Cultivation of *Chorella vulgaris* Using Wastewater as Nutrient Source for Biodiesel Production

by

Mohammad Iqram Bin Yusoff
16910

Dissertation submitted in partial fulfilment of
the requirements for the
Bachelor of Engineering (Hons)
(Chemical Engineering)

MAY 2015

Universiti Teknologi PETRONAS,
32610, Bandar Seri Iskandar,
Perak Darul Ridzuan

CERTIFICATION OF APPROVAL

Cultivation of *Chorella vulgaris* Using Wastewater as Nutrient Source for Biodiesel Production

by

Mohammad Iqram Bin Yusoff
16910

A project dissertation submitted to the
Chemical Engineering Programme
Universiti Teknologi PETRONAS
in partial fulfilment of the requirement for the
BACHELOR OF ENGINEERING (Hons)
(CHEMICAL ENGINEERING)

Approved by,

_____

(Dr. Lam Man Kee)

UNIVERSITI TEKNOLOGI PETRONAS
SERI ISKANDAR, PERAK
May 2015

CERTIFICATION OF ORIGINALITY

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

_____

(MOHAMMAD IQRAM BIN YUSOFF)

ABSTRACT

Cultivation of microalgae using wastewater as nutrient sources provides an alternative and sustainable solution for the production of biodiesel as renewable fuel. This is because wastewater contain high amount of nutrients that are essential for the growth of microalgae. Hence, in the present study, the potential of using domestic wastewater as nutrient source to cultivate *Chorella vulgaris* was investigated. With the utilization of domestic wastewater as nutrient source for the growth of *Chorella vulgaris*, it was found that the microalgae favoured to grow at the following condition: 20 mL amount of wastewater, initial pH of 3, and 30 mL initial amount of microalgae seed with 24 hours of continuous illumination. Then, the microalgae lipid was extracted with chemical solvent (Bligh & Dyer Method) and it was found that high lipid content of 32.7 % was embedded within microalgae cells. Besides, from the analysis of fatty acid methyl ester (FAME) profile, it can be concluded that the FAME produced from the transesterification of the *Chorella vulgaris* lipid is suitable for biodiesel production.

ACKNOWLEDGEMENT

First and foremost, the author would like to express the highest gratitude to God the Almighty for his blessing in giving him chance to complete the dissertation and successfully performed the final year project (FYP) which is the requirement for all the students upon graduation. Next, the author would like to express his gratitude to Dr. Lam Man Kee, as FYP supervisor for his guidance and support throughout the two semester period despite his busy schedule as lecturer. The author was able to complete the project with a proper planning and constant supervision from the dedicated supervisor.

The author also would like to thanks the member of Centre of Biofuel Research (CBBR) for their greatest cooperation in assisting the author conducted the project. Lastly, heartfelt appreciation goes to the author beloved parents (Yusoff Bin Hassan and Azifah Binti Abdul Rahman), family and friends for their endless support and encouragement in completing the research project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The oil and gas resources on Earth is depleting day by day due to the daily consumption of fossil fuel for transportation and for the various industries that require energy to perform their daily operation. Thus, alternative source of energy need to be explored to overcome the energy crisis in the future and to ensure the sustainability of the environment. This is mainly due to the extremely usage of fossil fuel as primary source of energy that has caused huge amount of greenhouse gases mainly CO_2 is released to the atmosphere that subsequently lead to global warming (Brennan and Owende, 2010). Besides, the occurrence of flash flood, extreme heat and cold wave, frequent occurrence of drought and desertification are mainly due to the alleviation of Earth temperature. The overall implication has triggered a need for global strategies for energy security and mitigation of CO_2-energy related emission. Hence, many countries has taken initiatives to develop green and renewable source of energy in order to reduce the emission of greenhouse gases to the atmosphere and for energy security purpose. There are a lot of renewable energy resources such as solar, wind, tidal, and biomass that can be harvested as energy sources to replace the needs of fossil fuel. However, the main downfall of these renewable energy is the sources are inconsistent and varies based on regional and local condition (Lam and Lee, 2012b). For a country that has significant amount of agriculture activities such as Malaysia, biomass can be a promising alternative source of renewable energy for the production of biofuels.

Biofuels can be categorized as renewable source of energy because it is mainly derived from biodegradable resources such as corn kernels, woody biomass, soy beans,

microalgae and many other suitable feedstock (Yusuf *et al.*, 2011). Two commercially available type of biofuels in the market are bioethanol and biodiesel, in which bioethanol is derived from sugar cane or corn starch while biodiesel is produced from oil crops, such as oilseed rape and soybean (Hill *et al.*, 2006). However, there are some social concerns on using the food crops for biodiesel production, which in turn deliberated the food versus fuel issue. Thus, in the recent years, aquatic microorganism such as microalgae is proposed as an alternative feedstock for the production of biodiesel (G. Chen *et al.*, 2015). Microalgae are photosynthetic microorganisms that only required CO_2, water and inorganic nutrients (e.g. nitrogen, phosphorus and kalium) for growing purpose. The produced microalgae biomass contained large amount of lipid (fatty acid), which can be extracted for subsequent biodiesel production. It was reported that, approximately about 50% of their biomass is lipids and sometimes may goes up to 80% by cultivating them at certain conditions (e.g. heterotrophic) (Chisti, 2007). In addition, oil productivity which is the mass of oil produced per unit volume of the microalgae broth produced per day depends on the microalgae species, nutrient sources and cultivation conditions (Chisti, 2007).

For the purpose of environmental sustainability in microalgae biofuel production, wastewater can be used an alternative nutrient sources to cultivate microalgae, while at the same time purifying the wastewater. Wastewater is rich in nitrogen and phosphorus content which if left to flow into the waterways can spawn unwanted algae bloom. Hence, by using wastewater to cultivate microalgae provides mutual benefit of producing biofuels and removing nitrogen and phosphorus as well as organic carbon from the wastewater (Mostafa *et al.*, 2012). Conventional wastewater treatment plant that involved primary and secondary treatment only managed to remove a fraction of nitrogen and phosphorus in the wastewater. Thus, cultivating microalgae using wastewater offers an inexpensive alternative to tertiary treatment and able to generate additional revenue (microalgae biomass and biofuel) to the wastewater treatment plant.

1.2 Problem Statement

Biodiesel is an alternative fuel to substitute the heavy reliance on petroleum derived diesel oil. However, the production of biodiesel from edible oils sources such as rapeseeds, soybeans, sunflower and crude palm oil has created food versus fuels feud that brings negative impacts to the food industry and society, especially the bottom billions. The use of edible oil as biodiesel feedstock will create competition with food production which causing the food price to increase and also leads to unsustainable production of biodiesel. Due to this concern, an alternative oil feedstock for biodiesel production is urgently required.

Biodiesel derived microalgae biomass has been proposed as an alternative approach that does not brings impact on the food industry. Microalgae are able to generate substantial amount of biomass and oil that can be utilized for the production of biodiesel. In addition, it is estimated that microalgae have higher biomass productivity compared to plant crops especially in term of land area required for cultivation. Moreover, the production of biodiesel by microalgae also could replace the needs of fossil fuels and thus, reducing the greenhouse gas emission (Schenk *et al.*, 2008). Nevertheless, the cultivation of microalgae requires substantial amount of nutrients to grow and the nutrients are usually referred to chemical fertilizer (synthetic). The production of chemical fertilizer is classified as energy intensive industry which is not sustainable for the mass production of microalgae for long term. Alternatively, wastewater offered a unique opportunity to grow microalgae due to its nature nutrients-rich content; while microalgae are acting as purifying agent to purify the wastewater (Mostafa *et al.*, 2012).

Thus, this work aims to evaluate the laboratory cultivation of microalgae strain in domestic wastewater for biomass and biodiesel production. The effect of various cultivation parameters such as amount of nutrients, illumination duration and pH value to the growth of microalgae will be systematically investigated.

1.3 Objectives

The objectives of the present research are as follow:

1. To study the effect of cultivation parameters such as amount of nutrients, the initial pH value and the amount of microalgae seeds of the cultivation medium to the growth of microalgae.
2. To harvest and to extract lipid from dried microalgae biomass.
3. To convert extracted microalgae lipid to biodiesel through transesterification reaction.

1.4 Scope of Study

In order to achieve the affirmation of the research objectives, the present research work is conducted to determine the effectiveness of different cultivation parameters such as amount of nutrients, the initial pH value and amount of microalgae seeds towards the growth of microalgae. The content of the wastewater collected from the wastewater treatment plant will be analysed prior to be used as nutrients for the cultivation of the microalgae. The amount of biomass yield (g/L) and the specific growth of the microalgae will be determined throughout the experiments. Nevertheless, the effect of photobioreactor configuration, CO_2 flow rate and illumination intensity is not within the scope of the present work.

Lipid extraction will be performed on the dried microalgae biomass by using Soxhlet extractor method using mixed methanol-chloroform with volume ratio of 2:1 as solvent. The lipid yield will be determined by gravitational method. The effect of different solvent towards the lipid extraction efficiency is not covered in the present study. Then, the lipid extracted will undergo transesterification reaction to convert into biodiesel.

1.5 Relevancy and Feasibility of the Project

The present study is relevant to the sustainable energy development issue as the project aims to find new source of renewable energy that can reduce the dependency on fossil fuels. This study aims to optimize the cultivation conditions of *Chorella vulgaris* for optimum biomass productivity. In addition, this study includes the feasibility of using wastewater as nutrient source for microalgae cultivation, in which the microalgae biomass will be subsequently used for biodiesel production. The high biomass productivity of wastewater-grown microalgae could offer real potential for sustainable biodiesel production.

CHAPTER 2

LITERATURE REVIEW

Literature review was performed in this chapter to support the research in the present thesis. This literature review explores three dominant themes of the research question: 1) cultivation of microalgae as feedstock for biodiesel production and 2) the utilization of wastewater as nutrient source for the growth of microalgae 3) biodiesel production.

2.1 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganism that can grow rapidly due to their simple multicellular structure (Mata, Martins & Caetano, 2010). Microalgae are present in all existing Earth ecosystem that covered a wide range of environmental condition that not just aquatic environment but also terrestrial. Microalgae representing a variety of species and it are estimated that more than 50,000 species exist but only around 30,000 species of microalgae have been studied and analysed (Richmond, 2004). The production biodiesel from microalgae biomass have received much attention recently as the third generation feedstock for advance biofuels production as well as contribution to the quality of the environment. The production of biodiesel that is derived from microalgae biomass have better advantages over crops and plant sources such as; higher photosynthetic efficiency, shorter reproduction cycle, high in nutrients absorption efficiency, high amount of lipid content and biomass productivity (Jaimes-Duarte *et al.*, 2012).

Details description on the potential conversion of microalgae biomass to biodiesel are discussed as following:

1. Microalgae are capable to be produced throughout the year, thus the productivity of oil from microalgae biomass exceeds the yield produced from the oilseed crops, e.g.: biodiesel yield of 12,000 L/ha for microalgae compared with 1190 L/ha for rapeseed (Schenk *et al.*, 2008).
2. Microalgae grow in aqueous media but the growth of microalgae require less water than terrestrial crop, thus reducing the load on freshwater source (Dismukes *et al.*, 2008).
3. The cultivation of microalgae may not incur land-use change because microalgae can be cultivated in brackish water on non-arable land thus able to minimize environmental impacts (Searchinger *et al.*, 2008).
4. Microalgae have a rapid growth rate and have oil content in the range of 20-50% of their dry weight of biomass (Chisti, 2007).
5. Wastewater can be used as nutrients provider for microalgae cultivation, therefore, other than providing growth medium for microalgae, there is potential of treatment of organic effluent from agriculture and food industry (Cantrell *et al.*, 2008)
6. In term of air quality maintenance and improvement, microalgae biomass production can help in bio-fixation of CO_2 (1kg of dry algal biomass utilized about 1.83 kg of CO_2) (Chisti, 2007).

In order for the microalgae strain to contribute a high oil yield, microalgae species can be induced to accumulate substantial quantities of lipid (Sheehan *et al.*, 1998). The average lipid content of a species of microalgae varies between 1% to 70% but some species can reach about 90% of lipid content of their per dry weight under certain growing conditions (Chisti, 2007). The lipid content and lipid productivity of certain marine and freshwater microalgae species are shown in the Table 2.1.

TABLE 2.1. Lipid Content and Productivities of Some Microalgae Species

Microalgae species	Alga type	Lipid content (% dry weight biomass/Oil content)	Lipid productivity (mg/L/day)	Reference
Botryococcus braunii	Green	25.0–75.0	–	(Chisti, 2007).
Chaetoceros muelleri	Diatom	33.6	21.8	(Rodolfi *et al.*, 2009).
Chlorella emersonii	Green	25.0–63.0	10.3–50.0	(Luisa Gouveia and Oliveira, 2009).
Chlorella minutissima	Green	57	–	(Luisa Gouveia and Oliveira, 2009).
Chlorella protothecoides	Green	14.6–57.8	1214	(Miao and Wu, 2004).
Chlorella sorokiniana	Green	19.0–22.0	44.7	(Rodolfi *et al.*, 2009).
Chlorella vulgaris CCAP 211/11b	Green	19.2	170	(Rodolfi *et al.*, 2009).
Chlorella vulgaris	Green	5.0–58.0	11.2–40.0	(Rodolfi *et al.*, 2009).
Chlorella sp.	Green	18.0–57.0	18.7	(L. Gouveia *et al.*, 2009).
Nannochloris sp.	Green	20.0–56.0	60.9–76.5	(Chisti, 2007).
Nannochloropsis oculata.	Green	22.7–29.7	84.0–142.0	(Chisti, 2007).
Nannochloropsis sp.	Eustigmatophytes	12.0–68.0	37.6–90.0	(Chisti, 2007).
Neochloris oleoabundans	Green	29.0–65.0	90.0–134.0	(Chisti, 2007).

Spirulina platensis	Green	10.30 ± 0.10	–	(Peng *et al.*, 2001).
Spirulina maxima	Green	4.0–9.0	–	(Mata *et al.*, 2010).

2.2 Cultivation of Microalgae

Microalgae are unicellular microorganisms that can be found in freshwater as well as marine environments. Microalgae are using the sunlight as an energy source and CO_2 as a carbon source for cells production (Demirbas and Demirbas, 2011). The growth of a microalgae species follows the growth curve that presenting different phase, such as lag phase, exponential growth phase, linear growth phase, stationary growth phase and death phase (Andersson *et al.*, 2011). When sufficient of nutrients and sunlight are provided to microalgae cultivation, the microalgae could double their biomass within 24 hours. Figure 1 shows microalgae growth curve in a batch culture (solid line) and nutrients concentration (dashed line) represents in Figure 2.1. The curve also shows that the availability of the nutrients for the growth of microalgae decreases as the amount of biomass increases.

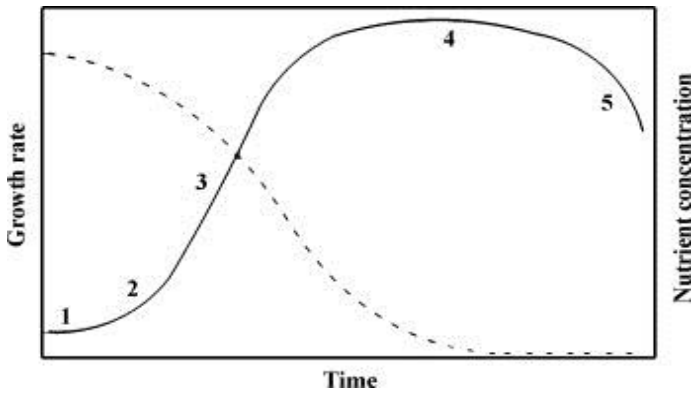


FIGURE 2.1. The graph of microalgae growth curve in batch culture (solid line) and nutrients concentration (dashed line); (1) lag phase, (2) exponential growth phase, representing the maximum growth rate under the specific conditions; (3) linear growth phase; (4) stationary growth phase; (5) decline or death phase (Mata *et al*., 2010)

The conversion of light energy into chemical energy involves a two-step process known as the dark reaction and light reactions. Dark reaction is the process of carbon fixation reaction that can occur in the presence or absence of light, while light reaction needs illumination for the reaction to occur. The conversion of energy occurs follows the following reaction (Ho *et al.,* 2011):

$$6CO_2 + 6H_2O + sunlight \rightarrow C_6H_{12}O_6 + 6O_2 \quad (1)$$

The growth of microalgae depends on various factors such as physical, chemical and biological where a drastic variation in one of the factors could affect the microalgae productivity and treatment efficiency. Examples of physical factors include light and temperature while for chemical factors such as the availability of nutrients and carbon dioxide and example for biological factors are competition between species and virus infections (Rathod, 2014).

Nutrients are necessary for the growth of microalgae. Two essential elements that are needed for the growing process are nitrogen and phosphorus (Andersson *et al.*, 2011). Nitrogen source such as nitrate, nitrite, ammonia and urea are absorbed by microalgae to regulate the synthesis of protein and growth metabolism (Rashid *et al.*, 2014). The form of nitrogen uptake is depends on the species of microalgae, but most of microalgae species prefer to use ammonia as nitrogen source (Rashid *et al.*, 2014). The pH of microalgae culture is altered by the uptake of nitrogen which influences the growth rate of the species. Another essential element for the cell growth and metabolism of microalgae is phosphorus. Phosphorus is absorbed by microalgae for the construction of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), cell membrane materials and it is important for the energy transfer during cellular processes (e.g. photophosphorylation) (B. Wang *et al.*, 2008). Moreover, for the photosynthesis process to occur requires large amount of proteins that are synthesized by phosphorus-rich ribosomes (Ågren, 2004). Thus, the deficiency of phosphorus may have severe impact on the microalgae metabolism, including reducing photosynthesis efficiency and lipid accumulation (B. Wang *et al.*, 2008).

Besides, some reports highlighted that if microalgae are cultivated in an environment that lack of nitrogen, it will promotes higher amount of lipid accumulation in the microalgae cells but low microalgae biomass growth (Lam and Lee, 2012a). The synthesis of starch is favoured in high nitrogen content in the culture medium, however, when the nitrogen nutrients is limited, the starch synthesis pathway is blocked and photosynthetically fixed carbon is redirected into fatty acid that leads to high lipid accumulation in microalgae cells (Yantao Li *et al.*, 2010). Consequently, the starch which is the carbon and the source of energy for the microalgae cells will be exhausted and reduces the photosynthetic efficiency. This will retarded the microalgae growth and thus less biomass are produced (Li *et al.*, 2008)

2.3 Methods of Microalgae Cultivation

Microalgae can be cultivated by using three different methods which are: 1) phototrophic, 2) heterotrophic and 3) mixotrophic. Phototrophic cultivation is utilizing light as energy source and CO_2 as inorganic carbon source, while heterotrophic only utilize organic substrate such as glucose, acetate and glycerol as the source for energy and carbon (Mata *et al*., 2010). According to Borowitzka (1999), phototrophic is the only cultivation method that is technically and economically feasible for commercial scale, typically at outdoor environment where light energy can be obtained in abundance and free. In addition, phototrophic microalgae can acts as carbon sink because of their ability to capture CO_2 from flue gas which is an added advantages to the culture system (Lam & Lee, 2012). Despite the feasibility and advantages of phototrophic method, the method has its limitation for the temperate countries where sunlight intensity is not available throughout the year.

On the other hand, heterotrophic cultivation overcome the limitation of phototrophic cultivation as some of microalgae strain able to grow under dark environment. The ability of microalgae to be less dependent on light energy (heterotrophic) allows for a simple scale-up possibilities as smaller reactor surface to volume ratio may be used for the cultivation of microalgae (Brennan and Owende,

2010). Moreover, heterotrophic cultivation provides a higher degree of control growth and lower harvesting costs due to higher cell densities achieved (G.-Q. Chen and Chen, 2006). However, for heterotrophic cultivation to be used for large scale production, several issues need to be addressed:

1. Only several species of microalgae strains can grow heterotrophically. According to Lam & Lee (2012), only *C. protothecoides, C. vulgaris, Crypthecodinium cohnii* and *Schizochytrium limacinum* are able to grow in total darkness and produced high quantity of lipid.
2. The presence of organic substrate (e.g. glucose, acetate, glycerol) can cause serious contamination by other microorganism (C.-Y. Chen *et al.*, 2011).
3. Increase in the energy consumption and cost due to addition of organic substrate. The most suitable organic substrate is glucose due to the high energy content; however, the consumption of glucose could trigger food versus fuel crisis as glucose is obtained from sugar-based plant that is also important for human consumption (Lam and Lee, 2012).
4. CO_2 is released through microalgae respiration from the heterotrophic cultivation thus does not able to synergize CO_2 mitigation from atmosphere (Li *et al.*, 2008a).

Some microalgae species are able to grow either using autotrophic or heterotrophic method depending on the concentration of organic carbon sources and light intensity available for the cultivation (Mata *et al.*, 2010). The cell growth of a mixotroph is not strictly depends on photosynthesis thus light energy is not an absolute limiting factors for the growth as its either light or organic carbon can support the growth of a mixotroph (Andrade and Costa, 2007). According to Brennan and Owende (2010), *Spirulina platensis* and *Chlamyddomonas reinhardtii* can be cultivated via mixotrophic method. The growth of the microalgae is influenced by the availability of glucose in the culture medium during the light and dark phase and therefore there will be less biomass loss during the dark phase (Andrade and Costa, 2007). From a study conducted by Chojnacka and Noworyta (2004), *Spirulina sp.* was grown in phototrophic, heterotrophic and mixotrophic cultivation. It was found that mixotrophic cultivation improved the growth rates of the microalgae species over phototrophic and heterotrophic cultures. The successful production of mixotrophic culture will reduces

the impact of biomass loss during dark respiration and at the same time decreases the intake of organic substances during growth phase (Brennan and Owende, 2010).

2.4 Microalgae Cultivation System

2.4.1 Open Pond Production System

The cultivation of microalgae in open pond production system has been practiced since 1950 (Borowitzka, 1999). The open pond system are made of a closed loop, oval shaped recirculation channels which has 0.2 to 0.5m in depth in order to allow sufficient of sunlight available for microalgae for optimum photosynthesis process (Brennan and Owende, 2010). In a continuous production cycle, both algae broth and nutrients are introduced in front of paddlewheel. The paddlewheel is functioning to prevent sedimentation of microalgae biomass and to circulate the culture medium through the loop until the harvest point (Brennan and Owende, 2010). Moreover, in order to have direct access of CO_2 from the flue gas, some open ponds are built on non-arable lands adjacent to the power plants and there are some ponds are built near the wastewater treatment facility for direct supply of nutrients (Cai *et al.*, 2013).

The open pond are commonly being used in most commercial scale of algae production because the system are relatively inexpensive to build and easy to scale up. However, disadvantages exists in some aspects of the system. The fact that it is an open pond, the system is exposed to the atmosphere which causes the increase in water loss in the system by evaporation especially when the surrounding temperature increases (Cai *et al.*, 2013). According to Richmond (2004), the water level in the pond cannot be lower than 15cm for the microalgae in the system to receive enough sunlight for growth. In addition, open pond system is dependent on the weather condition which does not allow for control of water temperature, evaporation and lighting for the system (Mata *et al.*, 2010). Moreover, in case the wastewater is used as nutrient source for large algae ponds, the wastewater needs to be sterilized prior to be used in the system in order to reduce the negative effects of pathogens and bacteria on microalgae

growth, which could increases the capital cost of open pond cultivation system (Cai *et al.*, 2013).

2.4.2 Closed Photobioreactor Systems

The photobioreactor cultivation system is designed to overcome some problems encountered by open pond cultivation system such as pollution and contamination risk. Closed system includes tubular, flat plate and column photobioreactor which allows a single species of microalgae culture for prolonged durations and lower the risk of contamination (Chisti, 2007). The design of photobioreactor consists of an array of straight glass or plastic tubes, generally 0.1m or less in diameter which functions to capture sunlight and can be arranged horizontally, vertically, inclined or as helix (Brennan and Owende, 2010). Mechanical pump or airlift system is used in photobioreactor to recirculate the algae cultures to allow for CO_2 and O_2 exchange between the liquid medium and the aeration gas as well as for mixing purpose (Eriksen, 2008). The agitation and mixing are very important process in the system to ensure gas exchange in the tubes.

Flat plate photobioreactor is the earliest form of technology for closed system and has received much research attention because of large surface area that are exposed to illumination (Ugwu *et al.*, 2008). This type of reactor is made up of transparent materials to maximize solar energy capture and a thin layer of dense culture flows across the flat plate that allows for absorbance radiates in the millimetres of thickness (Q. Hu *et al.*, 1998).

Tubular photobioreactor is not suitable to be scaled up because of the design limitation on the length of tubes and the design will highly resulted to O_2 accumulation, CO_2 depletion and pH variation in the systems (Eriksen, 2008). Therefore, the only possible way for large scale production of tubular photobioreactor system is by integrating multiple reactor units. However, tubular photobioreactor is suitable for

outdoor mass culture because the system expose to a larger surface area to sunlight (Ugwu *et al.*, 2008).

On the other hand, column photobioreactor provides the most efficient mixing, high volumetric mass transfer rates and controllable growth conditions for the cultivation of microalgae (Eriksen, 2008). The building of column photobioreactor is low cost, compact and easy to operate which make it favourably with tubular photobioreactor. Table 2.2 summarized the advantages and limitation of various photobioreactor for microalgae cultivation.

TABLE 2.2. Prospects and Limitations of Various Culture Systems for Algae (Ugwu *et al.*, 2008)

Culture systems	Prospects	Limitations
Open ponds	-relatively economical compare to other systems. -easy to clean up after cultivation. -good for mass cultivation of algae.	-less control of culture conditions. -difficulty in growing algal cultures for long periods. -occupy large land area. -limited to few strains of algae. -cultures are easily contaminated.
Flat-plate photobioreactor	-large illumination surface area. -suitable for outdoor cultures. -good biomass productivities. -relatively cheap, easy to clean up.	-scale-up require many compartments and support materials. -difficulty in controlling culture temperature.
Tubular photobioreactor	-large illumination surface area.	-gradients of pH, dissolved oxygen and CO_2 along the tubes, fouling.

Culture systems	Prospects	Limitations
	-suitable for outdoor cultures. -fairly good biomass productivities and relatively low cost.	-some degree of wall growth. -requires large land space.
Vertical-column photobioreactor	-high mass transfer. -good mixing with low shear stress. -low energy consumption, high potentials for scalability.	-small illumination surface area. -their construction require sophisticated materials. -decrease of illumination surface area upon scale-up.

2.5 Wastewater as Nutrients Provider for Microalgae Growth

2.5.1 Feasibility of Wastewater

According to World Bank (2012), annual freshwater consumption was estimated at 3,908.3 billion m^3 globally in the year 2009, and most of the consumed water turned into wastewater. The total nitrogen and phosphorus concentration in wastewater is depends on the type of wastewater where the concentration of the nutrients in municipal wastewater can be as high as 10-100 mg/L whereas for agriculture effluent the concentration of the nutrients can be more than 1000 mg/L (de la Noüe *et al.*, 1992; M. Singh *et al.*, 2011). Thus, without a proper treatment of the wastewater could lead to eutrophication and subsequently harmful to the ecosystem in the downstream watersheds area (Cai *et al.*, 2013). One of the example of freshwater eutrophication was recorded in Ohio's largest inland lake, Grand Lake St. Marys. The eutrophication was due to animal manure that was applied on the crop land surrounding the lake watershed area and the wastewater treatment facility that also located surrounding the lake contributed 5-10% of the phosphorus load into the lake (Cai *et al.*, 2013). The algae bloom in the lake affected the fishing and tourism industries surrounding the area as well as raised concerns from the residents on the

health issue. Economical approach for reducing the impacts of nutrients in the wastewater need to be urgently conducted.

The current conventional wastewater treatment technologies mainly are using chemical and physical method which are not economical for the treatment of agriculture wastewater. Extensive researches have been done to study the effectiveness of cultivation of microalgae and nutrients removal in various sources of wastewater, such as municipal, agriculture and industrial wastewater and it has been proven that substantial amount of algae biomass was produced with nutrient reduction in the wastewater (Cai *et al.,* 2013). Therefore, microalgae cultivation has the potential to be developed as biological method for wastewater treatment in order to substitute the current conventional wastewater treatment technology.

The utilization of microalgae for wastewater treatment could provide some advantages over conventional wastewater treatment (Oilgae, 2009):

1. Cost effective way in removing biochemical oxygen demand, pathogens, phosphorus and nitrogen than activated sludge process and other secondary treatment processes.
2. Traditional wastewater treatment requires high energy for mechanical aeration to provide oxygen to aerobic bacteria to consume the organic compound in the wastewater, whereas in algae based wastewater treatment, the oxygen for the aerobic bacteria will be provided by the algae. According to Oswald (2003), one kg of biochemical oxygen demand (BOD) reduced by the photosynthetic oxygenation requires no energy input and produces enough algal biomass that can produce one kWh of electric power.
3. Reductions in sludge formation as microalgae wastewater treatment technology avoid the use of chemicals and the entire process of effluent treatment is simplified. The resulting sludge with microalgae biomass is energy rich which can be further processed to make biofuels or fertilizers.
4. Reduction in greenhouse gases (GHG) emission as CO_2 that is being released by the wastewater treatment plant can be consumed by the algae for their

growth and thus reducing the carbon footprint of the wastewater treatment plant.

2.5.2 The benefits of Cultivation of Microalgae Using Wastewater

The cultivation of microalgae at industrial scale requires substantial amount of nutrients for the growth of microalgae usually obtained from chemical fertilizer. However, the production of chemical fertilizer has been recognized as energy intensive industry, in which about 37 to 40 GJ of low heating value of natural gas is consumed in producing 1 tonne of ammonia (Rafiqul *et al.,* 2005). According to Kim and Dale (2005), 1.2 kg of CO_2 is released for every 1 kg of ammonia produced. It is recorded that the highest source of industrial CO_2 emission in the United States is contributed from ammonia manufacturing plant (S. Kim and Dale, 2005). Therefore, the usage of chemical fertilizer as nutrient provider for the growth of microalgae is not sustainable for long term. In addition, if production of biofuels derived from microalgae requires large amounts of national fertilizer surpluses, this would impact on other markets such as food production as each year Food and Agriculture Organization (FAO) of the United Nations provides the reports on synthetic fertilizers world surplus values (Canter *et al.*, 2015). According to Canter *et al.*, (2015), the world surplus of nutrients cannot meet the requirement for production of microalgae based biofuels and thus, alternative source of nutrients are required for microalgae cultivation.

In order to ensure the long term sustainability, an alternative and low cost nutrient sources need to be identified to replace chemical fertilizer which imposes severe impact towards overall energy balance in microalgae cultivation. Wastewater appears to be an attractive and low cost nutrient sources and has the potential to be used for microalgae cultivation (Lam & Lee, 2012). In normal wastewater treatment facility, the primary treatment does not completely remove the nutrients. Hence, secondary and tertiary wastewater still contain significant amount of nitrogen and phosphorus. In order to completely remove the nutrients, about 60% to 80% of energy need to be consumed in the wastewater treatment plant (Clarens *et al.,* 2010). Treated

wastewater that containing nitrogen and phosphorus can cause unwanted algae bloom that could resulted in eutrophication if it is allowed to flow into waterways (Aslan and Kapdan, 2006). The nutrients available in the wastewater can be utilized by the microalgae for their growth and therefore provides mutual benefit of producing biofuels and removing nutrients from the wastewater (Mostafa *et al.*, 2012). Furthermore, microalgae cultivation using wastewater is more environmental friendly and sustainable because it does not generate additional pollutants such as sludge as by-products and provides opportunity for efficient recycling of nutrients where the nitrogen and phosphorus that contained in the wastewater can be utilized by the microalgae for their growth (Munoz and Guieysse, 2006).

Generally, most of the research and analysis on microalgae cultivation using wastewater was conducted on a laboratory and pilot scale. The growth of microalgae have been analysed on wide range of studies consisted of variety of wastewater conditions which mainly growth in municipal (urban) sewage wastewater, agriculture wastewater, industrial wastewater and artificial wastewater (Pittman *et al.*, 2011). Most of the studies are mainly focussed on the potential of microalgae for removing nutrients in various sources of wastewater. These initial experimental studies, particularly those that have assessed the variables for maximizes the algae biomass production are truly beneficial for evaluating the wastewater grown microalgae for biodiesel production.

However, wastewater-based algae cultivation is still covered with many uncertainties and challenges including the variation of wastewater composition due the sources, infrastructure, weather conditions and pre-treatment method. In addition, the high turbidity due to the presence of pigments and suspended solid particles that affects the light transmission in microalgae culture and the presence of competing micro flora and toxic compounds could inhibit the growth of microalgae (Zhou *et al.*, 2014). Cultivation of microalgae in municipal and agricultural wastewaters have been extensively studied due to the availability of the wastewater resources and contain less variables compare to other types of wastewater, such as industrial wastewater. The

following section further discussed the growth of microalgae and nutrient removal from various sources of wastewater.

2.5.2.1 Municipal Wastewater

In a conventional municipal wastewater treatment facility usually consisting of primary treatment where the process of sedimentation of solid materials occur. Then, continued by secondary treatment where the suspended and dissolved organic materials are removed. This followed by tertiary treatment in which the final treatment of the water is performed before being discharged to the environment (Pittman *et al.*, 2011). The tertiary treatment is the phase where the removal of many dissolved organic compounds including nitrogen (N) and phosphorus (P) take place and the potential of N and P removal using microalgae has been extensively assessed during the tertiary phase.

Up-to-date, most of studies have evaluated the growth performance of two microalgae species, namely *Chorella* and *Scenedesmus* genus. This is because both of the species showed a high tolerant to the contaminated environment (L. Wang *et al.*, 2010). For example, according to a recent study, *Chorella* and *Scenedesmus* recorded a very high (>80%) removal of ammonia, nitrate and total phosphorus from secondary treated wastewater (Ruiz-Marin *et al.*, 2010). Many of these experiments were conducted under laboratory based batch culture conditions where the microalgae showed higher growth rates over the batch growth period.

In another study, *Chorella minutissima* was found to be able to grow well in high concentration of raw sewage in wastewater treatment oxidation ponds in India (Bhatnagar *et al.*, 2010). The study also had discovered that the species could grow heterotrophically in the dark and mixotrophically in the light by using variety of carbon substrates in varies pH condition and in the presence of salt. This algae species recorded the highest growth under mixotrophic condition with biomass productivity if

379 mgL^{-1} compared to 73.03 mgL^{-1} under phototrophic condition after 10 days of growth (Bhatnagar *et al.*, 2010).

From the examples highlighted previously, it can be concluded that the *Chorella* and *Scenesdesmus* species could be good candidates for domestic wastewater treatment, especially in high-rate pond system. In addition, cultivation of algae in nutrient-rich municipal wastewater has the potential to enhance algae biomass productivity and serve as mutual roles of nutrient removal and cost effective biodiesel feedstock production (Zhou *et al.*, 2014).

2.5.2.2 Agricultural Wastewater

A comparison of mineral composition between mass culture media and animal manure wastewater showed that animal manure wastewater appeared to be more suitable medium for the growth of microalgae. A few researches had reported that specific species of microalgae are efficient nutrient removal from manure-based wastewater (Zhou *et al.*, 2014).

For example, *Botryococcus braunii*, a green algae grew well in swine manure wastewater that contained 788 mgL^{-1} NO_3 and removed about 80% of the initial NO_3 content (An *et al.*, 2003). From the study, it was clearly indicated that the pre-treated piggery wastewater has high potential to be a good culture medium for the growth of *B. Braunii*. Furthermore, mixotrophic type of microalgae also was evaluated to cultivate in digested poultry litter effluent and high biomass productivity of 76 mgL^{-1} was observed, but with relatively low lipid (M. Singh *et al.*, 2011). An alternative medium was developed in which deep seawater was mixed with fermented swine urine and cow compost water in order to enhanced algae biomass production and stimulate a faster algae growth through nutrients supplement (M. K. Kim and Jeune, 2009).

2.5.2.3 Industrial Wastewater

Industrial wastewater are commonly categorized as unsuitable for cultivation of microalgae due to variable constituents of wastewater from different industries which have the potential impact of toxic compound that could inhibit the microalgae growth. Thus, most research on algae-based treatment on industrial wastewater is mainly focused on the removal of heavy metals pollutants (cadmium, chromium, zinc, etc.) and organic chemical toxins (hydrocarbons, biocides and surfactants) rather than utilized the wastewater for microalgae cultivation for biofuels purposes (Ahluwalia and Goyal, 2007). Generally, the wastewater that are coming from industrial sites contain low amount of N and P and high toxin concentration that may retard the growth of microalgae. Therefore, industrial wastewater is not suitable to be utilized for large scale microalgae cultivation compare to municipal and agriculture which are more uniform in the constituents of the wastewater and are widely available.

However, one study proved the suitability of industrial wastewater in providing resources for the generation of microalgae biomass. Two freshwater microalgae *B. Braunii* and *Chorella saccharophilla*, and a marine algae *Pleurochrysis carterae* was recorded to be able to sustain growth in wastewater from carpet mill effluent that contained process chemicals and pigments that used in the process mills, in addition to inorganic elements including low concentrations of metals and relatively low concentration of N and P (S. Chinnasamy *et al.*, 2010). Table 2.3 summarized the recent research works in growing microalgae using different type of wastewater.

TABLE 2.3. Lipid Content and Lipid Productivity of Microalgae Species in Different Wastewater Resources

Wastewater type	Microalgae species	Biomass (DW) productivity (mg/L.day)	Lipid content (% DW)	Lipid productivity (mg/L.day)	References
Municipal (primary treated)	Nil	25	Nil	Nil	(Ip *et al.*, 1982).
Municipal (secondary treated)	*Scenedesmus obliquus*	26	31.4	8	(Martınez *et al.*, 2000).
Municipal (secondary treated)	*Botryococcus braunii*	345.6	17.85	62	(Órpez *et al.*, 2009).
Municipal (primary treated + CO_2)	Mix of *Chlorella sp.*, *Micractinium sp.*, *Actinastrum sp.*	270.7	9	24.4	(Woertz *et al.*, 2009).
Agricultural (piggery manure with high NO_3-N)	*B. braunii*	700	Nil	69	(An *et al.*, 2003).
Agricultural (dairy manure with polystyrene foam support)	*Chlorella sp.*	2.6 gm^{-2} day^{-1}	9	230 mg m^{-2} day^{-1}	(Johnson and Wen, 2010).
Agricultural (fermented swine urine)	*Scenedesmus sp.*	6	0.9	0.54	(M. Kim *et al.*, 2007).
Agricultural (swine effluent, maximum manure loading rate)	*R. hieroglyphicum*	10.7 gm^{-2} day^{-1}	0.7	72 mg m^{-2} day^{-1}	(Mulbry *et al.*, 2008).

Agricultural (dairy effluent + CO_2, maximum manure loading rate)	*R. hieroglyphicum*	17.9 g m^{-2} day^{-1}	1.2	210 mg m^{-2} day^{-1}	(Mulbry *et al.*, 2008).
Agricultural (digested dairy manure, 20× dilution)	*Chlorella sp.*	81.4	13.6	11	(L. Wang *et al.*, 2010).
Agricultural (dairy wastewater, 25% dilution)	Mix of *Chlorella sp.*, *Micractinium sp.*, *Actinastrum sp*.	59	29	17	(Woertz *et al.*, 2009).
Industrial (carpet mill, untreated)	*B. braunii*	34	13.2	4.5	(Senthil Chinnasamy *et al.*, 2010).
Industrial (carpet mill, untreated)	*Chlorella saccharophila*	23	18.1	4.2	(Senthil Chinnasamy *et al.*, 2010).
Industrial (carpet mill, untreated)	*Dunaliella tertiolecta*	28	15.2	4.3	(Senthil Chinnasamy *et al.*, 2010).
Industrial (carpet mill<, untreated)	*Pleurochrysis carterae*	33	12	4	(Senthil Chinnasamy *et al.*, 2010).

2.6 Biodiesel Production: An Introduction

The extensive usage of fossil fuels and natural gas as the source of energy for various industry and transportation sector had caused the depletion in the primary energy resources on Earth. In addition, increasing demand for energy, fluctuation in the price of crude oil in the market, global warming phenomena due to the emission of greenhouse gases and environmental pollution also contributes to the factor that

encourage the effort in finding alternative source of energy. Currently about 86% of energy being consumed worldwide and nearly about 100% of supply of energy for transportation sector comes from non-renewable fossil fuels (Abbaszaadeh *et al.*, 2012). One of the feasible ways to support the increasing demand for energy is by utilizing renewable and sustainable energy sources such as biofuels which is derived from biomass. Several countries such as Brazil, United States, Germany, Australia and Italy are already utilized biofuels especially bioethanol and biodiesel as their source of energy due to the advancement in technology and the availability of feedstock in the respective countries (Yusuf *et al.*, 2011).

Looking back in the history, biodiesel has been used in the industrial activities starting in the year 1880s where Rudolf Diesel designed a prototype of diesel engine that received a German Patent in the year 1892 and demonstrated a workable engine in 1897 by using peanut oil as fuel. Rudolf Diesel viewed that his engine will be utilizing the fuel derived from biomass particularly plant oils and animals fats. However, in 1920s, the advancement in technology at that time had made possible a much smaller diesel engine which required the used of fuel that has lower viscosity and injected by small injectors (Salvi and Panwar, 2012). In addition, a relatively cheap medium-weight diesel engine using diesel fuels originated from fossil had put a break to the commercial viability of biofuels. So the research activities on the vegetable-oil fuels were not extensively pursued. Since 1930s, the diesel engine has been designed to be compatible running on diesel fraction of crude oil that consists mainly of saturated hydrocarbon (Salvi and Panwar, 2012). Nowadays, the fuel trend has been shifted towards the usage of renewable sources like biofuels for the purpose of energy security and environmental protection. Among all the biofuels that are available to date, biodiesel is the one that received the most attention due to its similarity with the conventional diesel in terms of its chemical structure and energy content (Yusuf *et al.*, 2011). In addition, the biodiesel can be used in the existing diesel engine without any modification and is currently blended with fossil diesel as transportation in countries like Germany, Italy and Malaysia (Yusuf *et al.*, 2011).

Biodiesel is a mono alkyl ester made up of long chain of fatty acids that is derived vegetable oils and animal fats. Commercially biodiesel is produced by the transesterification of the vegetable oils with short chain such as methanol or ethanol

(Salvi & Panwar, 2012). There are various renewable raw materials that have the potential to be used as feedstock for biodiesel production such as edible and non-edible vegetables oils, animal fats, recycled or waste oils, by-products of the edible oil and dairy industries and other saturated and unsaturated fatty acid that varies in carbon chain length and degree off saturation. The criteria of the selection of vegetable oils include the availability, cost, oil quality, and product shelf life. The physical properties of biodiesel are given in Table 2.4. Biodiesel is a clear amber yellow liquid with the viscosity is similar to petroleum based diesel. Biodiesel is also biodegradable and non-toxic where it significantly reduces toxic and other emissions to the atmosphere when burned as fuel.

TABLE 2.4. Physical Properties of Biodiesel (Yusuf *et al.*, 2011)

Parameter	Characteristic Properties
Common chemical name	C_{14}-C_{24} methyl ester or $C_{15\text{-}25}H_{28\text{-}48}O_2$
Density range (kg/m^3 at 288K)	860-894
Boiling point range (K)	>457
Flash point range (K)	420-450
Distillation range (K)	470-600
Vapor pressure (mmHg at 295K)	<5
Solubility in water	Insoluble in water
Physical appearance	Light to dark yellow, clear liquid
Biodegradability	More degradable than petroleum diesel
Reactivity	Stable but avoid strong oxidizing agent

Although biodiesel cannot entirely replace current the portion of petroleum-based diesel fuel usage, there are a number of reasons to justify the development of technology in the production of biodiesel (Van Gerpen, 2005):

1. The production of biodiesel providing a market for excess production of vegetable oils and animal fats.
2. The utilization of biodiesel reduces the country dependency on imported petroleum as fuels.
3. Biodiesel is renewable and does not contribute to global warming due to the properties of closed carbon cycle. A life cycle analysis of biodiesel showed that

overall CO_2 emissions were reduced by 78% compared to the petroleum based diesel fuel.

4. The emission of carbon monoxide, unburned hydrocarbons and particulate emission from the burning of biodiesel is lower than the emission from the regular diesel fuel. However, there is slight increases in oxides of Nitrogen (NO_x) in most emissions tests that have been conducted.
5. The mixing of biodiesel to regular diesel fuel in an amount equal to 1-2% can convert fuel with poor lubrication properties into an acceptable fuel.

2.7 Biodiesel Production Technology

A lot of researches have been made in order to develop the process of converting vegetable oil derivatives into approximately the properties and performance of petroleum based diesel fuels. However, the problems in substituting triglycerides for diesel fuels are mostly due to the high viscosity, low stability against oxidation and low volatility which influences the formation of high amount of ashes due to incomplete combustion (Robles-Medina *et al.,* 2009). The conversion of triglycerides to biodiesel can be performed through several technologies as discussed in the following section.

2.7.1 Direct Use and Blending

Vegetable oil can be mixed directly with diesel fuel to be used directly for running an engine. Several researches have been conducted and positive result proved that the blending of vegetable oils with diesel fuels able to power up a diesel engine. In 1980, Caterpillar Brazil Company tested pre-combustion chamber engine with a mixture of 10% vegetable oil to maintain total power without any modification to the engine. A blend of vegetable oil up to 20% and 80% of diesel was found to be successful to power up the engine (Narayan, 2002).

According to a study conducted by Pramanik (2003), to investigate the effect of mixing of jatropha curcas oil with diesel on the performance of compression ignition engine. The study found that about 50% of jatropha oil can be mixed with diesel without any major operating difficulties of the engine. However, the result of the study can be further improved by using higher purity of jatropha oil which required the pre-treatment of the oil. Moreover, study on the durability of the engine to consume biodiesel for long term also needs to be conducted to justify the utilization of biodiesel as fuel for normal engine (Pramanik, 2003). Nevertheless, for long term operation, the direct mixing of vegetable oils to the diesel is found to be impractical for both direct and indirect diesel engines. The main issue is due to the high viscosity, acid composition, free-fatty acid content, gum formation due to oxidation, carbon deposit and lubricating oil thickening which heavily damaging the diesel engine (Yusuf *et al.*, 2011).

2.7.2 Microemulsion

Microemulsion is thermodynamically stable dispersions of oil, water, surfactant and a small amphiphilic molecule called a co surfactant. The diameters of the droplet in the microemulsion ranging from 100 to 1000Å (Yusuf *et al*., 2011). Microemulsion can be made of vegetable oils with an ester and dispersant or of vegetable oils, an alcohol and a surfactant. The mixing can be with or without diesel fuels. In addition, microemulsion have lower volumetric heating values than diesel fuels because of the alcohol content but the alcohol provides high latent heat of vaporization and tend to cool the combustion chamber (Yusuf *et al.*, 2011). A microemulsions of methanol with vegetable oils can perform nearly as good as petroleum based diesel.

2.7.3 Thermal Cracking (Pyrolysis)

Pyrolysis is a process of converting one organic substance into another by process of heating in the presence of catalyst. The pyrolyzed material can be vegetable

oil, animal fat, natural fatty acids or methyl esters of fatty acids. A lot of researches have been conducted to study on the pyrolysis of triglycerides to obtain products that is suitable to be used in diesel engine. Alkanes, alkenes, alkadienes, aromatic and carboxylic acids are products that can be produced by the thermal decomposition of triglycerides (Yusuf *et al.*, 2011). The mechanism for the thermal decomposition of triglycerides is complex because of many structures and possibility reaction of mixed triglycerides. The mechanism is shown in Figure 2.2.

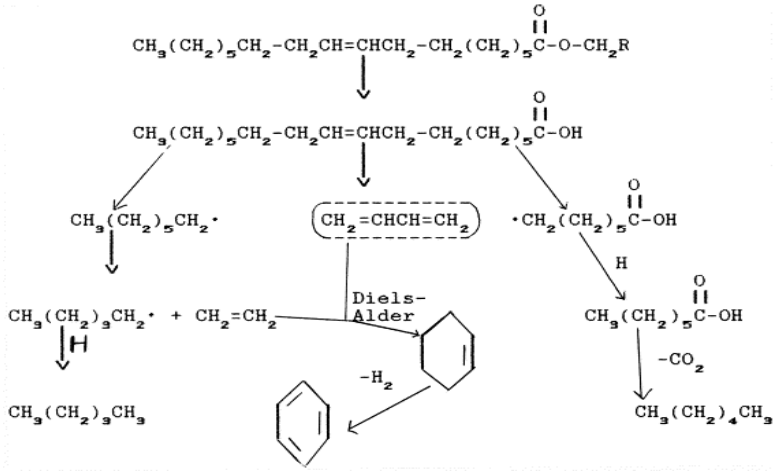


FIGURE 2.2. The Mechanism of Thermal Decomposition of Triglycerides (Schwab *et al.*, 1988)

The pyrolysis reaction has been studied on soybean, palm and castor oil and from the study, there is possibility to isolate fuels with physical and chemical properties comparable to specification of petroleum based diesel by having adequate choice of distillation temperature ranges (Lima *et al.*, 2004).

2.7.4 Transesterification

Transesterification is the most common process in producing biodiesel. Transesterification is a process of reacting triglyceride with an alcohol in the presence of alkaline or acid catalyst to produce fatty-acid methyl ester (FAME) as the main product and glycerol as the by-product (Yusuf *et al.*, 2011). Methanol and ethanol are the common alcohols that are being used for transesterification process due to their low cost and easily dissolved and react quickly with triglycerides. An alkaline

catalysed transesterification process is commonly used for biodiesel production because alkaline alkoxides and hydroxides are more reactive than acid catalyst. A 3:1 molar ratio of alcohol to triglycerides is needed in order to complete a transesterification stoichiometrically (Ma and Hanna, 1999). The general transesterification reaction is illustrated in Figure 2.3.

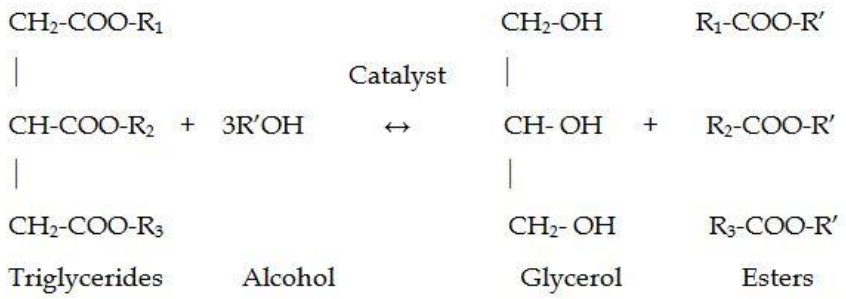


FIGURE 2.3. Transesterification Reaction of Triglycerides with Alcohol (Chisti, 2007)

In a study of the kinetics of palm-oil transesterification in a batch reactor conducted by Darnoko and Cheryan (2000), the study proved that the overall conversion of the transesterification process did not affect by the change in temperature. However, the rate of the transesterification process was increased with the increased in temperature. The overall kinetic reactions are dependent on the individual rate constant for the conversion of triglycerides to diglycerides, monoglycerides and alcohol ester. From the result that was obtained, the conversion of triglycerides to diglycerides was the slowest reaction for the whole process. The study concluded that the rate of transesterification was high at high temperature because of the time taken for the mass transfer to occur was shortened as the temperature increased (Darnoko and Cheryan, 2000).

CHAPTER 3

METHODOLOGY

This chapter outlines the general methodology of the study, the research design and research instruments that will be involved in conducting the research project. The details of the methodology is further explain in the following section.

3.1 Pure Microalgae Strain and Culture Conditions

Chorella vulgaris was provided by Prof. Lee Keat Teong (School of Chemical Engineering, Universiti Sains Malaysia). Prior to the experiments, the microalgae was preserved and grown in Bold's Basal Medium (BBM) consisting of: (1) 10 mL per liter of culture medium with the following chemicals: $NaNO_3$ (25 g/L), $CaCl_2 \cdot 2H_2O$ (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (7.5 g/L), K_2HPO_4 (7.5 g/L), KH_2PO_4 (17.5 g/L), NaCl (2.5 g/L) and (2) 1 mL per litre of culture medium with the following chemicals: EDTA anhydrous (50 g/L), KOH (31 g/L), $FeSO_4 \cdot 7H_2O$ (4.98 g/L), H_2SO_4 (1 mL), H_3BO_3 (11.4 g/L), $ZnSO_4 \cdot 7H_2O$ (8.82 g/L), $MnCl_2 \cdot 4H_2O$ (1.44), MoO_3 (0.71 g/L), $CuSO_4 \cdot 5H_2O$ (1.57 g/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.49 g/L) (Lam and Lee, 2012b). The initial pH of the medium was adjusted to 3.5. The seed culture was grown in a 100 ml Erlenmeyer flask containing 50 ml of medium, aeration with compressed air, surrounding temperature ranging from 25-28^{o}C and illuminated with cool-white fluorescent light (Philip TL-D 36W/865, light intensity of 60-70 μmol m^{-2} s^{-1}) continuously.

3.2 Cultivating *Chorella vulgaris* with Wastewater

The wastewater samples were collected from two points (inlet and outlet from primary/secondary treatment point) of wastewater treatment plant at Universiti Teknologi Petronas. The samples were collected in 5 L of polyethylene bottles. Then, the samples were kept undisturbed for the settling of large particles initially for 24 hours and after that the samples were analysed for various parameters such as nitrite, nitrogen, nitrate nitrogen, ammonia nitrogen and total phosphorus following standard methods (American Public Health Association, 2005). Prior being applied for microalgae cultivation, all wastewater samples were filtered using 1.2 μm-pore size GF/C filter (Whatman co.). The process will able to remove most eukaryotes but a fraction of bacteria and cysts of protozoa will remain.

Then, a pre-determined volume of the wastewater samples were introduced into the 1L of Erlenmeyer flask with tap water and the pH of the medium was changed according to the pre-determined value. Subsequently, *Chorella vulgaris* was introduced into the photobioreactor.

3.3 Measurement of Microalgae Growth

Dry cell weight is commonly used expression to determine the biomass production in biological process and it is obtain by measuring the total suspended solid concentration in cultures medium. However, the measurement of a small volume of low concentration of biomass is error-prone. Alternative method to determine the dry cell weight is by measuring the optical density (OD) at 688 nm by the spectrophotometer (Shimadzu UV mini-1240). 10 mL of sample was centrifuged at 10x1000 g for 5 min. The microalgae biomass was dried in an oven at 100^{o}C for 24 hours and the supernatant was decanted back into the culture medium. The correlation between the dry cell weights and optical density was established.

3.4 Measurement of Nitrate Content in the Medium

A sample of 1 mL collected from the flask and the sample was centrifuged at 10x1000 g for 5 min. The optical density was measured at 275 nm and 220 nm using spectrophotometer (Shimadzu UV mini-1240). Then, the absorbance reading at 275 nm subtracted two times by the reading obtained at 220 nm to obtain the actual absorbance that is caused by the presence of nitrate (Lam and Lee, 2012b). Dry potassium nitrate (KNO_3) at different concentrations was used for calibration purposes.

3.5 Microalgae Harvesting and Biomass Collection

After the cultivated microalgae had achieved the stationary growth phase, the air aeration to the culture medium was stopped. The microalgae was left for two days to it settle naturally to the bottom of photobioreactor for two days. Two distinguished layers were formed at the bottom of photobioreactor. Water with suspended microalgae cells formed the upper layer and microalgae biomass deposited at the bottom layer. The upper layer was carefully poured out and the microalgae biomass left in the flask was dried in an oven at 80^{o}C for 24 hours. Next, the dried microalgae biomass was scrapped from the bottom of the flask and kept in empty container.

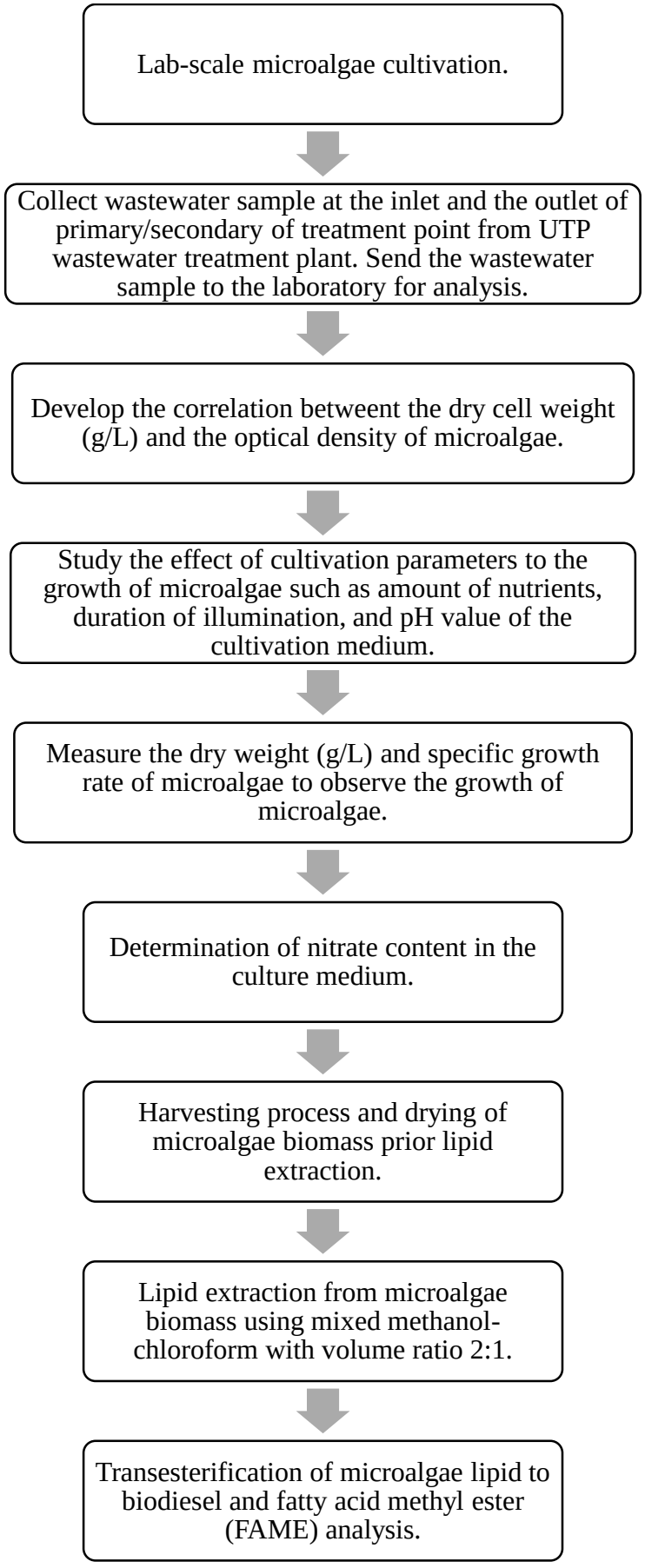
3.6 Microalgae Lipid Extraction

The dried *Chorella vulgaris* biomass was placed in a cellulose thimble and Soxhlet extractor was used for the extraction process. Methanol-chloroform with volume ratio of 2:1 used as solvent for the lipid extraction process (Bligh and Dyer, 1959). A total of 250 mL of solvent was placed in the Soxhlet extractor and heated to 60-65^{o}C for 24 hours. Then, rotary evaporator was used to further evaporated the solvent the leftover lipid was collected. The residue left after the process was extracted using the same solvent twice.

3.7 Transesterification Reaction and Fatty Acid Methyl Esters (FAME) Analysis

1 g of crude *Chorella vulgaris,* 15:1 of methanol to lipid molar ratio and 3 wt.% of concentrated sulphuric acid reacted for transesterification process. The reaction carried out in a water bath shaker at 60ºC for 3 hours. 1 μL of the reaction product was subjected to gas chromatography-mass spectrometry (GC–MS; PerkinElmer Clarus 600) analysis after the reaction has complete. The Gas Chromatography was equipped with flame ionization detector (FID) and Elite 5-MS column (30 m × 0.25 mm × 0.25 mm). The initial oven temperature was 65°C, held for 2 min and raised to 280°C at ramping rate of 8°C/min and held at 280°C for 10 min (Lam and Lee, 2012b).

3.8 Process Flow of the Research Project



3.9 Gantt Chart of FYP I

Week / Date	1 14/1	2	3	4	5	6	7	8 4/3	9	10 18/3	11	12 1/4	13 8/4	14
Selection of Final Year Project title	X													
Preliminary research work: Perform literature review related to the research project.														
Prepare the methodology of the experimental work.														
Identify the chemicals and equipment to be used in the project														
Submission of Extended Proposal to the SV and Coordinator.								X						
Prepare the presentation slide for Project Proposal Defence.														
Project Proposal Defence										X				
Developing lab scale microalgae cultivation system.														
Collecting wastewater sample and analyse the content.														
Develop the correlation between the dry cell weight and the optical density of microalgae.														
Interim report writing.														
Submission of Draft Interim Report to SV.												X		
Submission of Final Interim Report (after correction).													X	

3.10 Gantt Chart of FYP II

Week / Date	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Optimization study of microalgae biomass productivity in different culture parameters: • Nutrients content • Duration of illumination • pH value of the culture medium														
Determination of nitrate content in culture medium.														
Harvesting and microalgae biomass collection.														
Lipid extraction process.														
Transesterification of microalgae lipid to biodiesel.														
Fatty acid profile analysis on the microalgae biodiesel produced.														
Submission of progress report.							X							
Pre-SEDEX										X				
Submission of Draft Final Report.														
Submission of Dissertation (soft bound)														
Submission of Technical Paper.											X			
Viva												X		
Submission of Project Dissertation (hard bound)														X

3.11 Key Milestones

FYP	Date Start	Date End	Period	Milestones
FYP I	20/1/2015	20/1/2015	1 day	Selected the title for the research project.
	30/1/2015	27/1/2015	4 week	Completed writing the extended proposal report and submitted to the supervisor to be reviewed.
	2/3/2015	4/3/2015	3 days	Made correction to the extended proposal report and submitted to the supervisor to be evaluated.
	3/3/2015	18/3/2015	2 weeks	Completed and presented Proposal Defence.
	2/3/2015	20/4/2015	7 weeks	Completed preliminary experimental work: 1. Lab-scale microalgae cultivation 2.Wastewater collection and analysis 3. Establish the dry cell weight and optical density correlation.
	11/3/2015	8/4/2015	4 weeks	Completed the interim report writing.
FYP II	18/5/201	15/6/2015	4 weeks	Completed optimization study on biomass productivity from microalgae using wastewater as nutrients source.
	15/6/2015	6/7/2015	3 weeks	Microalgae biomass collection and lipid extraction process.
	29/6/2015	27/7/2015	4 weeks	Transesterification of microalgae lipid and fatty acid profile analysis of microalgae biodiesel.
	24/7/2015	21/8/2015	4 weeks	Completed and submission of project dissertation.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Calibration and Preliminary Study

In the preliminary study, a correlation between the optical density of *Chorella vulgaris* and biomass weight was pre-determined. The optical density was measured daily at 688 nm by using spectrophotometer. 5 ml sample were centrifuged at 10 x 1000 g for 5 minutes and the supernatant was slowly decanted back into the culture medium. Then, the microalgae biomass was dried in an oven at of the microalgae biomass (g/L) collected. The correlation obtained is as shown in Figure 4.1.

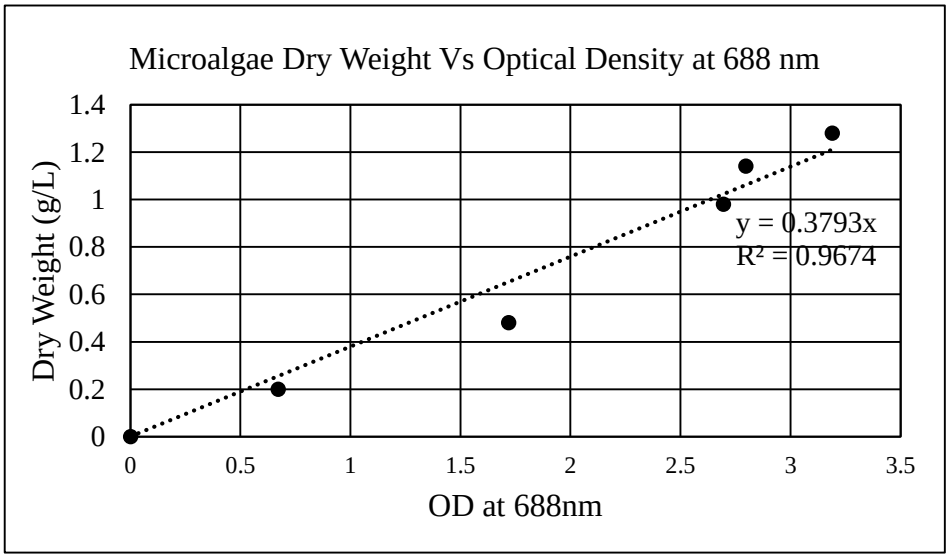


FIGURE 4.1. Graph of Microalgae Dry Weight Vs Optical Density at 688 nm

The obtained linear correlation from figure 4.1 is as shown in the equation below:

$$\text{Dry weight (g/L)} = 0.3793 \times OD_{688nm},\ R^2 = 0.9674 \quad (2)$$

The specific growth (μ) was measured using the equation below:

$$\mu(\text{day}^{-1}) = \frac{\ln \frac{N_2}{N_1}}{t_2 - t_1} \quad (3)$$

Where N_1 and N_2 are the biomass (g/L) at time t_1 and t_2 respectively.
While the biomass productivity was measured using the equation:

$$\text{Biomass productivity } \left(\frac{g/L}{day}\right) = \frac{N_f - N_i}{\text{Total cultivation time (day)}} \quad (4)$$

Where N_f is the final reading of biomass (g/L) and N_i is the initial biomass (g/L) reading.

4.2 Effects of Amount of Nutrients

The nutrient uptake by the microalgae is affected by the overall composition of the nutrients that are available in the culture medium where nitrogen and phosphorus are often the limiting nutrient. Thus an optimal N/P ratio exists where the maximum nutrients removal is determined (Kunikane *et al.*, 1984). The N/P ratio of the culture medium should be in a proper range in order to ensure the simultaneous utilization of both nutrients by the microalgae that are essential for the growth (Xin *et al.*, 2010).

In addition, there are some limitations that need to be addressed when wastewater is used as nutrients for microalgae culture medium even though many previous studies suggested that there is high potential in utilization of these nutrient resources for cost-effective biodiesel production (Chiu *et al.*, 2015). However, the

utilization of wastewater as culture medium required the consideration of resistant of the microalgae species to biotic pollution such as bacteria, fungi and zooplanktons that are exist in wastewater. Thus, the wastewater need to be treated by autoclave sterilization or centrifugation before it is applied for microalgae as control technique of biotic pollution (Chiu *et al.*, 2015).

In the present study, different amount of wastewater was filled in 5 Erlenmeyer flask to study the ability of *Chorella vulgaris* in taking up the nutrients and the effects of the amount of nutrients provided towards the growth of microalgae. Figure 4.2 depicts the growth of *Chorella vulgaris* with different amount of wastewater. From the figure, *Chorella vulgaris* was found to grow better in low amount of wastewater compared to higher amount of wastewater that were added to the culture medium. This is because high amount of wastewater leads to high tendency in containing biotic pollutants in the microalgae cultivation medium which subsequently inhibits the growth of microalgae. The results is further supported in Figure 4.3, in which the highest amount of biomass produced (0.0375 g/L/day) was the cultivation that contained minimum amount of wastewater (20 mL). Furthermore, *Chorella vulgaris* in the medium containing 20 mL of wastewater also recorded the highest specific growth which is at 0.3043 day^{-1} followed by 50 mL, 100 mL, 200 mL and 150 mL with specific growth of 0.2783 day^{-1}, 0.2893 day^{-1}, 0.2457 day^{-1} and 0.2346 day^{-1} respectively.

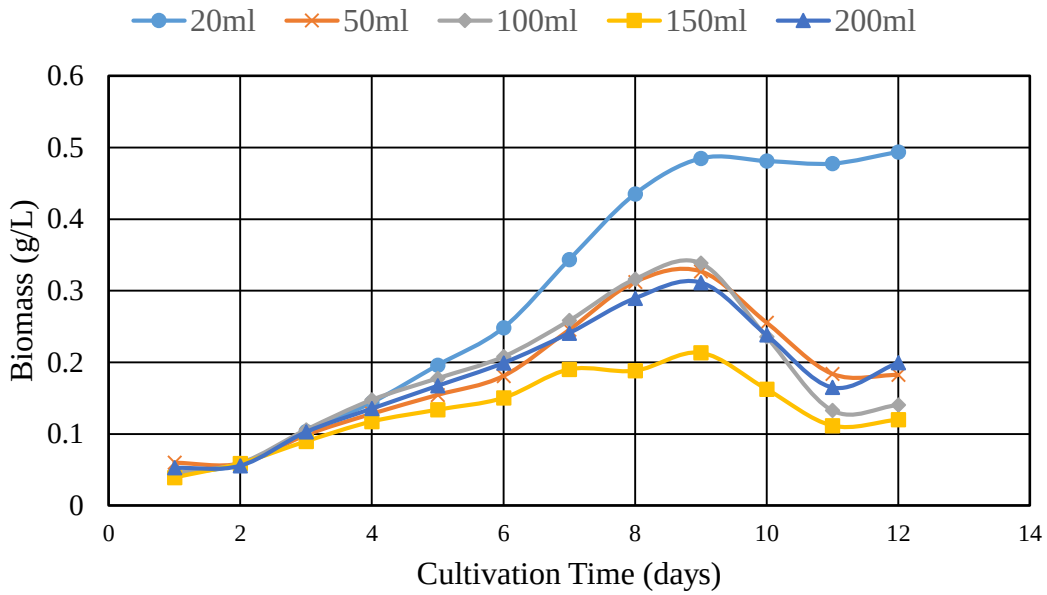


FIGURE 4.2. Effect of Amount of Wastewater towards the Growth of *Chorella vulgaris*. Other Culture Condition: Initial pH = 3, Amount of Seed = 100 mL and Illuminated for 24 hours Continuously

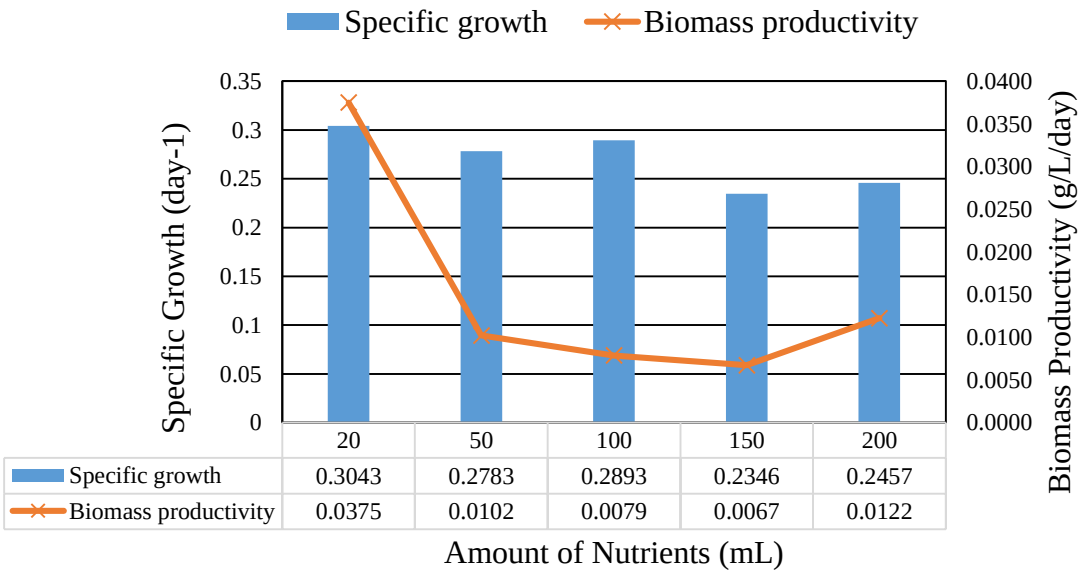


FIGURE 4.3. Specific Growth and Biomass Productivity of Chorella vulgaris in Different Amount of Wastewater

4.3 Effects of initial pH

One of the important factors in cultivation of microalgae is the pH of the culture medium because the variation in pH can affect the metabolism and growth of microalgae which could altering the equilibrium of inorganic carbon species, varied the availability of nutrients and affecting the cell physiology (Mostafa *et al.*, 2012). The range for the optimum growth and photosynthesis for most microalgae species is in the neutral to alkaline range, but there are some species that are able to grow in acidic condition as low as pH 1 (Raven, 1990).

According to Azov (1982), biomass production of green algae such as *Chorella vulgaris* in intensive laboratory continuous cultures was significantly affected by the pH of the cultures medium. The pH tolerance limits of the microalgae is determined either by chemical influence on the growth medium or by the metabolic effects on the cells (Goldman *et al.*, 1982). Goldman et al. (1981), concluded that the maximum tolerable pH is not influenced by the availability of inorganic carbon whereas, pH is the major determinants of the carbon system species in water that affect the availability of carbon for microalgae photosynthesis. The effect of pH level to the cultivation of microalgae is crucial information in the near future when flue gases are used to cultivate microalgae in industrial scale where the suitable microalgae strains should tolerable to the inconsistent concentration of CO_2 in the flue gases that results in pH variation. (Tang *et al.*, 2011).

Figure 4.4 shows effects of initial pH value of the culture medium towards the growth of *Chorella vulgaris*. Based on the results that were obtained, *Chorella vulgaris* showed a similar trend and almost a linear growth at initial pH of 3 and 5 with maximum biomass obtained ranging from 0.18 to 0.25 g/L after 12 days of cultivation. For higher initial pH of the culture medium, the microalgae did not exhibited a satisfactory growth curves. The growth of microalgae at pH 7, 9 and 12 was stagnated and showed no significant increment of biomass produced even after 12 days of cultivation. From Figure 4.5, the sample with initial pH of 3 recorded the highest specific growth which is at 0.1756 day^{-1}. From these results, it was found that *Chorella vulgaris* in the present study favoured the culture medium at initial pH of 3 as it

recorded the highest biomass productivity. This results also indicates that the *Chorella vulgaris* used in this study can adapt very well at low pH medium which is an advantage to inhibits other contaminants such as fungus that are exist in the culture medium and enable the microalgae to sustain their growth naturally.

Besides, it also presume that *Chorella vulgaris* in the present study preferred to utilize carbonic acid (H_2CO_3) as the carbon source to grow. This is because, at pH below than 4.5, the cultivation medium is dominated with free CO_2 molecules or H_2CO_3 when CO_2 dissolve in water (Devgoswami *et al.*, 2013). Thus, the microalgae had utilized carbonic acid available in the cultivation medium as carbon source for its growth.

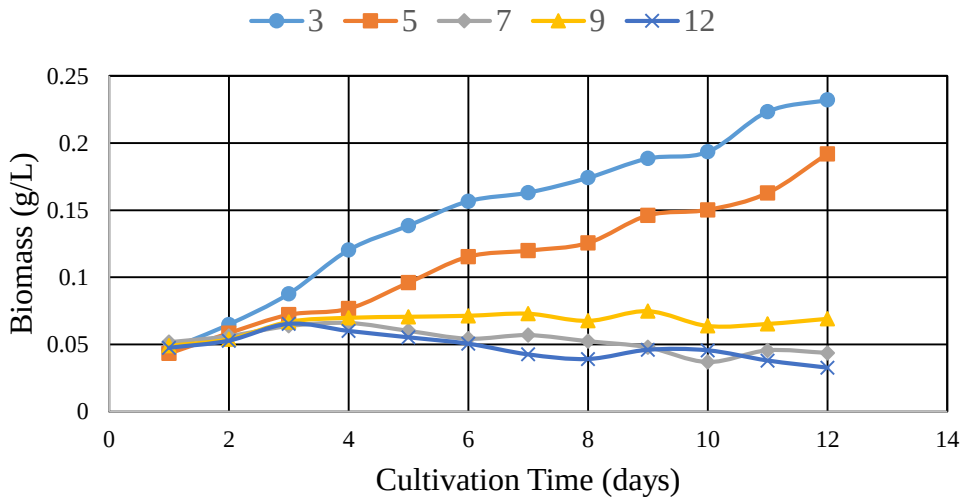


FIGURE 4.4. Effects of Initial pH Value towards the growth of *Chorella vulgaris*. Other Culture Condition: Amount of Wastewater = 20 mL, Amount of Seed = 100 mL and Illuminated for 24 hours Continuously

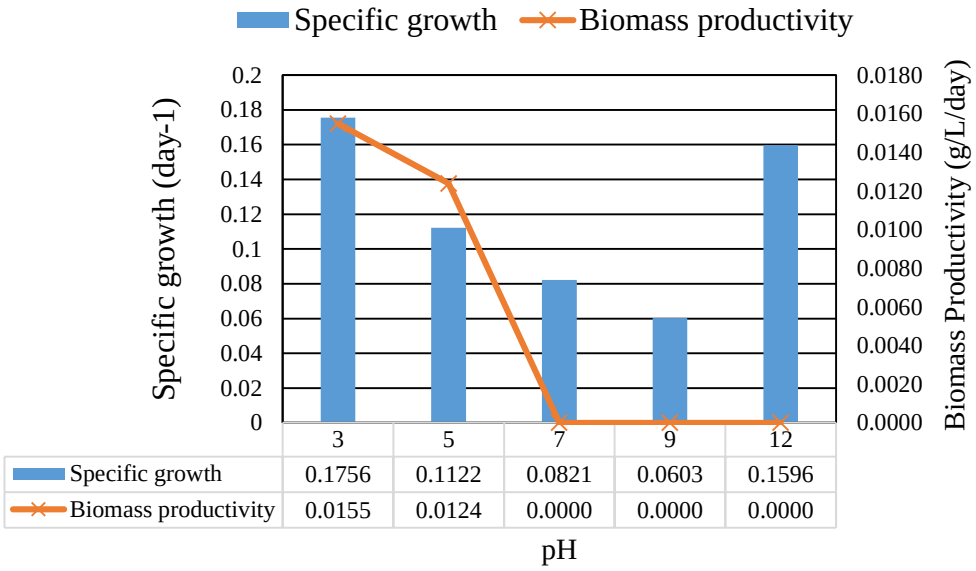


FIGURE 4.5. Specific Growth and Biomass Productivity of Chorella vulgaris in Different Initial pH Value

4.4 Effect of Amount of Seed

As the microalgae population increases in density as time pass, it will affect the growth of the microalgae as the growth becomes slower due to the depletion of nutrients and decreases in light penetration because of self-shading. Thus, stirring apparatuses and cause water circulation are very important to agitate the microalgae for the light to reach the dense growth to ensure even distribution of light (Q Hu *et al.*, 1998). In addition, highly dense population of microalgae could possibly affect the growth of microalgae due to competition for nutrients as well as light that are essential for the growth of microalgae. According to Darley (1982), a higher microalgae density would lead to self-shading, accumulation of auto inhibitors and causing reduction in photosynthetic efficiency.

Figure 4.6 shows the effect of amount of seed to the growth of *Chorella vulgaris*. From the results that were obtained, all the 5 samples exhibited a similar growth patterns during the 12 days of cultivation. From Figure 4.7, the highest biomass productivity was recorded in the sample contained 200 mL of seed which is 0.201

g/L/day followed by 150 mL, 100 mL, 50 mL and 30 mL which recorded biomass productivity of 0.0197, 0.0149, 0.0123 and 0.0079 g/L/day respectively. However, *Chorella vulgaris* showed the highest specific growth in the culture medium that contained the lowest amount of seed which is at rate 0.3198 day^{-1}.

With respect to the results that were obtained, it provides an option either to have a high biomass productivity or to have a high specific growth rate for the cultivation of microalgae. Based on the results, cultivation medium with low amount of microalgae seed could provide a higher specific growth rate which could be an advantage for nutrients removal or pollutant removal from the wastewater. Besides, cultivation medium with high amount of microalgae seed could provide a higher biomass productivity which are essential to obtain high amount of microalgae biomass for biodiesel production.

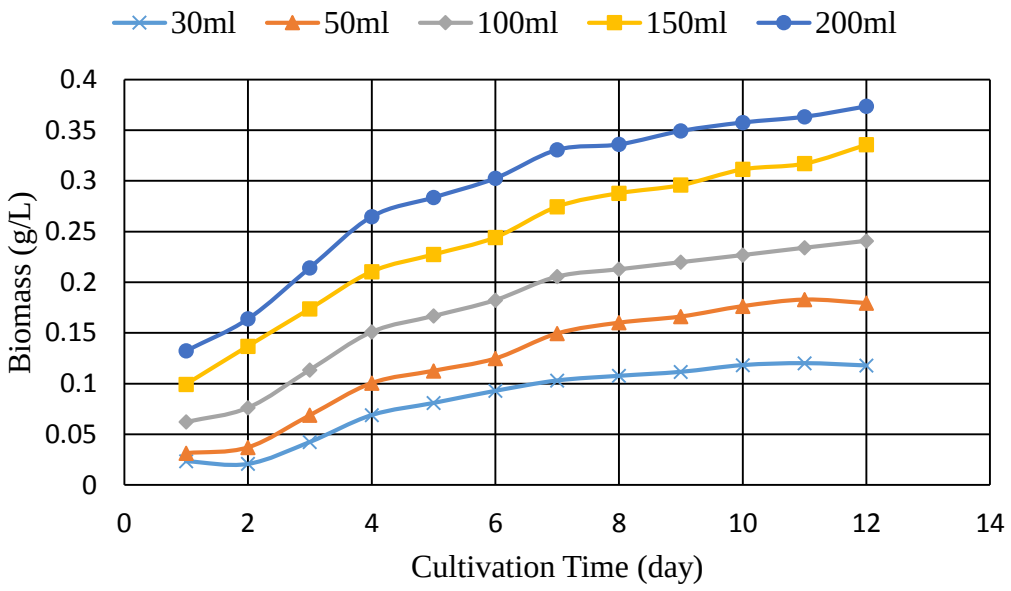
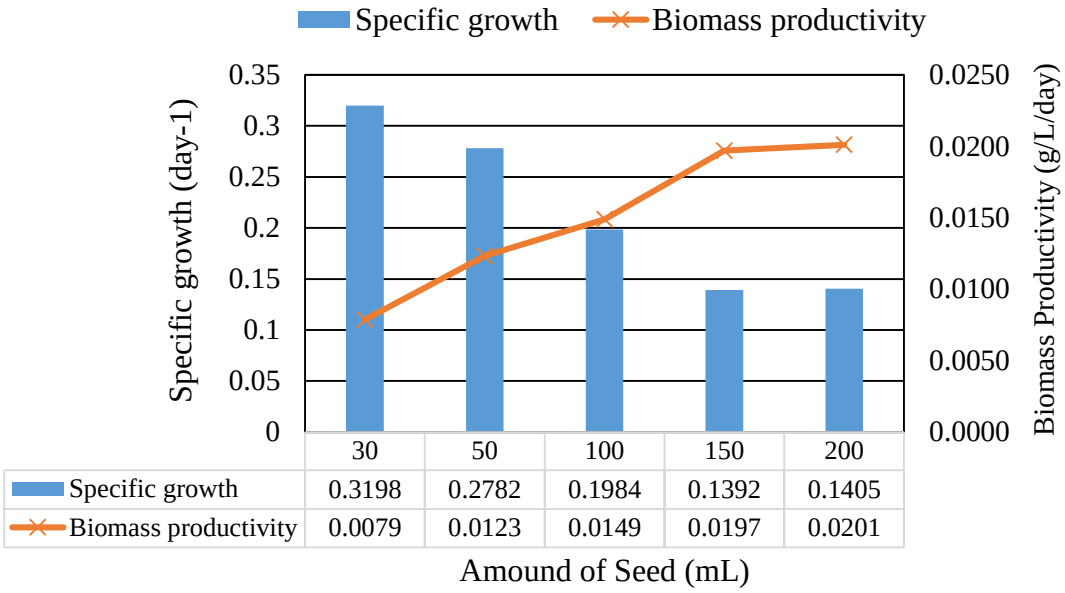


FIGURE 4.6. Effects of Amount of Seeds towards the Growth of *Chorella vulgaris*. Other Culture Conditions: Amount of Wastewater = 20 mL, Initial pH = 3, and Illuminated for 24 hours Continuously



	30	50	100	150	200
Specific growth	0.3198	0.2782	0.1984	0.1392	0.1405
Biomass productivity	0.0079	0.0123	0.0149	0.0197	0.0201

FIGURE 4.7. The Specific Growth and Biomass Productivity of *Chorella vulgaris* in Different Amount of Seed

4.5 Nutrients Removal

The presence of high concentration of nutrients in particular nitrogen and phosphorus can possibly causing eutrophication if the nutrients accumulate in water bodies and thus, requires the wastewater to be treated. Nitrogen could present in wastewater in the form of ammonia (NH_4^+), organically bound nitrogen or even nitrite (NO_2^-) and nitrate (NO_3^-) while phosphorus could present in the wastewater in the form of phosphates (PO_4^{3-}) (Chiu *et al.*, 2015). Thus, the nutrients available in the wastewater can be utilized by the microalgae for their growth and for that reason *Chorella sp.* has been used in numerous studies due to the effectiveness in removing nitrogen and phosphorus from different wastewater streams (Cai *et al.*, 2013).

Figure 4.8 depicts the result for the total nitrogen testing of the wastewater sample before and after the cultivation period. The concentration of total nitrogen in the wastewater sample was reduced from 2.7 mg/L to 0.4 mg/L was accounted for about 85% of removal efficiency. Furthermore, Figure 4.9 also showed the result for total phosphorus testing for wastewater sample. The total phosphorus content in the

wastewater sample was reduced from 24.19 mg/L to 15.67 mg/L. The removal efficiency of the total phosphorus content in the wastewater sample was about 35% throughout the cultivation period. The reduction in the amount of the nutrients was corresponding with the findings from the literature where *Chorella vulgaris* recorded an average removal efficiency of nitrogen and phosphorus of 72% and 28% from respectively wastewater sample (Aslan and Kapdan, 2006).

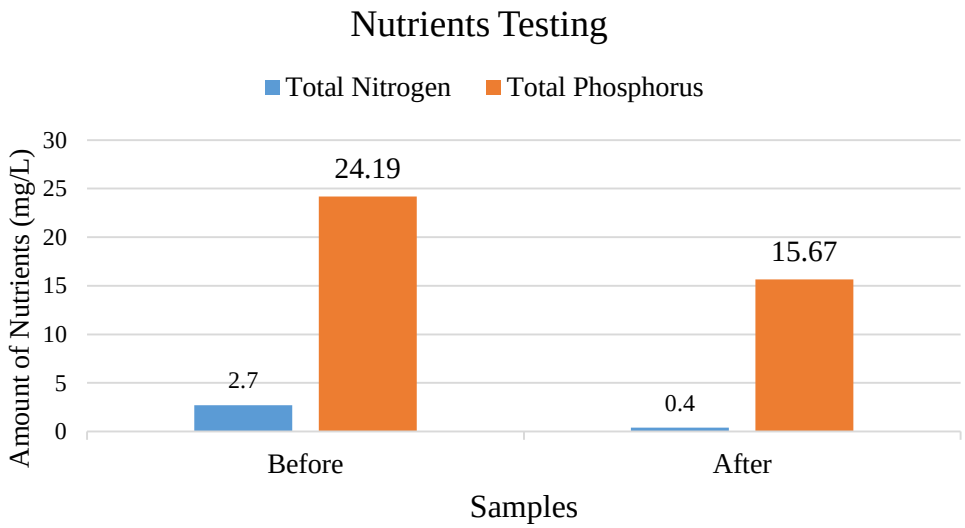


FIGURE 4.8. Results of Nutrients Testing

4.6 Lipid Extraction and Biodiesel Production from *Chorella vulgaris*

Various microalgae species have been identified to have high content of lipids which could be converted to biodiesel and thus providing alternatives to petroleum based diesel (Chisti, 2007). Although the main focus of the present study is to determine the suitability of utilizing wastewater as nutrient sources for cultivation of microalgae, the study also focused on the amount of lipid produce by *Chorella vulgaris.* According to a study, higher lipid contents could be achieved under wastewater cultivation that contains low concentration of nitrogen and phosphorus. This is because, at low concentration of nitrogen during microalgae cultivation creating a medium with nitogen deficient condition that contributes to lipid accumulation (Chiu *et al.*, 2015).

Generally, there are two methods for extraction of lipid from microalgae biomass which are by using chemical solvent or mechanical method. One of the main advantages of using mechanical method is that the method is suitable for most microalgae species, nevertheless, for large scale extraction required a thorough evaluation due to energy efficiency (J. Kim *et al.*, 2013). Besides, extraction of lipid by using chemical solvent is identified as the most reliable method to determine the overall lipid content of microalgae due to the high polarity of fatty acids towards the chemical solvent used for the extraction (Lam and Lee, 2012b).

In the present study, the dried microalgae biomass that was harvested undergo lipid extraction process by using a mixture of methanol and chloroform with volume ratio of 2:1 (Bligh & Dyer method). The percent of microalgae lipid extracted by using Bligh and Dyer method was 32.7% (Table 4.1). The result is within the range of total lipid of *Chorella vulgaris* reported in the literature which is between 5% to 40% under phototrophic cultivation condition (Senthil Chinnasamy *et al.*, 2010). It was found that high lipid content from microalgae biomass was obtained in the present study even though the nitrogen content in the wastewater was extremely low. This is because at low nitrogen content, starch synthesis pathway is blocked within microalgae cells and therefore photosynthetically fixed carbon is redirected into fatty acid production which leads to high accumulation of lipid in microalgae cells (Y. Li *et al.*, 2010).

TABLE 4.1. Lipid Extraction of Dried Microalgae Biomass

Total dried microalgae biomass collected	0.2346 g
Weight of beaker (before)	16.8981 g
Weight of beaker (after)	16.9749 g
Percent of microalgae lipid extracted	$= \frac{(\text{weight after} - \text{weight before})}{\text{Total weight}} \times 100\%$ $= \frac{(16.9749 - 16.8981)}{0.2346} \times 100\%$ $= 32.7\%$

Then, the dried microalgae biomass that was harvested from the cultivation using wastewater was converted to biodiesel through transesterification reaction. Transesterification of lipids is the basic chemical reaction that is required to produce biodiesel by utilizing alcohol and high pH of the reaction condition. In addition, the chemical reaction is sensitive to water as the presence of water in the reaction could cause saponification reaction which will affects the yield and quality of biodiesel (A. Singh *et al.*, 2011). Figure 4.9 shows the biodiesel that was produced from the dried microalgae biomass through transesterification reaction.

FIGURE 4.9. Biodiesel Produced from Dried Microalgae Biomass through Transesterification Reaction

4.7 Fatty Acid Methyl Ester Content Analysis

Fatty Acid Methyl Ester **(**FAME) is the main component of biodiesel and the chemical structure of distinctive FAME profile plays a critical role in determining the properties of biodiesel produced. It is important to determine the profiles of fatty acids as different environmental condition in which the microalgae is exposed during the cultivation period is possible to create significant changes in the biosynthesis of fatty acids (Los and Murata, 2004). Figure 4.10 shows the microalgae FAME profile detected by GC and Table 4.2 shows the fatty acid composition.

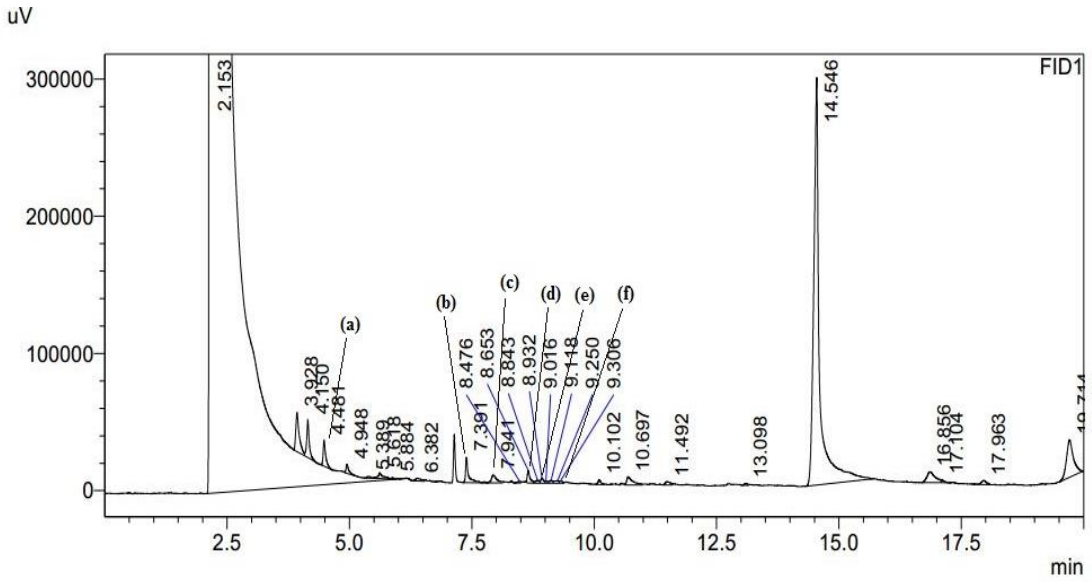


FIGURE 4.10. Microalgae FAME Profile: (a) M. Dodecanoate (b) M. Palmitoleate (c) M. Heptadecanoate (d) M. Stearate (e) M. Oleate (f) M. Linoleate

TABLE 4.2. Content of FAME

Constituents	Fatty Acid Methyl Ester	Amount (%)
C10:0	M. Dodecanoate	25.57
C16:1	M. Palmitoleate	30.54
C17:0	M. Heptadecanoate	17.17
C18:0	M. Stearate	13.65
C18:1	M. Oleate	10.25
C18:2	M. Linoleate	2.82

Based on the analysis that has been done, the overall FAME composition of *Chorella vulgaris* mostly contained C10:0 (Methyl Dodecanoate), C16:1 (Methyl Palmitoleate) C17:0 (Methyl Heptadecanoate), C18:0 (Methyl Stearate), C18:1 (Methyl Oleate) and C18:2 (Methyl Linoleate). As depicts from Table 4.2, C16:1, C17:0, C18:0, C18:1 and C18:2 which covered major portion of FAME composition (74.43%) of *Chorella vulgaris* in the present study has been discovered as essential components in producing an ideal biodiesel (Schenk *et al.*, 2008). In addition, these fatty acids (C16:0, C17:0, C18:0, C18:1, C18:2) also can be found in other oil bearing

crops such as soybean, sunflower and palm oil which are suitable for the production of biodiesel (Lam and Lee, 2012b). Besides, the high amount of unsaturated fatty acid which accounted for total of 74.43% from the overall FAME composition is essential for production of biodiesel as it able to reduce the pour point of biodiesel that will allows it to be used in cold climate countries (Abdelmalik *et al.*, 2011). Thus, it can be concluded that microalgae biomass cultivated using wastewater is suitable for biodiesel production.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The depletion of the oil and gas resources and the negative impacts of burning of fossil fuels to the environment has initiates the effort to discover alternative sources of renewable energy. The production of biodiesel using microalgae biomass not only providing plausible solution to diversify the current renewable biomass feedstock but also substantially improve the life cycle of biodiesel. In addition, by utilizing wastewater as nutrients source to cultivate microalgae could further enhance the sustainability of this renewable feedstock. This is because, microalgae will purify the wastewater by absorbing the available nutrients while generating biomass for biodiesel production. In term of environmental and economic perspective, the mutual benefits will be the most plausible option to commercialize the microalgae biodiesel production.

However, issues such as contamination, inconsistent wastewater components and unstable biomass production could affect the growth of microalgae. The microalgae resistance towards biotic pollution that exist in the wastewater must be considered as an important issue for utilization of wastewater for cultivation of microalgae. Thus, in order to ensure stable microalgae growth using wastewater as nutrient source, it is necessary to develop control method of biotic pollutants.

Hence, the results that were obtained in the present study showed that the *Chorella vulgaris* was able to grow optimally in the following growth condition: 20 mL amount of wastewater, initial pH of 3, and 30 mL initial amount of microalgae

seed with 24 hours of continuous illumination. The highest specific growth rate that was achieved by the *Chorella vulgaris* was 0.3198 day^{-1} during the test for amount of microalgae seed while the highest biomass productivity that was achieved by the *Chorella vulgaris* was 0.0375 g/L/day during the test for amount of wastewater. Besides, the microalgae biomass that was collected successfully undergo lipid extraction process by using chemical solvent (Bligh & Dyer Method) and the percentage of lipid extracted was 32.7%. Furthermore, based on the analysis that has been done, the overall FAME composition of *Chorella vulgaris* mostly contained high percentage of unsaturated fatty acids. Thus, it can be concluded that the FAME produced from the transesterification of the *Chorella vulgaris* lipid is suitable for making biodiesel.

5.2 Recommendations for Future Work

There are several recommendations that can be made in order to improve the present study in the future:

1. Use different species of microalgae in the study to determine the most effective microalgae species that can be grown in specific source of wastewater.
2. Treated the wastewater by autoclave sterilization or centrifugation before using it for the cultivation of microalgae.
3. Immobilize the microalgae cells into alginate beads to facilitate the biomass harvesting process.
4. Determine the properties of biodiesel that is produced from microalgae biomass cultivated using wastewater and make comparison with the standard properties of biodiesel used in the market.

REFERENCES

Abbaszaadeh, A., Ghobadian, B., Omidkhah, M. R., & Najafi, G., 2012, "Current biodiesel production technologies: a comparative review", *Energy Conversion and Management* **63:** 138-148

Abdelmalik, A. A., Abbott, A. P., Fothergill, J. C., Dodd, S., & Harris, R. C., 2011, "Synthesis of a base-stock for electrical insulating fluid based on palm kernel oil", *Industrial Crops and Products* **33(2):** 532-536

Ågren, G. I., 2004, "The C: N: P stoichiometry of autotrophs–theory and observations", *Ecology Letters* **7(3):** 185-191

Ahluwalia, S. S., & Goyal, D., 2007, "Microbial and plant derived biomass for removal of heavy metals from wastewater", *Bioresource Technology* **98(12):** 2243-2257

An, J.-Y., Sim, S.-J., Lee, J., & Kim, B., 2003, "Hydrocarbon production from secondarily treated piggery wastewater by the green alga Botryococcus braunii", *Journal of Applied Phycology* **15(2-3):** 185-191

Andersson, V., Broberg, S., & Hackl, R. (2011). Integrated Algae Cultivation for Biofuels Production in Industrial Clusters: Program Energisystem.

Andrade, M. R., & Costa, J. A., 2007, "Mixotrophic cultivation of microalga Spirulina platensis using molasses as organic substrate", *Aquaculture* **264(1):** 130-134

Aslan, S., & Kapdan, I. K., 2006, "Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae", *Ecological Engineering* **28(1):** 64-70

Bhatnagar, A., Bhatnagar, M., Chinnasamy, S., & Das, K. C., 2010, "Chlorella minutissima--a promising fuel alga for cultivation in municipal wastewaters", *Appl Biochem Biotechnol* **161(1-8):** 523-536

Bligh, E. G., & Dyer, W. J., 1959, "A rapid method of total lipid extraction and purification", *Canadian journal of biochemistry and physiology* **37(8):** 911-917

Borowitzka, M. A., 1999, "Commercial production of microalgae: ponds, tanks, tubes and fermenters", *Journal of Biotechnology* **70(1–3):** 313-321

Brennan, L., & Owende, P., 2010, "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products", *Renewable and Sustainable Energy Reviews* **14(2):** 557-577

Cai, T., Park, S. Y., & Li, Y., 2013, "Nutrient recovery from wastewater streams by microalgae: Status and prospects", *Renewable and Sustainable Energy Reviews* **19(0):** 360-369

Canter, C. E., Blowers, P., Handler, R. M., & Shonnard, D. R., 2015, "Implications of widespread algal biofuels production on macronutrient fertilizer supplies: Nutrient demand and evaluation of potential alternate nutrient sources", *Applied Energy* **143(0):** 71-80

Cantrell, K. B., Ducey, T., Ro, K. S., & Hunt, P. G., 2008, "Livestock waste-to-bioenergy generation opportunities", *Bioresource Technology* **99(17):** 7941-7953

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., & Chang, J.-S., 2011, "Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review", *Bioresource Technology* **102(1):** 71-81

Chen, G.-Q., & Chen, F., 2006, "Growing Phototrophic Cells without Light", *Biotechnology Letters* **28(9):** 607-616

Chen, G., Zhao, L., & Qi, Y., 2015, "Enhancing the productivity of microalgae cultivated in wastewater toward biofuel production: A critical review", *Applied Energy* **137(0):** 282-291

Chinnasamy, S., Bhatnagar, A., Claxton, R., & Das, K. C., 2010, "Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium", *Bioresour Technol* **101(17):** 6751-6760

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

Chisti, Y., 2007, "Biodiesel from microalgae", *Biotechnology advances* **25(3):** 294-306

Chiu, S.-Y., Kao, C.-Y., Chen, T.-Y., Chang, Y.-B., Kuo, C.-M., & Lin, C.-S., 2015, "Cultivation of microalgal Chlorella for biomass and lipid production using wastewater as nutrient resource", *Bioresource Technology* **184(0):** 179-189

Clarens, A. F., Resurreccion, E. P., White, M. A., & Colosi, L. M., 2010, "Environmental Life Cycle Comparison of Algae to Other Bioenergy Feedstocks", *Environmental Science & Technology* **44(5):** 1813-1819

Darnoko, D., & Cheryan, M., 2000, "Kinetics of palm oil transesterification in a batch reactor", *Journal of the American Oil Chemists' Society* **77(12):** 1263-1267

de la Noüe, J., Laliberté, G., & Proulx, D., 1992, "Algae and waste water", *Journal of Applied Phycology* **4(3):** 247-254

Demirbas, A., & Demirbas, M. F., 2011, "Importance of algae oil as a source of biodiesel", *Energy Conversion and Management* **52(1):** 163-170

Devgoswami, C. R., Kalita, M., Talukdar, J., Bora, R., & Sharma, P., 2013, "Studies on the growth behavior of Chlorella, Haematococcus and Scenedesmus sp. in culture media with different concentrations of sodium bicarbonate and carbon dioxide gas", *African Journal of Biotechnology* **10(61):** 13128-13138

Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., & Posewitz, M. C., 2008, "Aquatic phototrophs: efficient alternatives to land-based crops for biofuels", *Curr Opin Biotechnol* **19(3):** 235-240

Eriksen, N., 2008, "The technology of microalgal culturing", *Biotechnology Letters* **30(9):** 1525-1536

Goldman, J. C., Azov, Y., Riley, C. B., & Dennett, M. R., 1982, "The effect of pH in intensive microalgal cultures. I. Biomass regulation", *Journal of Experimental Marine Biology and Ecology* **57(1):** 1-13

Gouveia, L., Marques, A. E., da Silva, T. L., & Reis, A., 2009, "Neochloris oleabundans UTEX #1185: a suitable renewable lipid source for biofuel production", *J Ind Microbiol Biotechnol* **36(6):** 821-826

Gouveia, L., & Oliveira, A. C., 2009, "Microalgae as a raw material for biofuels production", *Journal of industrial microbiology & biotechnology* **36(2):** 269-274

Hill, J., Nelson, E., Tilman, D., Polasky, S., & Tiffany, D., 2006, "Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels", *Proceedings of the National Academy of Sciences* **103(30):** 11206-11210

Hu, Q., Kurano, N., Kawachi, M., Iwasaki, I., & Miyachi, S., 1998, "Ultrahigh-cell-density culture of a marine green alga Chlorococcum littorale in a flat-plate photobioreactor", *Applied Microbiology and Biotechnology* **49(6):** 655-662

Hu, Q., Zarmi, Y., & Richmond, A., 1998, "Effects of light intensity, light path and culture density on output rate of Spirulina platensis (Cyanobacteria)", *Eur J Phycol* **33:** 165 - 171

Ip, S., Bridger, J., Chin, C., Martin, W., & Raper, W., 1982, "Algal growth in primary settled sewage: the effects of five key variables", *Water Research* **16(5):** 621-632

Jaimes-Duarte, D.-L., Soler-Mendoza, W., Velasco-Mendoza, J., Muñoz-Peñaloza, Y., & Urbina-Suárez, N.-A., 2012, "Characterization Chlorophytas microalgae with potential in the production of lipids for biofuels", *CT&F-Ciencia, Tecnología y Futuro* **5(1):** 93-102

Johnson, M. B., & Wen, Z., 2010, "Development of an attached microalgal growth system for biofuel production", *Applied Microbiology and Biotechnology* **85(3):** 525-534

Kim, J., Yoo, G., Lee, H., *et al.*, 2013, "Methods of downstream processing for the production of biodiesel from microalgae", *Biotechnology advances* **31(6):** 862-876

Kim, M., Park, J., Park, C., *et al.*, 2007, "Enhanced production of Scenedesmus spp.(green microalgae) using a new medium containing fermented swine wastewater", *Bioresource Technology* **98(11):** 2220-2228

Kim, M. K., & Jeune, K. H., 2009, "Use of FT-IR to identify enhanced biomass production and biochemical pool shifts in the marine microalgae, Chlorella ovalis, cultured in media composed of different ratios of deep seawater and fermented animal wastewater", *J Microbiol Biotechnol* **19(10):** 1206-1212

Kim, S., & Dale, B. E., 2005, "Environmental aspects of ethanol derived from no-tilled corn grain: nonrenewable energy consumption and greenhouse gas emissions", *Biomass and Bioenergy* **28(5):** 475-489

Kunikane, S., Kaneko, M., & Maehara, R., 1984, "Growth and nutrient uptake of green alga, Scenedesmus dimorphus, under a wide range of nitrogen/phosphorus ratio—I. Experimental study", *Water Research* **18(10):** 1299-1311

Lam, M. K., & Lee, K. T., 2012a, "Microalgae biofuels: A critical review of issues, problems and the way forward", *Biotechnology advances* **30(3):** 673-690

Lam, M. K., & Lee, K. T., 2012b, "Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production", *Applied Energy* **94(0):** 303-308

Li, Y., Han, D., Hu, G., Sommerfeld, M., & Hu, Q., 2010, "Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii", *Biotechnology and bioengineering* **107(2):** 258-268

Li, Y., Han, D., Hu, G., Sommerfeld, M., & Hu, Q., 2010, "Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii", *Biotechnol Bioeng* **107(2):** 258-268

Li, Y., Horsman, M., Wang, B., Wu, N., & Lan, C. Q., 2008, "Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans", *Applied Microbiology and Biotechnology* **81(4):** 629-636

Lima, D. G., Soares, V. C., Ribeiro, E. B., *et al.*, 2004, "Diesel-like fuel obtained by pyrolysis of vegetable oils", *Journal of Analytical and Applied Pyrolysis* **71(2):** 987-996

Los, D. A., & Murata, N., 2004, "Membrane fluidity and its roles in the perception of environmental signals", *Biochim Biophys Acta* **1666(1-2):** 142-157

Ma, F., & Hanna, M. A., 1999, "Biodiesel production: a review", *Bioresource Technology* **70(1):** 1-15

Martınez, M., Sánchez, S., Jimenez, J., El Yousfi, F., & Munoz, L., 2000, "Nitrogen and phosphorus removal from urban wastewater by the microalga Scenedesmus obliquus", *Bioresource Technology* **73(3):** 263-272

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

Miao, X., & Wu, Q., 2004, "High yield bio-oil production from fast pyrolysis by metabolic controlling of Chlorella protothecoides", *Journal of Biotechnology* **110(1):** 85-93

Mostafa, S. S., Shalaby, E. A., & Mahmoud, G. I., 2012, "Cultivating microalgae in domestic wastewater for biodiesel production", *Notulae Scientia Biologicae* **4(1):** 56-65

Mulbry, W., Kondrad, S., & Buyer, J., 2008, "Treatment of dairy and swine manure effluents using freshwater algae: fatty acid content and composition of algal biomass at different manure loading rates", *Journal of Applied Phycology* **20(6):** 1079-1085

Munoz, R., & Guieysse, B., 2006, "Algal-bacterial processes for the treatment of hazardous contaminants: a review", *Water Res* **40(15):** 2799-2815

Órpez, R., Martínez, M. E., Hodaifa, G., El Yousfi, F., Jbari, N., & Sánchez, S., 2009, "Growth of the microalga Botryococcus braunii in secondarily treated sewage", *Desalination* **246(1):** 625-630

Peng, W., Wu, Q., Tu, P., & Zhao, N., 2001, "Pyrolytic characteristics of microalgae as renewable energy source determined by thermogravimetric analysis", *Bioresource Technology* **80(1):** 1-7

Pittman, J. K., Dean, A. P., & Osundeko, O., 2011, "The potential of sustainable algal biofuel production using wastewater resources", *Bioresource Technology* **102(1):** 17-25

Pramanik, K., 2003, "Properties and use of Jatropha curcas oil and diesel fuel blends in compression ignition engine", *Renewable energy* **28(2):** 239-248

Rafiqul, I., Weber, C., Lehmann, B., & Voss, A., 2005, "Energy efficiency improvements in ammonia production—perspectives and uncertainties", *Energy* **30(13):** 2487-2504

Rashid, N., Ur Rehman, M. S., Sadiq, M., Mahmood, T., & Han, J.-I., 2014, "Current status, issues and developments in microalgae derived biodiesel production", *Renewable and Sustainable Energy Reviews* **40(0):** 760-778

Raven, J. A., 1990, "Sensing pH?", *Plant, Cell & Environment* **13(7):** 721-729

Robles-Medina, A., González-Moreno, P., Esteban-Cerdán, L., & Molina-Grima, E., 2009, "Biocatalysis: towards ever greener biodiesel production", *Biotechnology advances* **27(4):** 398-408

Rodolfi, L., Chini Zittelli, G., Bassi, N., *et al.*, 2009, "Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor", *Biotechnology and bioengineering* **102(1):** 100-112

Ruiz-Marin, A., Mendoza-Espinosa, L. G., & Stephenson, T., 2010, "Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater", *Bioresource Technology* **101(1):** 58-64

Salvi, B. L., & Panwar, N. L., 2012, "Biodiesel resources and production technologies – A review", *Renewable and Sustainable Energy Reviews* **16(6):** 3680-3689

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., *et al.*, 2008, "Second generation biofuels: high-efficiency microalgae for biodiesel production", *Bioenergy research* **1(1):** 20-43

Schwab, A., Dykstra, G., Selke, E., Sorenson, S., & Pryde, E., 1988, "Diesel fuel from thermal decomposition of soybean oil", *Journal of the American Oil Chemists' Society* **65(11):** 1781-1786

Searchinger, T., Heimlich, R., Houghton, R. A., *et al.*, 2008, "Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change", *Science* **319(5867):** 1238-1240

Sheehan, J., Dunahay, T., Benemann, J., & Roessler, P. (1998). *A look back at the US Department of Energy's aquatic species program: biodiesel from algae* (Vol. 328): National Renewable Energy Laboratory Golden.

Singh, A., Nigam, P. S., & Murphy, J. D., 2011, "Mechanism and challenges in commercialisation of algal biofuels", *Bioresource Technology* **102(1):** 26-34

Singh, M., Reynolds, D. L., & Das, K. C., 2011, "Microalgal system for treatment of effluent from poultry litter anaerobic digestion", *Bioresource Technology* **102(23):** 10841-10848

Tang, D., Han, W., Li, P., Miao, X., & Zhong, J., 2011, "CO2 biofixation and fatty acid composition of Scenedesmus obliquus and Chlorella pyrenoidosa in response to different CO2 levels", *Bioresource Technology* **102(3):** 3071-3076

Ugwu, C. U., Aoyagi, H., & Uchiyama, H., 2008, "Photobioreactors for mass cultivation of algae", *Bioresource Technology* **99(10):** 4021-4028

Van Gerpen, J., 2005, "Biodiesel processing and production", *Fuel processing technology* **86(10):** 1097-1107

Wang, B., Li, Y., Wu, N., & Lan, C. Q., 2008, "CO2 bio-mitigation using microalgae", *Applied Microbiology and Biotechnology* **79(5):** 707-718

Wang, L., Li, Y., Chen, P., *et al.*, 2010, "Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp", *Bioresource Technology* **101(8):** 2623-2628

Woertz, I., Feffer, A., Lundquist, T., & Nelson, Y., 2009, "Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock", *Journal of Environmental Engineering* **135(11):** 1115-1122

Xin, L., Hu, H. Y., Ke, G., & Sun, Y. X., 2010, "Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp", *Bioresour Technol* **101(14):** 5494-5500

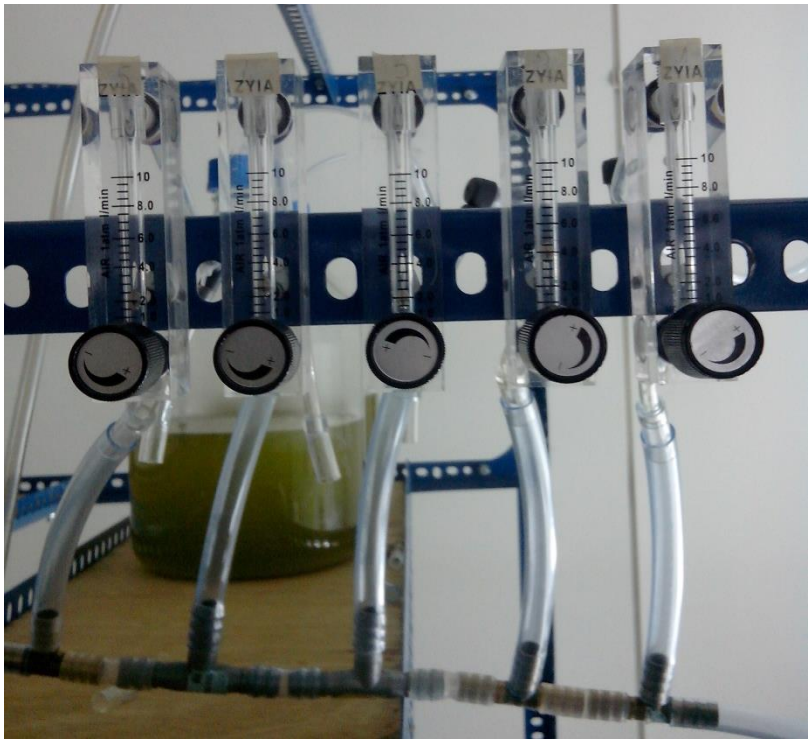
Yusuf, N., Kamarudin, S., & Yaakub, Z., 2011, "Overview on the current trends in biodiesel production", *Energy Conversion and Management* **52(7):** 2741-2751

Zhou, W., Chen, P., Min, M., *et al.*, 2014, "Environment-enhancing algal biofuel production using wastewaters", *Renewable and Sustainable Energy Reviews* **36(0):** 256-269

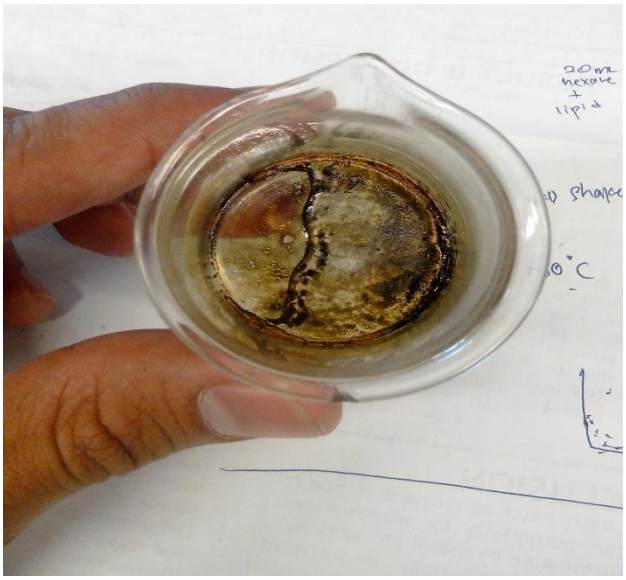
APPENDICES

APPENDIX 7.1. Setup of the Experiment

APPENDIX 7.2. Air Supply for the Cultivation of *Chorella vulgaris*

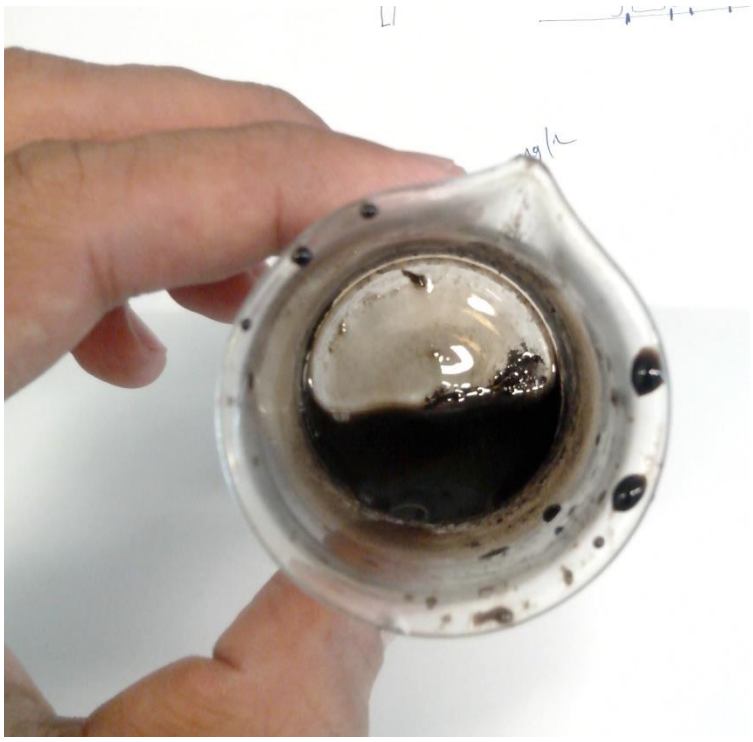


APPENDIX 7.3. Dried *Chorella vulgaris* Lipid after Lipid Extraction Process



APPENDIX 7.4. Transesterification Reaction by Using the Incubator Shaker

APPENDIX 7.5. Biodiesel Produced from the Transesterification of *Chorella vulgaris* Lipid



APPENDIX 7.6. Sample of Data Extracted from Spectrophotometer