalgae, *Botryococcus* sp. growth:

$$\frac{dX}{dt} = \mu_{max}(1 - \frac{X}{X_m})X$$

where dx/dt is the microalgae growth rate and X is the cell concentration of microalgae in the medium.

$$\frac{dX}{dt} = \mu_{max} X (\frac{X_{max} - X}{X_{max}})$$

$$\int \frac{1}{X(X_{max} - X)} dx = \int \frac{\mu_{max}}{X_{max}} dt$$

By partial fraction,

$$\frac{1}{X(X_{max} - X)} = \frac{A}{X} + \frac{B}{X_{max} - X}$$

$$1 = A(X_{max} - X) + BX$$

When $X = 0$

$$1 = AX_{max}$$

$$A = \frac{1}{X_{max}}$$

When $X = 1$,

$$1 = A\left(X_{\max} -\right) + B$$

$$1 = \frac{1}{X_{\max}}\left(X_{\max} - 1\right) + B$$

$$X_{\max} = X_{\max} - 1 + BX_{\max}$$

$$B = \frac{1}{X_{\max}}$$

$$\int \frac{1}{X\left(X_{\max} - X\right)} dx = \frac{1}{X_{\max}} \int \left(\frac{1}{X} + \frac{1}{X_{\max} - X} \right) dx$$

$$\frac{1}{X_{\max}} \left[\ln X - \ln \left| X_{\max - X} \right| \right]$$

$$\frac{1}{X_{\max}} \left[\ln X - \ln \left| X_{\max} - X \right| \right] = \frac{\mu_{\max}}{X_{\max}} t + C$$

$$\ell^{\ln \left| \frac{X}{X_{\max} - X} \right|} = \ell^{\mu_{\max} t + C}$$

$$\frac{X}{X_{\max} - X} = \ell^{\mu_{\max} t} . \ell^{c}$$

$$A = \ell^{c}$$

$$X = \left(X_{\max} - X\right) A \ell^{\mu_{\max} t}$$

$$X = AX_{\max} \ell^{\mu_{\max} t} - AX \ell^{\mu_{\max} t}$$

$$X + AX\ell^{\mu_{max}t} = AX_{max}\ell^{\mu_{max}t}$$

$$X\left(1 + A\ell^{\mu_{max}t}\right) = AX_{max}\ell^{\mu_{max}t}$$

$$X = \frac{AX_{max}\ell^{\mu_{max}t}}{1 + A\ell^{\mu_{max}t}}$$

When t = 0,

$$X_0 = \frac{AX_{max}}{1 + A}$$

$$X_0 + AX_0 = AX_{max}$$

$$-AX_0 + AX_{max} = X_0$$

$$A\left(-X_0 + X_{max}\right) = X_0$$

$$A = \frac{X_0}{-X_0 + X_{max}}$$

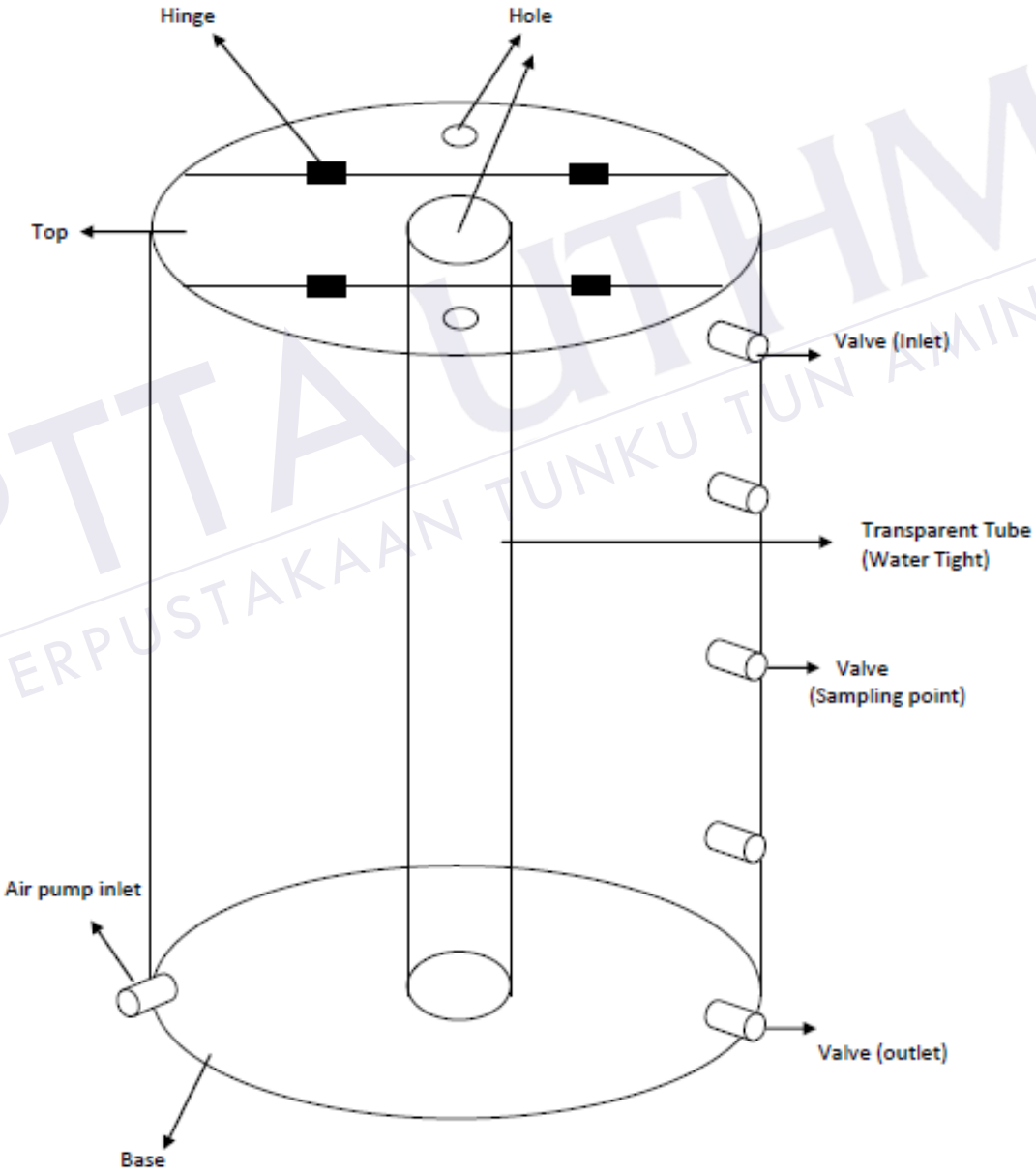
$$X = \frac{\frac{X_0}{-X_0 + X_{max}} X_{max}\ell^{\mu_{max}t}}{1 + \frac{X_0}{-X_0 + X_{max}}\ell^{\mu_{max}t}}$$

$$X = \frac{X_0 X_{max}\ell^{\mu_{max}t}}{-X_0 + X_{max} + X_0\ell^{\mu_{max}t}}$$

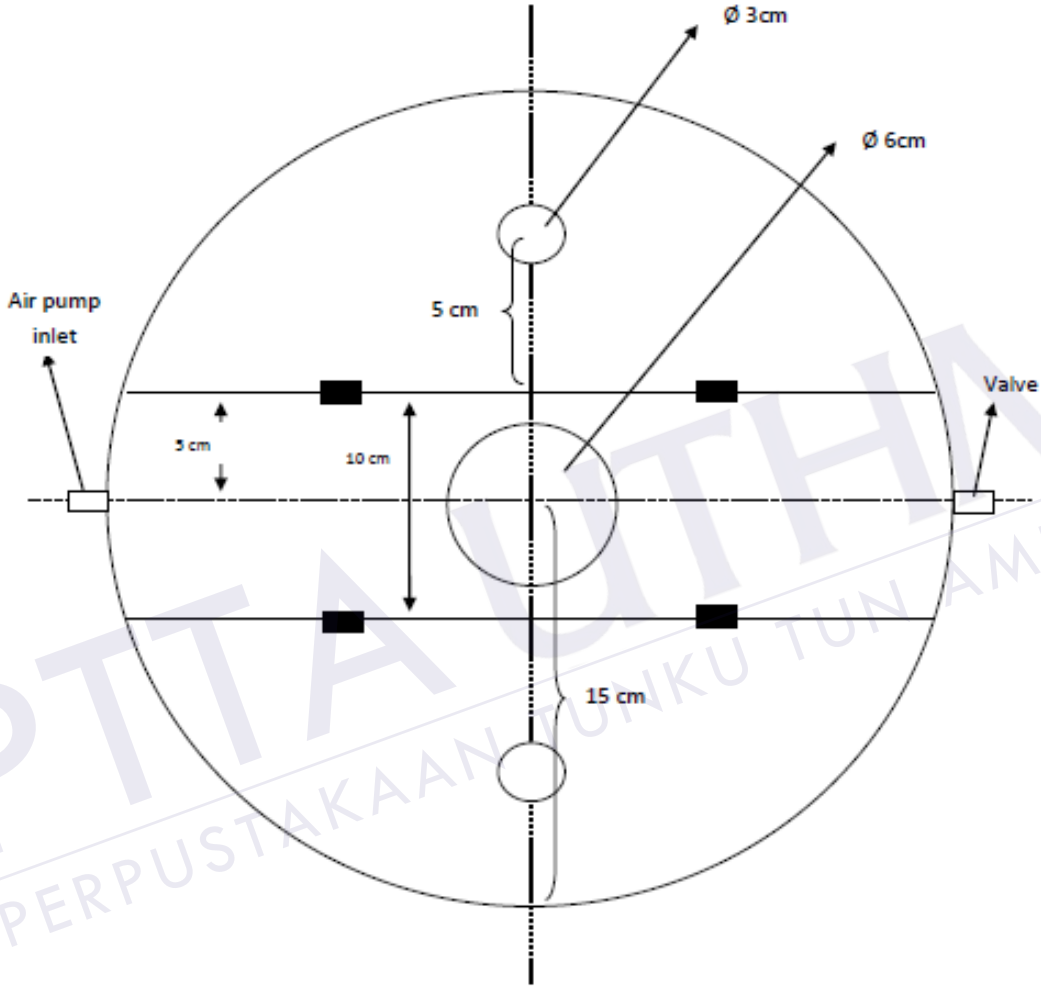
APPENDIX D

Closed tubular photobioreactor diagrams

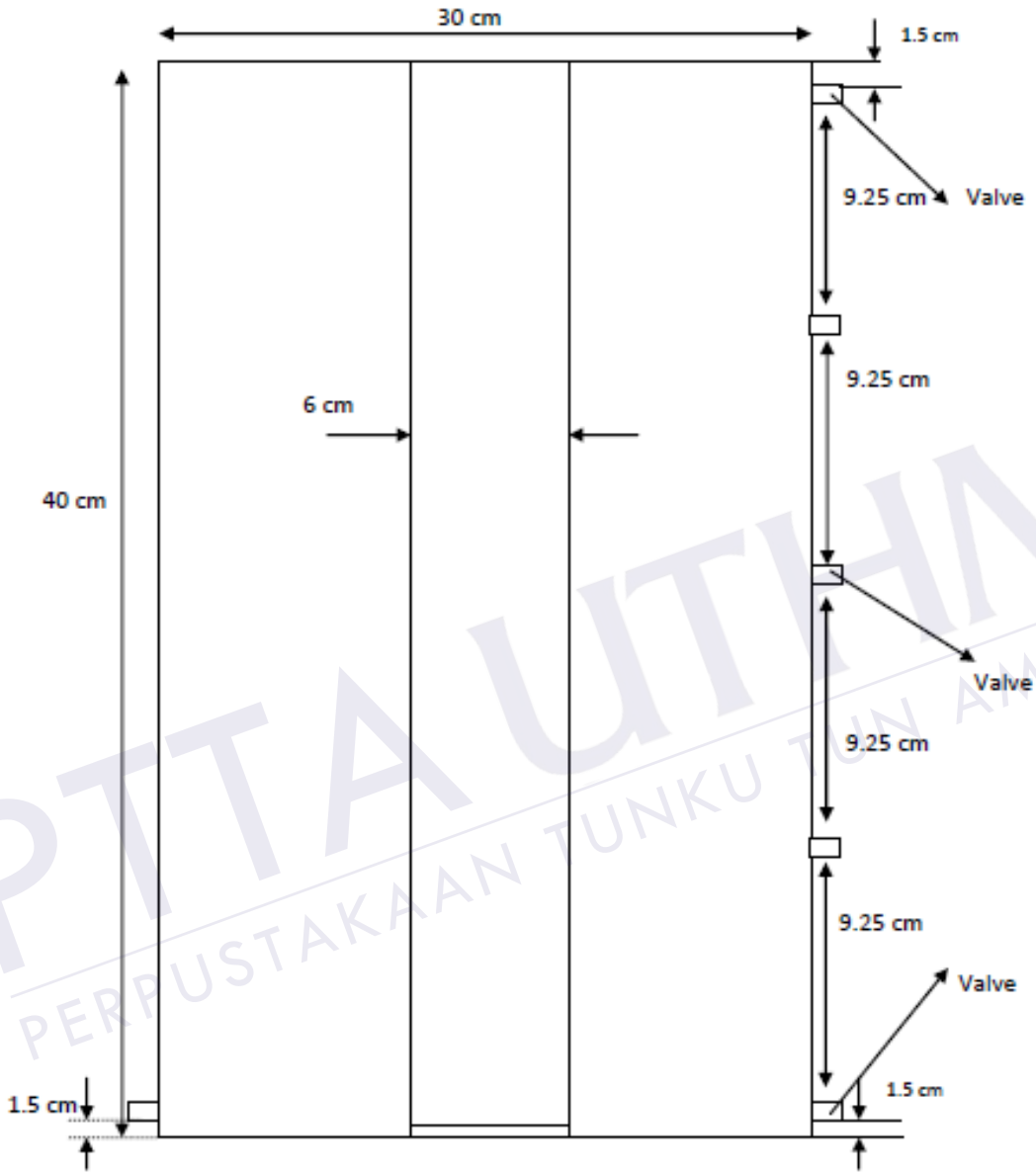
The construction of photobioreactor according to Yen *et al*., (2014)



3D View



Top View



Side View

APPENDIX E

Raw data of lab-scale phycoremediation

1) First-order kinetic coefficient

Domestic wastewater (Outdoor culture)

Total phosphorus (TP)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0629x + 2.1677	0.0629	0.8976
10^4	y = -0.0621x + 2.0204	0.0621	0.8435
10^5	y = -0.1018x + 2.3116	0.1018	0.9098
10^6	y = -0.0958x + 2.2588	0.0958	0.9096
10^7	y = -0.099x + 2.2834	0.0990	0.9330

Total nitrogen (TN)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0538x + 3.1859	0.0538	0.9051
10^4	y = -0.0517x + 3.0337	0.0517	0.9164
10^5	y = -0.079x + 3.1353	0.0790	0.9262
10^6	y = -0.157x + 3.3845	0.1570	0.9008
10^7	y = -0.0719x + 3.2044	0.0719	0.9076

Total organic carbon (TOC)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0343x + 2.5993	0.0343	0.9027
10^4	y = -0.0252x + 2.6606	0.0252	0.9343
10^5	y = -0.0284x + 2.6041	0.0284	0.9161

10^6	y = -0.0362x + 2.6331	0.0362	0.9155
10^7	y = -0.0641x + 2.7153	0.0641	0.9413

Domestic wastewater (Indoor culture)

Total phosphorus (TP)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.1053x + 2.1678	0.1053	0.9327
10^4	y = -0.1086x + 2.0141	0.1086	0.9687
10^5	y = -0.1221x + 1.935	0.1221	0.9194
10^6	y = -0.1479x + 2.0628	0.1479	0.8786
10^7	y = -0.1238x + 2.1023	0.1238	0.9185

Total nitrogen (TN)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0521x + 3.1382	0.0521	0.9242
10^4	y = -0.0507x + 3.0666	0.0507	0.9562
10^5	y = -0.0709x + 2.9148	0.0709	0.9257
10^6	y = -0.0952x + 2.7292	0.0952	0.9612
10^7	y = -0.0112x + 2.8028	0.0112	0.4556

Total organic carbon (TOC)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0278x + 2.6652	0.0278	0.9417
10^4	y = -0.037x + 2.6751	0.0370	0.9400
10^5	y = -0.0739x + 2.1881	0.0739	0.9444
10^6	y = -0.109x + 2.3521	0.1090	0.9016
10^7	y = 0.003x + 2.5582	0.0030	0.0035

Food processing wastewater (Outdoor culture)

Total phosphorus (TP)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.028x + 1.6285	0.0280	0.8418
10^4	y = -0.0309x + 1.6093	0.0309	0.8937
10^5	y = -0.0499x + 1.7837	0.0499	0.9678
10^6	y = -0.0508x + 1.8026	0.0508	0.9182
10^7	y = -0.0224x + 1.7096	0.0224	0.8902

Total nitrogen (TN)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0536x + 5.1266	0.0536	0.9023
10^4	y = -0.0666x + 5.0897	0.0666	0.9722
10^5	y = -0.1151x + 5.3023	0.1151	0.9172
10^6	y = -0.1349x + 5.3755	0.1349	0.8755
10^7	y = -0.065x + 5.0522	0.0650	0.936

Total organic carbon (TOC)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	*-k*	R^2
10^3	y = -0.0369x + 2.7413	0.0369	0.936
10^4	y = -0.0268x + 2.6753	0.0268	0.9544
10^5	y = -0.0349x + 2.7512	0.0349	0.9181
10^6	y = -0.1164x + 3.0152	0.1164	0.9119
10^7	y = -0.0322x + 2.6936	0.0322	0.9483

Food processing wastewater (Indoor culture)

Total phosphorus (TP)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	$-k$	R^2
10^3	y = -0.0113x + 1.7324	0.0113	0.923
10^4	y = -0.0252x + 1.7537	0.0252	0.9372
10^5	y = -0.0324x + 1.7801	0.0324	0.9023
10^6	y = -0.0759x + 1.8983	0.0759	0.9225
10^7	y = -0.0446x + 1.7761	0.0446	0.9502

Total nitrogen (TN)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	$-k$	R^2
10^3	y = -0.0435x + 5.1462	0.0435	0.9192
10^4	y = -0.0692x + 5.1258	0.0692	0.9427
10^5	y = -0.0766x + 5.2127	0.0766	0.9107
10^6	y = -0.0799x + 5.234	0.0799	0.9118
10^7	y = -0.0191x + 5.0224	0.0191	0.8942

Total organic carbon (TOC)

Concentration (cells/mL)	Integrated first-order kinetic equation $\ln[P] = -kt + \ln[P]_0$	$-k$	R^2
10^3	y = -0.0211x + 2.7121	0.0211	0.9194
10^4	y = -0.039x + 2.6523	0.0390	0.9402
10^5	y = -0.0482x + 2.6446	0.0482	0.9102
10^6	y = -0.0712x + 2.8131	0.0712	0.8966
10^7	y = -0.0537x + 2.5596	0.0537	0.8361

2) The maximum growth rate (μ_{max}) determination in wastewaters

Domestic wastewater (Outdoor culture)

Concentration(cells/mL)	Sample	μ_{max}	R^2
10^3	A	1.362	0.9562
	B	1.316	0.9483
	C	1.361	0.9386
10^4	A	0.686	0.9815
	B	0.700	0.9812
	C	0.686	0.9812
10^5	A	0.599	0.9704
	B	0.601	0.9679
	C	0.588	0.9697
10^6	A	0.266	0.9394
	B	0.259	0.9376
	C	0.262	0.9389
10^7	A	0	0
	B	0	0
	C	0	0

Domestic wastewater (Indoor culture)

Concentration(cells/mL)	Sample	μ_{max}	R^2
10^3	A	1.997	0.9932
	B	2.002	0.9891
	C	2.002	0.9946
10^4	A	1.237	0.9283
	B	1.222	0.9199
	C	1.237	0.9235
10^5	A	0.583	0.9947
	B	0.562	0.9825
	C	0.586	0.9923
10^6	A	0.270	0.9971
	B	0.271	0.9937
	C	0.270	0.9961
10^7	A	0	0
	B	0	0
	C	0	0

Food processing wastewater (Outdoor culture)

Concentration(cells/mL)	Sample	μ_{max}	R^2
10^3	A	0.537	0.9454
	B	0.536	0.9340
	C	0.556	0.9588
10^4	A	0.525	0.9569
	B	0.537	0.9673
	C	0.531	0.9638
10^5	A	0.158	0.9788
	B	0.150	0.9799
	C	0.153	0.9715
10^6	A	0.278	0.9907
	B	0.279	0.9902
	C	0.278	0.9958
10^7	A	0.094	0.9657
	B	0.094	0.9657
	C	0.094	0.9670

Food processing wastewater (Indoor culture)

Concentration(cells/mL)	Sample	μ_{max}	R^2
10^3	A	1.037	0.9853
	B	1.052	0.9762
	C	1.063	0.9697
10^4	A	0.312	0.9646
	B	0.318	0.9733
	C	0.315	0.9750
10^5	A	0.039	0.9904
	B	0.041	0.9777
	C	0.040	0.9815
10^6	A	0.170	0.9888
	B	0.173	0.9911
	C	0.176	0.9857
10^7	A	0.057	0.9797
	B	0.057	0.9769
	C	0.058	0.9782

APPENDIX F

Raw data of microalgae oil extraction experiments

Description	Domestic wastewater (DW)			Food processing wastewater (FW)		
	Sample A	Sample B	Sample C	Sample A	Sample B	Sample C
Thimble, g	5.718	5.805	5.69	5.567	5.679	5.70
Dry sample, g	2.790	3.422	3.889	3.484	2.915	2.681
Empty vial, g	14.778	14.675	14.733	14.750	14.78	14.887
Empty vial+Oil, g	16.805	17.031	17.485	16.658	16.404	16.196
Oil, %	72.65	68.85	70.64	54.76	55.70	48.83
Mean, %	70.71			53.10		

APPENDIX G

Statistical analysis (One-way ANOVA)

1. Influence of environmental factors on the growth of microalgae

a) Light intensities

ANOVA

Microalgae growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12975268.159	5	2595053.632	15.977	.000
Within Groups	21440064.656	132	162424.732		
Total	34415332.814	137			

b) Temperature

ANOVA

Microalgae growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	810180.246	4	202545.061	10.687	.000
Within Groups	2179562.007	115	18952.713		
Total	2989742.253	119			

c) Photoperiod

ANOVA

Microalgae growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	755207.919	4	188801.980	16.256	.000
Within Groups	1103372.573	95	11614.448		
Total	1858580.492	99			

d) Salinity

ANOVA

Microalgae growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	687207.701	4	171801.925	21.956	.000
Within Groups	743349.186	95	7824.728		
Total	1430556.887	99			

2. Phycoremediation of wastewaters

Domestic wastewater (Outdoor culture)

- Total removal of total phosphorus (TP)

ANOVA

TP_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5477.010	5	1095.402	2966.414	.000
Within Groups	4.431	12	.369		
Total	5481.441	17			

- Total removal of total nitrogen (TN)

ANOVA

TN_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8936.120	5	1787.224	2308.704	.000
Within Groups	9.289	12	.774		
Total	8945.410	17			

- Total removal of total organic carbon (TOC)

ANOVA

TOC_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2894.588	5	578.918	214.441	.000
Within Groups	32.396	12	2.700		
Total	2926.984	17			

Domestic wastewater (Indoor culture)

- Total removal of total phosphorus (TP)

ANOVA

TP_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8641.339	5	1728.268	172.483	.000
Within Groups	120.239	12	10.020		
Total	8761.578	17			

- Total removal of total nitrogen (TN)

ANOVA

TN_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10022.540	5	2004.508	444.842	.000
Within Groups	54.073	12	4.506		
Total	10076.613	17			

- Total removal of total organic carbon (TOC)

ANOVA

TOC_removal

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26195.111	5	5239.022	1491.263	.000
Within Groups	42.158	12	3.513		
Total	26237.269	17			

Food processing wastewater (Outdoor culture)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TP Total Removal (18 days of treatment)	Between Groups	3224.735	5	644.947	200.797	.000
	Within Groups	38.543	12	3.212		
	Total	3263.279	17			
TN Total Removal (18 days of treatment)	Between Groups	2343.737	5	468.747	122.674	.000
	Within Groups	45.853	12	3.821		
	Total	2389.590	17			
TOC Total Removal (18 days of treatment)	Between Groups	6230.175	5	1246.035	1308.980	.000
	Within Groups	11.423	12	.952		
	Total	6241.598	17			

Food processing wastewater (Indoor culture)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TP Total removal (18 days of phycoremediation)	Between Groups	8172.717	5	1634.543	1282.870	.000
	Within Groups	15.290	12	1.274		
	Total	8188.007	17			
TN Total removal (18 days of phycoremediation)	Between Groups	9804.696	5	1960.939	234.052	.000
	Within Groups	100.539	12	8.378		
	Total	9905.234	17			
TOC Total removal (18 days of phycoremediation)	Between Groups	7486.357	5	1497.271	456.628	.000
	Within Groups	39.348	12	3.279		
	Total	7525.705	17			

3. Growth of microalgae in wastewaters with different cell concentration

Domestic wastewater (Outdoor culture)

ANOVA

Cells

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7933171209478 48.900	4	1983292802369 62.220	202.308	.000
Within Groups	8822992766571 7.300	90	980332529619. 081		
Total	8815470486135 66.100	94			

Domestic wastewater (Indoor culture)

ANOVA

Cells

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8805090652698 88.900	4	2201272663174 72.220	480.311	.000
Within Groups	4124717974097 8.780	90	458301997121. 986		
Total	9217562450108 67.600	94			

Food processing wastewater (Outdoor culture)

ANOVA

Food wastewater (Outdoor culture)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1934731839638 217.800	4	4836829599095 54.440	248.629	.000
Within Groups	1750859516460 95.200	90	1945399462734 .391		
Total	2109817791284 313.000	94			

Food processing wastewater (Indoor culture)

ANOVA

Growth, FW (Indoor culture)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1750151880508 122.500	4	4375379701270 30.600	336.709	.000
Within Groups	1169509148918 17.640	90	1299454609909 .085		
Total	1867102795399 940.200	94			

Cultivation using photobioreactors

Comparison of growth of microalgae cultivated in domestic wastewater and food processing wastewater

ANOVA

Dry weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2816.232	1	2816.232	6.248	.020
Within Groups	10818.396	24	450.767		
Total	13634.628	25			

APPENDIX H

List of publications

No.	Title	Type of paper	Journal / conference	Publisher	Index		Status
					Scopus	ISI (IF)	
1	Phycoremediation of Dairy Wastewater by Using Green Microalgae: *Botryococcus* sp.	Conference paper	International Integrated Engineering Summit 2014	Trans Tech Publications	Yes	N/A	Published 2015, 773-774, pp: 1318-1323
2	Experimental Study for Phycoremediation of *Botryococcus* sp. on Greywater	Conference paper	International Integrated Engineering Summit 2014	Trans Tech Publications	Yes	N/A	Published 2015, 773-774, pp: 1312-1317
3	Phycoremediation of Wastewaters and Potential Hydrocarbon from Microalgae: A Review	Review paper	Advances in Environmental Biology	AENSI	N/A	N/A	Published 2015, 9(20), pp: 1-8
4	Influence of initial cell concentrations on the growth rate and biomass productivity of microalgae in domestic wastewater	Research article	Applied Ecology and Environmental Research	Szent Istvan University	Yes	0.50	Published 2016, 14(2), pp: 399-409
5	Screening of Sustainable Hydrocarbon Extracted from Microalgae via Phycoremediation	Research article	Journal of Engineering and Applied Sciences	ARPN	Yes	N/A	Published 2016, 11(12), pp: 7431-7436
6	Application of Phycoremediation Technology in the Treatment of Food Processing Wastewater by Freshwater Microalgae Botryococcus sp.	Research article	Journal of Engineering and Applied Sciences	ARPN	Yes	N/A	Published 2016, 11(11), pp: 7288-7292
7	The influence of photoperiod, light intensity, temperature, and salinity on the growth rate and biomass productivity of green microalgae *Botryococcus* sp.	Research article	Sains Malaysiana	Universiti Kebangsaan Malaysia	Yes	0.35	Under review

8	Effect of pH and alum dosage on the efficiency of microalgae harvesting via flocculation technique	Research article	International Journal of Green Energy	Taylor and Francis	Yes	1.60	Accepted (In Press)
9	Extraction of hydrocarbons from freshwater green microalgae (*Botryococcus* sp.) biomass after phycoremediation of domestic wastewater	Research article	International Journal of Phytoremediation	Taylor and Francis	Yes	2.08	Accepted (In Press)
10	An overview of environmental factor's effect on the growth of microalgae	Review paper	Jurnal Teknologi	Universiti Teknologi Malaysia	Yes	N/A	Under review
11	Growth of microalgae *Botryococcus* sp. in domestic wastewater and application of statistical analysis for the optimisation of flocculation using alum and chitosan	Research article	Preparative Biochemistry and Biotechnology	Taylor and Francis	Yes	1.11	Accepted (In Press)
12	Effects of different culture conditions on the phycoremediation efficiency of domestic wastewater	Research article	Journal of Environmental Chemical Engineering	Elsevier	Yes	N/A	Published 2016, 4(4PA), pp: 4744-4753
13	Outdoor phycoremediation and biomass harvesting optimisation of microalgae *Botryococcus* sp. cultivated in food processing wastewater using an enclosed photobioreactor	Research article	Chemical Engineering Journal	Elsevier	Yes	5.31	Under review
14	Green approach in the bio-removal of heavy metal in wastewater	Conference paper	International conference on sustainable environment and water research 2016	MATEC Web of Conference	Yes	N/A	Accepted (In Press)
15	The potential of biodiesel production from Botryococcus sp. biomass after phycoremediation of domestic and industrial wastewater	Conference paper	International Conference on Sustainable and Green Technology 2016	IOP Conference Series: Materials Science and Engineering	Yes	N/A	Published 2016, 160, pp: 012048

APPENDIX I

Achievements

1. International Invention, Innovation and Technology Exhibition 2014 (ITEX 2014), PWTC - **Gold Medal**, 8th -10th MAY 2014. **Titile:** *Bio-Renewal Plasticizer from Wastewater Phycoremediation*.
2. Research & Innovation Festival 2014 (R&I 2014), UTHM – **Gold Medal**, 2nd – 3rd Nov 2014. **Title:** *Bio-Renewal Hydrocarbon from Municipal Wastewater Phycoremediation*.
3. BioMalaysia Innovation 2014, PWTC - **Bronze Medal**, 19th – 20th Nov 2014. **Title**: *Susutainable Production of Bio-Renewal Hydrocarbon from Wastewater Phycoremediation*.
4. Seoul International Invention Fair 2014 (SIIF 2014), Seoul Korea – **Bronze Prize**, 28th Nov – 1st Dec 2014. **Title:** *Bio-Renewal Plasticizer from Wastewater Phycoremediation.*

VITA

The author was born on November 10 in Sarawak, Malaysia. He received primary education at SK Kedumpai, Simunjan from 1992 to 1997. He continued his studies at the secondary school at SMK Simunjan N0.1, Simunjan from 1998 to 2002. After completing Form Five, he continued his education at the Polytechnic Kuching Sarawak in certificate of Civil Engineering for two years starting from 2003 to 2005. In 2006, he continued his studies at the polytechnic in diploma at the same year. After completing his diploma, he started working as a lab technician in one of the private company in kuching, Sarawak. In 2009, he once again pursue the Bachelor and Master degree in Civil Engineering at Universiti Tun Hussein Onn Malaysia in Batu Pahat, Johor. Mr. Paran Gani graduated Master's Degree in 2014 with great success. Before pursuing master's degree study, he used to work as a civil engineer in kuching, Sarawak from February 2012 to September 2012. As someone who is passionate and creative, it is his aim to make education and academic pursuit a lifelong path and dedication. In March 2014, he pursued doctorate course in civil engineering specialized on environmental engineering in UTHM and expected to be completed in 2017. During the doctorate study, he participated and win gold medal in ITEX 2014 exhibition and R&I 2014 festival. In addition, he has also attended international conference in both overseas and local venue such as in Bali, Indonesia, Gyeongsan, South Korea and Melaka, Malaysia. With existing expertise, he contributed a lot in writing such publications in high impact scientific journals. Recently, he publishs in the top reputation publisher such as Elsevier and Taylor & Francis.