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THE AMERICAN UNIVERSITY IN CAIRO
SCHOOL OF SCIENCES & ENGINEERING

UTILIZING WASTEWATER AS NUTRITION SOURCE FOR THE CULTIVATION OF *CHLORELLA VULGARIS*

By

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A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science in Environmental Engineering

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ACKNOWLEDGMENT

First, I thank Allah for granting me this development opportunity by conducting a master's degree in environmental engineering at a reputable university as the American University in Cairo (AUC). Moreover, I thank Alexandria University for accommodating me in its biology lab during the early stage of my research and provide me with resources and materials to set up the initial cultivation phase for my research.

I want to thank also my company (BP) for offering me an education assistantship without which I would not be able to pay for my master's degree. Also, I want to thank my line manager Ahmed El Sherbiny who encouraged me to join the environmental program in the AUC and facilitated me all resources to progress with my master's degree.

I am also thankful to my academic supervisors Prof. Salah El-Hagar, Dr. Ahmed El-Gendy, and Prof. Eman Mohamed Fakhry for guiding more over my research course. Prof. Salah has always provided support, and guidance, and helped me a lot in managing my efforts to stay on track and follow the research plan. Assoc. Prof. Ahmed El-Gendy has helped me to reveal new potentials in myself and guided me to overcome the challenges I encountered through my research and encouraged me to expand my research to cover broader scope in microalgae research. Prof. Eman Mohamed Fakhry gave me the throttle to start my research and empowered me with academic knowledge to set up my experiments.

I want to thank also Assoc. Prof. Hala Mohamed Taha (researcher at hydrobiology, Marine Division, National Institute of Oceanography & Fisheries), and Eng. Ahmed Saad from the environmental lab in the AUC for educating me the required lab skills to conduct my research.

Last but not least, I am grateful to my family who incurred my absence through those years and supported me to complete my master's degree.

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ABSTRACT

Investigating alternatives for fossil fuels have always been an area of interest for scientist around the globe. The decline in the oil & gas stock along with the increasing demand for energy that accompanies the increase in population has created the need for an alternative energy solution. From the renewable energy solution, microalgae stand out as a very promising source for biofuel production due to its high lipid content. However, the production of biofuel from microalgae is still of a high cost compared to production of the same amount from fossil fuels. The unfeasibility commercial production for biofuel from microalgae goes back to the high cost in the cultivation process, mainly supply the cultivation medium with nutrients, extraction process, and transesterification process.

This research aimed to reduce the cultivation process cost by investigating the substitution of required nutrients in the synthetic Woods Hole MBL (MBL) medium by those available in wastewater streams. *Chlorella vulgaris* was selected for this research for its high biomass productivity and its ability for adaptation in various media. Different cultivation conditions were tested to reach to growth rate close to which was recorded from the cultivation on synthetic medium (MBL). The research reached to the conclusion that a mixture between synthetic medium (MBL) and non-sterilized agriculture wastewater under indirect sunlight (16:8 light to dark cycle) achieved a growth rate close to the growth rate from cultivation on a pure synthetic medium (MBL). Regarding total lipids, The non-sterilizer agriculture wastewater and MBL mixture achieved the highest results after fourteen cultivation days. Both growth rates and total lipid results prove that a mixture between agriculture wastewater and synthetic medium (MBL) can be utilized as a substitution for the pure MBL medium. This substitution will support the objective of reducing the total cost for producing biofuel from microalgae.

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CHAPTER 1 - INTRODUCTION

1.1 HISTORICAL BACKGROUND

Humankind relied on energy since the dawn of time to maintain its survival. At the beginning we needed heat to survive cold weather; sun, burned wood, straw, and dried dung were the primary sources of energy for heating. Humankind always has the ambition to explore the world and energy is needed to power transportation means for such exploration purpose. Muscle of horses and wind were the first forms of energy for transportation which helped humankind to explore the world. By the era of ancient Alexandria simple machines that used steam as a source of energy were developed which gradually reduced the reliance on animal power to do the work. The evolution of steam engines continued to ramp up till the mid-1700s when Thomas Newcomen and James Watt developed the primary form of modern steam engines. Coal extracted from mines in England was capable of powering steam engines for doing works of dozens of horses. Steam engines powered by coal started to provide energy for locomotives, factories, and farms.

In 1880, coal was first used to generate electricity when Thomas Edison provided electricity to Wall Street financiers and the New York Times. By the late 1800s, petroleum started to evolve. The spread of petroleum utilization was accompanied by concerns from the community as contamination of drinking wells was observed.

The turnout to petroleum oil grudgingly increased as the whale oil industry started to decline. Petroleum oil was initially used to light streets. By the early twentieth century, the processing of petroleum oil into gasoline was started, and the internal combustion engines era has begun. The first commercial well was drilled in 1859 by Edwin Drakes in Pennsylvania it was a first time to use steam engines in drilling.

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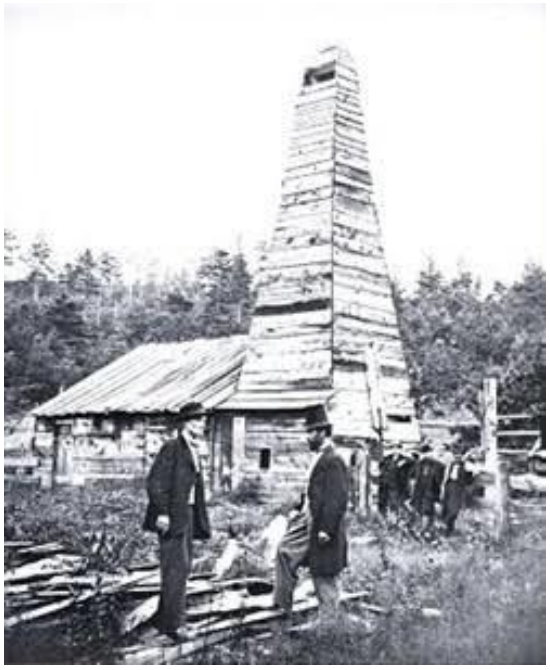

Figure 1: Edwin drake's oil well in Titusville

(American Oil & Gas Historical Society)

1.2 THE GLOBAL ENERGY SHORTAGE AND THE SOLUTION

As the world's population increases so do the global petroleum consumption. Figure two shows statistics from the Energy Information Administration presents the world total petroleum consumption over the years.

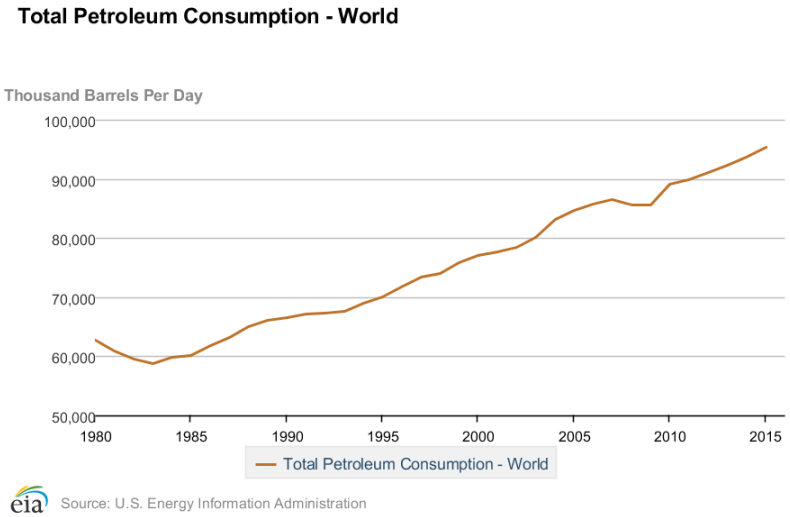

Figure 2: Total petroleum consumption

(U.S. Energy Information Administration)

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Humankind recognized that relying on petroleum-derived fuels is unsustainable since they are generated from depleting resources. Alternatively, fuel products that are generated from biological resources can be an excellent replacement to fossil fuels; not just because they have renewable nature, but they also help in reducing the greenhouse gases (GHGs) accumulation in the atmosphere (Posten & Schaub, 2009).

Although using terrestrial crops as biomass source to generate biofuel is considered as an environmentally friendly solution for power generation, yet it has its constraints such as the utilization of the available land for the cultivation of food crops versus crops for biofuel production. Microalgae overcome this constraint as it can generate more biomass than terrestrial crops for the same cultivation space. Algae can yield 61,000 L / ha compared to 200 L / ha from soya and 450 L / ha from canola crops (Duan & Savage, 2011). Microalgae have a high yield rate as it can double its biomass within 24 hrs.

1.3 MICROALGAE AS A RENEWABLE SOURCE FOR BIOFUEL

Researches over the years have proven microalgae to be a very promising renewable source for useful bio-products, and biofuels (Horsman et al., 2008). Microalgae can help to reduce the carbon footprint from different emission sources as microalgae rely on carbon dioxide (CO_2) in its photosynthetic process. Microalgae utilize the energy from the light to convert the carbon into lipid in a process called carbon fixation. It has been proven that for every 1 lb. Produced from microalgae a 1.8 lb. from CO_2 can be sequestered (Keffer & Kleinheinz, 2002). Since the 1970s, microalgae have been studied as an alternative source for fossil fuel; however, the excessive cost of production prohibited the enlargement to the commercial scale.

In 1980s research resumed, and nowadays different biofuel products can be produced by using microalgae such as Biodiesel, Bio-syngas, Bio-oil, and Bio-hydrogen (Horsman, Wu, Lan, & Dubois-Calero, 2008). When comparing biofuels that are produced from plants to those

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produced from microalgae, we will notice that the latest has better properties regarding caloric values.

Microalgae biofuels have low viscosities and low densities which gives them an advantage over plants biofuels (Miao & Wu, 2004). Recently microalgae have been considered as the most promising renewable source for the production of biofuel for many reasons; of which the most important are ((Campbell & Duncan, 1997); (Chisti, 2007); (Huntley & Redalje, 2007); (Schenk, et al., 2008); (Li Y. , Horsman, Wu, Lan, & Dubois-Calero, 2008); (Rodolfi, et al., 2009); (Khan, Rashmi, Prasad, & Banerjee, 2009)):

- Higher photon conversion efficiency in comparison to plants (approximately 3–8% against 0.5% for terrestrial plants) results in higher growth rate;
- High carbon fixation capacity;
- Ability to grow in salt & wastewaters which in returns reduces the requirement for fresh water;
- Microalgae can utilize nutrients, e.g., nitrogen & phosphorus, in agriculture, and municipal wastewater which can reduce the chemicals needed for cultivation along with bioremediation of wastewater;
- There are no particular specifications for the cultivation land which leave arable lands for the cultivation of feedstocks;
- The production of microalgae can be easily customized according to the operational skills, and technology available;
- Microalgae cultivation does not require fertilizers, or pesticides which reduce the number of wastes, and pollutants generated;
- Relying on microalgae for biofuel production will help in reducing the number of nitrogen oxides released (Li Y. , Horsman, Wu, Lan, & Dubois-Calero, 2008).

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1.4 CHLORELLA VULGARIS POTENTIAL FOR BIOFUEL PRODUCTION

The driving factor behind the cultivation process toward biofuel production is the lipid productivity which is the product of biomass productivity, and lipid content. Many types of research have been conducted around the world to find the microalgae strain with high cell growth, and high lipid content; this is necessarily required for the economic feasibility of biodiesel production from microalgae. Generally, microalgae that are used for biofuel production can be classified based on lipid productivity, and biomass productivity. Table (1) shows lipid and biomass productivity for two widely used strains.

Table 1: Lipid and biomass productivity for *Botryococcus braunii* and *Chlorella vulgaris*

(Dayananda, Sarada, et al., 2007 & Griffiths & Harrison, 2009)

Microalgae	Lipid Productivity	Biomass productivity
Botryococcus Braunii (Dayananda, Sarada, et al., 2007)	Hight (lipid content of 50%)	Low (28 mg L^{-1} d^{-1})
Chlorella vulgaris (Griffiths & Harrison, 2009)	Low ((lipid content of 20%)	High (short doubling time of 19 h)

Researches proved that utilizing microalgae with high lipid content, but with low mass productivity results ultimately in low oil productivity. On the other hand, *Chlorella vulgaris* has proven to be a very promising strain for the biodiesel production due to its high biomass productivity, and ease of cultivation process, along with its ability to adapt in various cultivation media (Huntley & Redalje, 2006).

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1.5 CULTIVATION OF CHLORELLA VULGARIS IN WASTEWATER

Like in any other medium, many variables shall be controlled to ensure efficient cultivation of *Chlorella vulgaris* in wastewater medium. The following is a list of parameters that need to be adjusted before cultivation: -

- pH
- Temperature
- Nutrients Concentration (Nitrogen, Phosphorus, and Organic Carbon) and the ration between them
- Lighting
- Oxygen, and CO_2

The main characteristic of wastewater that differentiates it from other cultivation medium is its high concentration of Nitrogen (NH_3, No_3^-, No_2^-) and Phosphorous nutrients. However, excess concentration of Nitrogen, usually in the form of Ammonia, lead to inhibition in the growth of Microalgae. (Ip, Bridger, Chin, Martin, & Raper, 1982).

The presence of other microorganisms in wastewater may compete with microalgae on available nutrients in the medium which in return lead to inhibition of the microalgae growth. Also, it was found that the starting density of microalgae in the wastewater medium affects the growth of the whole population (Lau, Tam, & Wong 1995)

There are variances in the tolerance of microalgae species for being cultivated in different wastewater medium. Chlorophytes microalgae, especially *Chlorella vulgaris* are very efficient in accumulating Nitrogen, and Phosphorus from wastewater (Travieso, Benitez, & Dupeiron, 1992)

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1.6 Other Applications & use of microalgae

1.6.1 Use of microalgae for wastewater treatment

Cultivating microalgae on wastewater combines the goals of providing microalgae with required nutrients for its growth and treat the wastewater by consuming pollutants from the wastewater stream (Andersen, 2005). Effluent streams from wastewater treatment plants are still rich with nitrogen, and phosphorous which if left to be discharged to waterways will generate unwanted algae blooms, and cause eutrophication effect (Sebnem, 2006). Utilizing algae for wastewater treatment has many advantages (Becker, 2004), of which are:

- It provides a feasible method for nutrients recycling as algae biomass which in return will reduce the treatment cost
- The discharged effluent streams to water bodies are much more abundant with Oxygen

The efficiency of utilizing *Chlorella vulgaris* in treating agriculture and municipal wastewaters is out of this research scope.

(Shijian Ge, 2018) proved that almost complete nitrogen and phosphorus could be removed from wastewater (> 99% for both total nitrogen and $PO4^{3-}$-P) through autotrophic, and mixotrophic cultivation with the addition of glucose during the exponential phase.

1.6.2 Use of microalgae for cosmeceutical application

Cosmeceuticals products are designed for the health and beauty of the skin. With the increasing demand for harmless Cosmeceuticals products, antioxidants generated from natural resources have to gain more prominent attention. Recently *Tetraselmis tetrathele* microalgae has been strongly nominated as a natural resource for Cosmeceuticals products due to its high antioxidant contents. (Farahin A. W., 2018) Concluded that *Tetraselmis tetrathele* is a very

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promising bioactive compound for the manufacturing of nano cosmeceutical products due to *Tetraselmis tetrathele* high homogeneity and stability.

1.7 RESEARCH MOTIVATION

One of the causes behind the high cost for producing biodiesel from microalgae is the cost of the chemicals that are required for the cultivation process. This research is motivated with ambitious to reduce the cultivation cost for microalgae by substitute the required chemicals with nitrogen, and phosphorus elements that exist in wastewater streams.

1.8 RESEARCH QUESTIONS

A. Can the optimization of cultivation conditions support *Chlorella vulgaris* to grow in agriculture and municipal wastewaters and reach high growth rates?

B. What are the cultivation conditions for *Chlorella vulgaris* to achieve high growth rates in both agriculture and municipal wastewaters?

1.9 RESEARCH OBJECTIVE

This research aims to maximize the growth rate of *Chlorella vulgaris* in agriculture and municipal wastewaters by investigating the parameters affecting the growth rate of *C. vulgaris*. The aim is to utilize agriculture and municipal wastewaters as an alternative to the pure synthetic medium (MBL) in which chemicals are added to provide microalgae with required nutrients. This substitution should support the reduction in the overall cost of producing biofuels from microalgae.

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CHAPTER 2 - LITERATURE REVIEW

2.1 FACTORS AFFECTING THE GROWTH RATE OF MICROALGAE

Like any other plant, microalgae use the photosynthetic process to utilize the energy in light for the conversion of carbon in the air into lipid. Different factors affect the photosynthetic process and the growth rate of the microalgae as following:

2.1.1 CARBON SOURCE

The carbon source is considered the most critical factor which affects the growth rate of microalgae. Carbon fixation through the photosynthetic process in microalgae can happen autotrophically in which microalgae extract the required carbon source from the inorganic carbon in carbon dioxide (Ren, et al., 2010) . Other microalgae species can perform the carbon fixation heterotrophically in which the microalgae rely on the organic carbon source in the growth medium with the presence or absence of light. Some microalgae can utilize carbon from both inorganic and organic sources which described as mixotrophic (Chojnacka & Noworyta, 2004). Regarding the enlargement of microalgae cultivation for commercial scale, autotrophic cultivation is still dominant due to the elimination of extra cost by adding a source for organic carbon to the growth medium.

2.1.2 NITROGEN SOURCE

Researches have proven that cultivating microalgae with the limitation of nitrogen source causes remarkable accumulation of lipid content in microalgae (Mandal & Mallick, 2009). In a comparison between cultivating microalgae of varied species under normal cultivation condition and nitrogen starvation cultivation conditions, (Hu, et al., 2008) proved that cultivation with nitrogen deficiency increased lipid content in microalgae by 10 - 20 %.

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Microalgae convert nitrogen from its starch form to lipid as a long-term storage mechanism for energy (Siaut, et al., 2011). However, it is very crucial to be aware that putting the microalgae on starvation mode to increase the lipid content does not come with no cost; the drawback of the nitrogen starvation mode is a deterioration in the microalgae growth rate. Therefore, to achieve a high growth rate with high lipid content, a balance between cultivation with regular nitrogen content, and deficient nitrogen content should be achieved.

2.1.3 LIGHTING

Lighting wavelength is one of the most affecting factors in the growth of microalgae (Terry, 1986). The intensity of light can significantly stimulate or inhibit the growth of microalgae. Researchers have proven that the effect of lighting intensity on the growth rate of microalgae can be divided into three phases: light limitation, light saturation, and light inhabitation (Ogbonna & Tanaka, 2000). Figure 3 shows the relation between the lighting intensity and the growth rate.

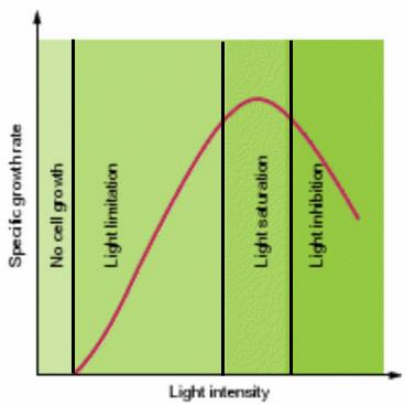

Figure 3: Effect of light intensity on the growth rate of microalgae

(Ogbonna and Tanaka, 2000).

For maximum biomass productivity, the light with saturation intensity needs to be distributed among the photobioreactor. However, this is impossible because the higher the distance from the source of light the lower the light intensity. This reverse proportional between the distance from lighting source and lighting intensity is due to the light shading effect that is

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caused by the increase in the cell concentration. To overcome the lighting distribution challenge inside the photobioreactor, mixing of the growth media can be used to reduce the effect of light shading inside the photobioreactor.

2.1.4 Carbon dioxide (CO_2) concentration

The concentration of CO_2 can affect the growth rate and the lipid accumulation in microalgae. When the CO_2 percentage increased from 0.035 % to .28%, the lipid content of *Nannochloropsis sp*. Increased (Hu & Gao, 2003). On the other hand, the concentration of CO_2 may adversely affect the growth rate, and lipid accumulation if increased above a certain percentage. (Hsueh, Li, Chen, & Chu, 2003) Reported that when the CO_2 % in the air (0.04%) increased to 8 % both the biomass, and the lipid content of *Nannochloropsis* increased, but when the CO_2 reached to 10 % a decline in the both was noticed. For *Chlorella vulgaris*, the optimum CO_2 % range that stimulates high lipid accumulation is 2% - 5% (Chiu, et al., 2008). Moreover, it has been reported by (Cheng, Zhang, Chen, & Gao, 2006) that the maximum carbon fixation rate in *Chlorella vulgaris* was achieved at a CO_2 concentration of 1%.

2.1. 5 Temperature

Temperature could impose a significant effect on the cultivation of microalgae on a commercial scale, especially with open ponds system. The temperature of the cultivation environment varies between day and night, and from season to another. The microalgae growth rate will be promoted when microalgae are provided with the appropriate temperature range; on the other hand, increase of temperature above the allowable range will inhabit the growth of microalgae due to the change in the protein/enzyme nature, and the cellular physiological changes (Pandey, Lee, Chisti, & Soccol, 2003). It has been reported that *Chaetoceros sp*. Showed a higher growth rate when the cultivation condition was controlled in a temperature range of 25 - 30 ^{o}C (Renaud, Thinh, Lambrinidis, & Parry, 2002). In a study for the influence

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of the temperature of *Chlorella vulgaris*, the results suggested that the highest mortality was achieved at a temperature range 20 – 28 ^{o}C (Serra-Maia, Bernard, Gonçalves, Bensalem, & Lopes, 2016).

2.1.6 pH

pH is one of the cultivation conditions that should be controlled to maintain a suitable environment for microalgae to grow. The optimal pH for most microalgae species falls in the range between 7 – 9 (Lavens & Sorgeloos, 1996). It is essential to maintain the pH within the accepted range to avoid disruption to the microalgae cell wall; moreover, pH affects the biochemical reaction in the microalgae. pH also is a crucial factor in utilizing CO_2 gas as a source of carbon for microalgae; as when the CO_2 gas is fed to the cultivation medium it dissolves, and form (HCO3-); this conversation is wholly depended on the pH of the medium.

2.1.7 Salinity

Many types of research on microalgae showed interest in studying its ability to grow in the marine environment. Microalgae can equalize the osmotic stress in the cultivation medium. It has been found that marine species of microalgae can tolerate salinity concentration in the surrounding medium up to 1.7 M (Pandey, Chisti, Lee, & Soccol, 2013). However, researchers concluded that salinity 35% or higher, which is standard in seawater, prohibit the growth of microalgae, and the photosynthesis process (Jacob, O.Kirst, Wiencke, & Lehmann, 1991).

2.2 The microalgae – bacteria symbiosis

Microalgae and bacteria have a synergistic effect to each other on both the physiological and metabolism scale. In the paste, bacteria were considered as a contamination in the microalgae cultivation media. However, this perception has changed with the discovery of

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microalgae – bacteria symbiosis, and its benefit for biotechnology. Recent studies have proven the positive effect of microalgae – bacteria symbiosis on the algae growth rate (Fuentes, 2016).

In the microalgae – bacteria symbiosis, microalgae give off dissolved organic matters (DOMs) as sources of carbon, Sulphur, nitrogen, or phosphorus to bacteria which in return remineralize these organic nutrients to its inorganic states which are required for the microalgae growth (Buchan et al., 2014). Moreover, the bacteria provide microalgae with B vitamins, while the algae provide the bacteria with the fixed carbon in the form of dissolved organic carbon (DOC) (Croft et al. 2005) as demonstrated in Figure (4).

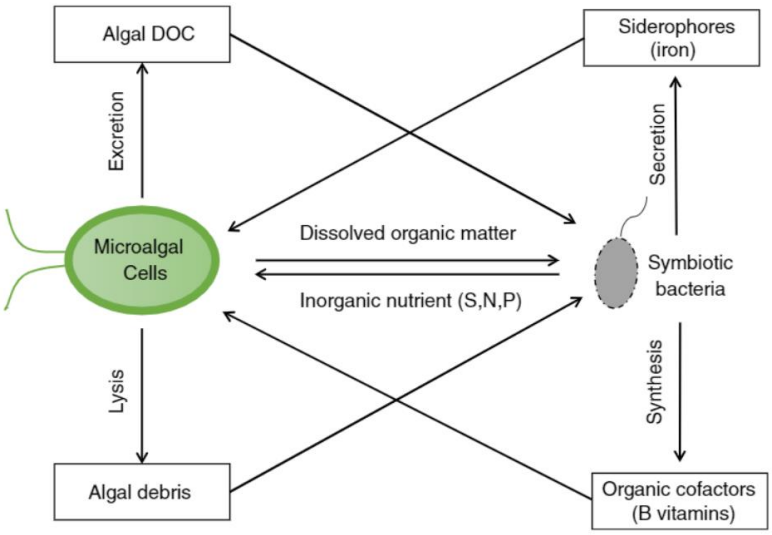

Figure 4: The microalgal – bacteria symbiosis effect

(Yao., et al., 2018)

2.3 MICROALGAE CULTIVATION SYSTEMS

The selection of the cultivation system has a significant impact on the production cost for the microalgae which is not commercially feasible to compete with diesel from fossil fuel. Of the reasons for this excessive cost is the design of the photobioreactor, the supporting

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systems, and the required energy input. The concept of photobioreactor for autotrophic cultivation of microalgae is familiar between opened and closed system which mainly to provide microalgae with proper mixing, lighting intensity, and gas transfer. Although the opened system has a lower cost in comparison to the closed system; however, it is much exposed to contamination due to the free gas exchange with the surrounding environment. Other downsides for opened systems are the cultivation conditions poorly controlled, and the growth rate is lower than the closed system. Table (2) shows the advantages, and disadvantages of both opened and closed systems.

Table 2: Advantages and disadvantages of cultivation systems for microalgae

(Pulz, 2001)

Parameter	Open Ponds (Raceway Ponds)	Closed Systems (PBR Systems)
Contamination risk	Extremely high	Low
Space required	High	Low
Water losses	Extremely high	Almost none
CO_2 losses	High	Almost none
Biomass quality	Not susceptible	Susceptible
Variability as to cultivatable species	Not given; cultivation possibilities are restricted to a few algal varieties	High; nearly all microalgal varieties
Flexibility of production	Change of production between the possible varieties nearly impossible	Change of production without any problems
Reproducibility of production parameters	Not given; dependent on exterior conditions	Possible within certain tolerances
Process control	Not given	Given
Standardization	Not possible	Possible
Weather dependence	Absolute; production impossible during rain	Insignificant because closed configurations allow production during bad weather
Period until net production is reached after start or interruption	Long; approx. 6–8 weeks	Relatively short; approx. 2–4 weeks
Biomass concentration during production	Low, approx. 0.1–0.2 g/L	High; approx. 2–8 g/L
Efficiency of treatment process	Low; time-consuming, large-volume flows due to low concentrations	High; short-term, relatively small-volume flows

On the other hand, opened systems are less involved regarding operation than in closed systems. For cultivating microalgae to achieve specific product specifications, a closed system can achieve better results as it allows better control of the cultivation conditions.

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2.3.1 OPENED SYSTEMS

Opened systems exist in the form of the raceway, shallow big, or circular (Masojídek & Torzillo, 2008). The oldest opened system is the circular type which has centrally rotating agitator like the water treatment tanks. The area of the circular type open pond is limited to 10,000 m2 as increasing the area above this limit will lead to uneven mixing by the rotating arms.

Figure 5: Circular open pond system

(Making Biodiesel Books)

Raceway ponds are the most conventional opened system due to its lower cost compared to circular ponds. Raceway ponds are constructed of closed loops with oval shape recirculation channels. The raceway is characterized by their shallow depth (0.2 – 0.5 m) to allow better penetration of sunlight to the cultivation medium (Ugwu, Aoyagi, & Uchiyama, 2008).

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Figure 6: Raceway open pond

(Meristem Journeys)

The opened system still has the privilege of producing microalgae products at a lower cost such as biofuel. However, for other products with a high value such as pharmaceutical, cosmetics products a controlled cultivation condition can only be achieved by closed systems.

2.3.2 CLOSED SYSTEM

The term Closed refers to the system in which no direct gas exchange with the surrounding environment which minimizes the possibility for contamination. One type of the closed system is the vertical column photobioreactor which consists of vertical tubing, mainly glass, to allow penetration of light through the cultivation medium. The vertical column photobioreactor has the gas system installed at its bottom which converts the gas into tiny bubbles which provide the cultivation medium with mixing, carbon dioxide, and removal of oxygen that is produced by microalgae in the photosynthetic process.

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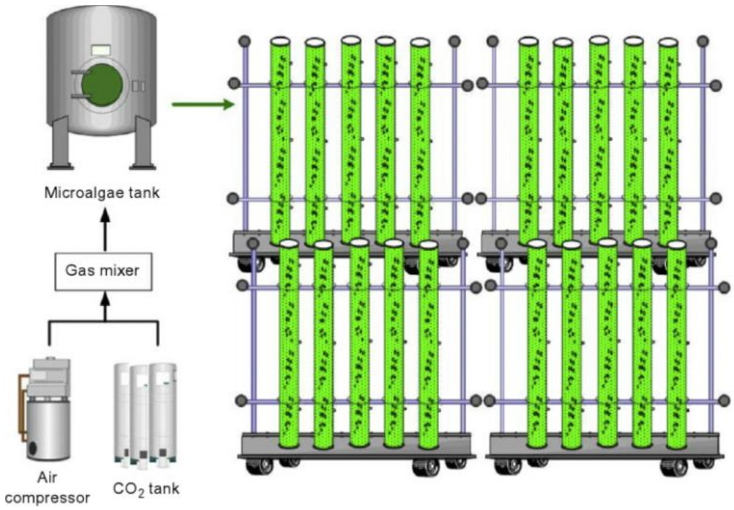

Figure 7: Vertical column photobioreactor

(Capital Energy)

Another type of closed system is the flat panel photobioreactor which consists of transparent flat plates that which illuminate on both sides, and mixing is provided by aeration as in the vertical column photobioreactor. The flat panel photobioreactor can be constructed to the desired light path; however, the downside of this system is it required large space, more lighting power than other systems, and it is difficult to be cleaned; never the less, it has lower efficiency of mass production (Slegers, 2011).

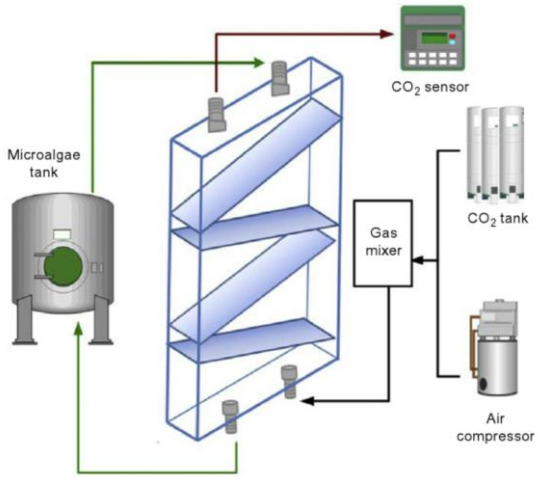

Figure 8: Flat plate photobioreactor

(Capital Energy)

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The most commercially used closed system is the Horizontal Tubular Photobioreactor which is made from small diameter tubes of polypropylene or polyvinylchloride.

The idea below the small diameter is to allow for efficient penetration of light through the cultivation medium, and like other closed systems, mixing is provided by aeration which is derived by an air pump. The most significant feature of the tubular system is its high air residence time which introduces more dissolved CO_2 to the cultivation medium. Also, the tubular system can utilize both artificial, and sunlight.

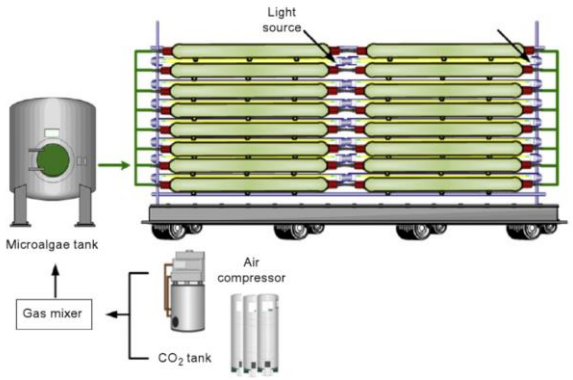

Figure 9: Horizontal tubular photobioreactor

(Capital Energy)

2.3.3 Overall look for the microalgae cultivation systems

Despite its low construction, and operation costs, opened systems require land space, and is more exposure to contamination risks; moreover, since there is no control especially for temperature in opened systems, it is widely dependent on the temperature of the weather; therefore, it is not possible to use it in cold regions of the world. On the other hand, closed systems provide more control on the cultivation environment, yet its excessive cost is the main barrier for being commercially feasible for mass production of microalgae products. Table (3) demonstrates the prospects, and limitations of the most commonly used cultivation systems (Ugwu, Aoyagi, & Uchiyama, 2008).

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Table 3: Prospects and limitations of commonly used microalgae cultivation systems

(Pandey, Chisti, Lee, & Soccol, 2013)

	Culture System	Prospects	Limitations
Open	Ponds	Relatively economical, easy to clean up after cultivation, good for mass cultivation of algae	Little control of culture conditions, difficulty in growing algae cultures for long periods, poor productivity, occupy large land mass, limited to few strains of algae, cultures are easily contaminated
	Raceway	Can be operated in a continuous mode.	High ratio of area/volume required, required high power of paddle to avoid algae precipitation
Closed	Vertical column photobioreactors	High mass transfer, good mixing with low shear stress, low energy consumption, high potentials for scalability, easy to sterilize, readily tempered, good for immobilization of algae, reduced photoinhibition and photo-oxidation	Small illumination surface area, their construction requires sophisticated materials, stress to algal cultures, decrease of illumination surface area upon scale-up
	Flat plate photobioreactors	Large illumination surface area, suitable for outdoor cultures, good for immobilization of algae, good light path, good biomass productivities, relatively cheap, easy to clean up, readily tempered, low oxygen buildup	Scale-up requires many compartments and support materials, difficulty in controlling culture temperature, some degree of wall growth, possibility of hydrodynamic stress to some algal strains
	Horizontal tubular photobioreactors	Large illumination surface area, suitable for outdoor cultures, fairly good biomass productivities, relatively cheap	Gradients of pH, dissolved oxygen and CO_2 along the tubes, fouling, some degree of wall growth, requires large land space

2.4 Lipid productivity and microalgae species selection

Lipid productivity is a factor of microalgae growth rate, and lipid content. Microalgae species designated for lipid production can be divided into two categories; first, microalgae with high lipid content but low growth rate such as e Botryococcus Braunii which has lipid content of 50 %, but low growth rate of 28 mg L-1 d-1, (Dayananda, Sarada, Rani, Shamala, & Ravishankar, 2007) Second, *Chlorella vulgaris* with low lipid content of 20 %, but its biomass is doubled every 19 hours (Griffiths & Harrison, 2009). Lipid content can be presented as a product of biomass productivity and lipid content

Lipid productivity = biomass productivity x lipid content

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In general cultivation condition of *Chlorella vulgaris*, the culture life cycle was 14 days, and the lipid productivity is 14.9 mg L-1 d-1 as reported by (Illman, Scragg, & Shales, 2000). However, (Lv, Cheng, Xu, Zhang, & Chena, 2010) enhanced the cultivation conditions for *Chlorella vulgaris* and could achieve lipid productivity of 40 mg L-1 d-1. Also in heterotrophic cultivation of Chlorella Protothecoides, (Xiong, Li, Xiang, & Wu, 2008) reached to lipid productivity of 1210 mg L-1 d-1.

The nitrogen deprivation stimulates the lipid accumulation in microalgae. Lipid content in *Chlorella vulgaris* can reach up to 40 % with deprivation in Nitrogen compared to 18 % with normal conditions (Illman, Scragg, & Shales, 2000). However, the decrees in Nitrogen concentration is accompanied by a decrease in the growth rate for the microalgae (Rodolfi, et al., 2009). The Nitrogen level of 5 mM considered as the minimum acceptable level for microalgae to grow (Li Y. , Horsman, Wang, Wu, & Lan, 2008). In studying the effect of Nitrogen level, (Li Y. , Horsman, Wang, Wu, & Lan, 2008) noticed that in cultivating *Neochloris Oleoabundans* the NaNO3 range of 3 – 20 mM was tested. It was noticed that the height lipid productivity was achieved at 5mM NaNO3, while the highest lipid content was achieved at three mM NaNO3.

2.5 THE EFFECT OF PESTICIDES IN AGRICULTURE WASTEWATER

Due to the varsity of food crops concerning the seasonality of the cultivation process, different pesticides may be used which in turn will be introduced into the agriculture wastewater stream. Below are the most used active ingredients in pesticides products:

Nonylphenol (NP) usually used as intermediate in the manufacturing of non-ionic surfactants nonylphenol ethoxylates which are used in pesticides. The existence of NP in the agriculture wastewater medium inhibits the growth rate of *C. vulgaris*, decrease chlorophyll

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content, and overproduce Reactive Oxygen Species (ROS) which destroy the *C. vulgaris* membrane system (Haifeng Qian, 2011)

Pentachlorophenol (PCP) is the most toxic member in the Chlorophenols (CPs) as it has a high number of chlorine atoms. PCP is widely used with pesticides and is considered a primary pollutant source due to its long half-life, and its harmful effect at low concentrations (Paulode Moraisa, 2014) proved that PCP inhibited the growth of C. vulgaris in all concentration levels above 0.99 µg L^{-1}.

Topramezone has been recently selected as herbicide due to its pyrazole structure, and its ability to eliminate different broadleaf weeds and annual grass. Applying Topramezone to agriculture crops cause a significant increase of such herbicide in the agriculture wastewater. Research has proven that Topramezone is capable of affecting the cell morphology, and photosynthetic process in *Chlorella vulgaris*; moreover, Topramezone induces Reactive Oxygen Species (ROS) cause damage to *C. vulgaris* membrane through lipid peroxidation (Fangfang Zhaoa, 2017)

Boscalid as the most widely used pesticide of Succinate dehydrogenase inhibitor (SDHI) has a significant role in protecting agriculture crops from many plant diseases through the inhibition of fungal respiration. However, (Le Qian, 2018) proved that Boscalid at a concentration of 1.6 mg/L had inhibited the *Chlorella vulgaris* growth rate and affected its content of chlorophyll and carotenoids.

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2.6 FEASIBILITY ASSESSMENT FOR GENERATING MICROALGAE FROM *CHLORELLA VULGARIS*

In a trial for an approximation for industrial operation (Juan J. Jaramillo et al., 2012) calculated the mass and energy balance for producing oil and cake from *Chlorella vulgaris* by simulation. In their simulation (Juan J. Jaramillo et al., 2012) utilized CO_2 emitted from the rice husk processing. Figure(10) proposes the algae processing system, and the mass and energy balance is presented in Table (4).

The simulation gave a yield of 0.37 kg of oil and 0.63 kg of cake per kilogram of microalgae biomass. The model utilized generated energy from the rice husk industry to supply the *Chlorella vulgaris* processing system with 86% of the required energy. The economic evaluation of the cultivation and extraction of *Chlorella vulgaris* are presented in Table (5).

The economic study for the model showed a production cost of 0.56 USD/kg for oil and 0.33 USD/kg for cake. Figure (11) represents the cost distribution for the cultivation of *Chlorella vulgaris* and oil extraction in which 33% of raw materials cost goes to the formulation of the growth media, 57.14% goes for the light cost and maintenance, and 9.52% for gas pretreatment. (Juan J. Jaramillo et al., 2012) flagged the importance of finding the inexpensive nutrient source as a replacement for the formulated media, e.g., utilizing wastewater. (Juan J. Jaramillo et al., 2012) concluded that the cost of microalgae oil generated from *Chlorella vulgaris* is 0.504 USD/L.

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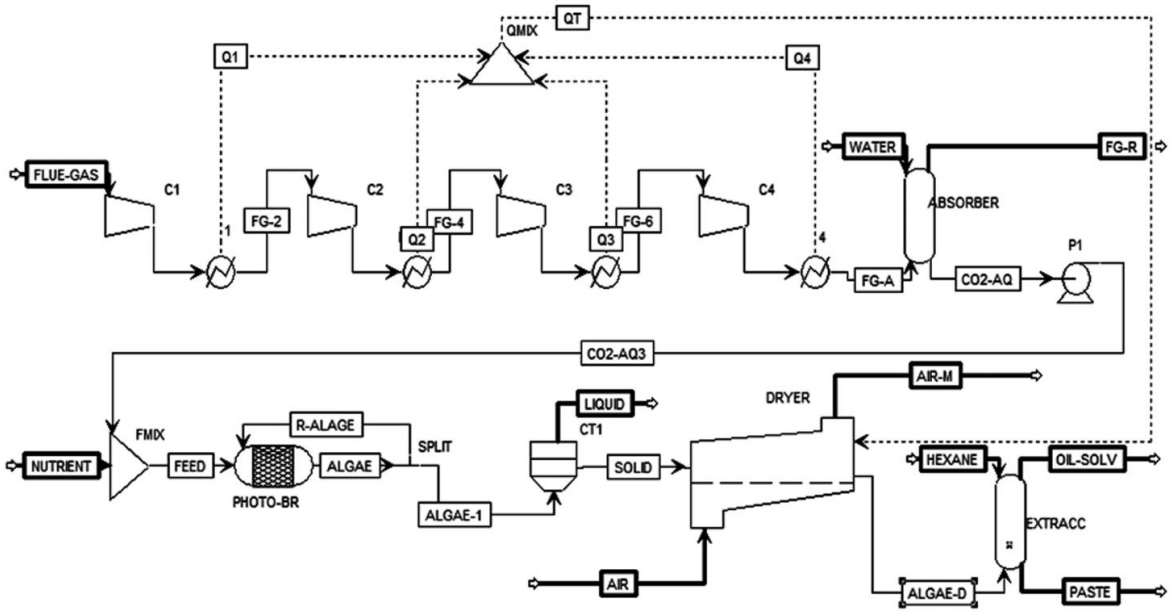

Figure 10: Cultivation and extraction flowsheet for *Chlorella vulgaris*

(Juan J. Jaramillo, et al, 2012)

Table 4: Mass and energy balances of the cultivation of *Chlorella vulgaris*

(Juan J. Jaramillo et al., 2012)

stream	feedstock	flow rate (kg/h)
flue gas	combustion gases	8621
water	water	9500
nutrients	nutrients	108.30
air	air	100000
hexane	hexane	12312.62
stream	products	flow rate (kg/h)
oil-solv	microalgae oil	347.38
paste	microalgae cake	593.11
air-m	humidified air	100752.39
oil-solv	solvent recuperated	12312.62
fg-r	gases emitted	6403.13
liquid	cell-free broth	10132.24
stream	energy	flow rate (MJ/h)
QT	exchange energy	3479.8
–	energy demand	3009.6

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Table 5: The economic evaluation of the cultivation and extraction of *Chlorella vulgaris*

(Juan J. Jaramillo et al., 2012)

	cost (USD/kg)		
category	biomass production (microalgal biomass dry basis total)	oil (microalgae oil dry basis)	cake (microalgae cake dry basis)
feedstock	0.10	0.27	0.16
utilities	0.03	0.08	0.05
operation and maintenance costs	0.03	0.08	0.05
operational charges	0.004	0.03	0.006
indirect costs	0.02	0.05	0.03
general and administrative costs	0.02	0.05	0.03
total cost	0.21	0.56	0.33

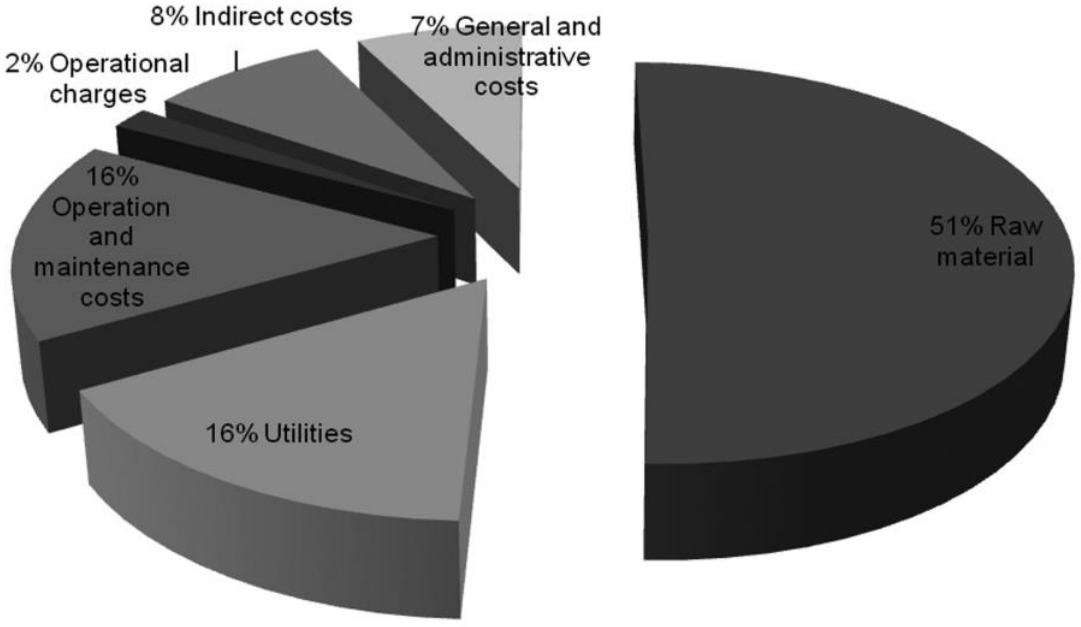

Figure 11: Cost distribution of *Chlorella vulgaris* cultivation
(Juan J. Jaramillo et al., 2012)

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2.7 CULTIVATING OTHER MICROALGAE STRAINS IN WASTEWATER

In a study for the growth of nine microalgae strains, blue and green microalgae, (Mostafa S. S. M., 2012) proved the concept of cultivating microalgae in wastewater for the combined objective of nutrients removal, and lipid production to be used as feedstock for biodiesel. The nine strains were cultivated on secondary treated municipal wastewater. Four streams were tested in (Mostafa S. S. M., 2012) as follows:

- T_1: wastewater without nutrients or sterilization
- T_2: wastewater with sterilization
- T_3: wastewater + nutrients with sterilization
- T_4: wastewater + nutrients without sterilization

Table (6) represents the dry weight of different microalgal species cultivated in different wastewater treatments. (Mostafa S. S. M., 2012) concluded that wastewater without nutrients or sterilization (T_1) are suitable for the cultivation of (*Nostoc humifusum),* wastewater with sterilization (T_2) are suitable for the cultivation of (*Oscillatoria sp*, and *Phormium fragile*), wastewater + nutrients with sterilization (T_3) are suitable for the cultivation of (*Nostoc muscorum, Anabaena flows aquae, Chlorella vulgaris, Spirulina platensis, Wollea saccate*), and wastewater + nutrients without sterilization (T_4) are suitable for the cultivation of (*Anabaena oryzae*)

Table 6: Dry weight of microalgal species cultivated in different wastewater treatments (Mostafa S. S. M., 2012)

Algal species	value									
	Control		T_1		T_2		T_3		T_4	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Nostoc muscorum	21.12	181.12	29.44	86.40	37.76	102.4	36.48	104.32	24.32	92.80
Anabaena flous aquae	30.08	216.32	11.52	83.20	54.40	97.28	44.16	236.80	12.80	89.54
Chlorella vulgaris	83.20	271.36	57.60	158.08	75.52	261.76	204.80	670.72	148.48	452.48
Oscillatoria sp	14.72	513.28	2.56	53.12	24.32	288.00	26.24	157.44	19.20	164.48
Spirulina platensis	25.60	303.36	6.40	57.60	32.00	202.24	25.60	272.00	57.60	167.68
Anabaena oryzae	19.84	320.00	14.72	192.00	60.80	236.80	51.20	232.96	18.56	238.08
Wollea saccata	44.80	103.68	22.40	71.68	39.40	156.80	51.20	698.88	24.96	656.64
Nostoc humifusum	47.36	400.00	10.88	572.80	33.92	487.68	29.44	90.88	21.76	295.04
Phormedium fragile	28.80	103.68	21.12	156.16	8.32	481.92	32.00	90.88	7.04	129.92
LSD		0.028		0.032		0.028		0.032		0.032

Each value is presented as mean of triplet treatments, LSD: Least different significantly at $P \leq 0.05$ according to Duncan's multiple range test
T_1: wastewater without treatment; T_2: wastewater after sterilization; T_3: wastewater+ nutrients with sterilization T_4: wastewater+ nutrients without sterilization

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2.8 THE EFFECT OF OTHER MICROORGANISMS ON MICROALGAE

Other microorganisms in non-sterilized cultivation media, such as fungal, bacterial and amoebic species, can cause a challenge for the cultivation of microalgae (Mendes, 2013; Carney, 2014). A very well know algal parasites are Fungi and fungal-like organism (oomycetes, labyrinthulids) (Carney, 2014). Figure (12) depict microscopic examination of algal culture contaminated with a protozoan. (A-C) The sequence of events depicting the predatory activity of protozoan strain over the alga glamor; (D-G) Motion of protozoan towards the green alga for feeding and finally away from it and (H-I) Presence of microalgal cells within the predatory protozoan (Wahi, 2018)

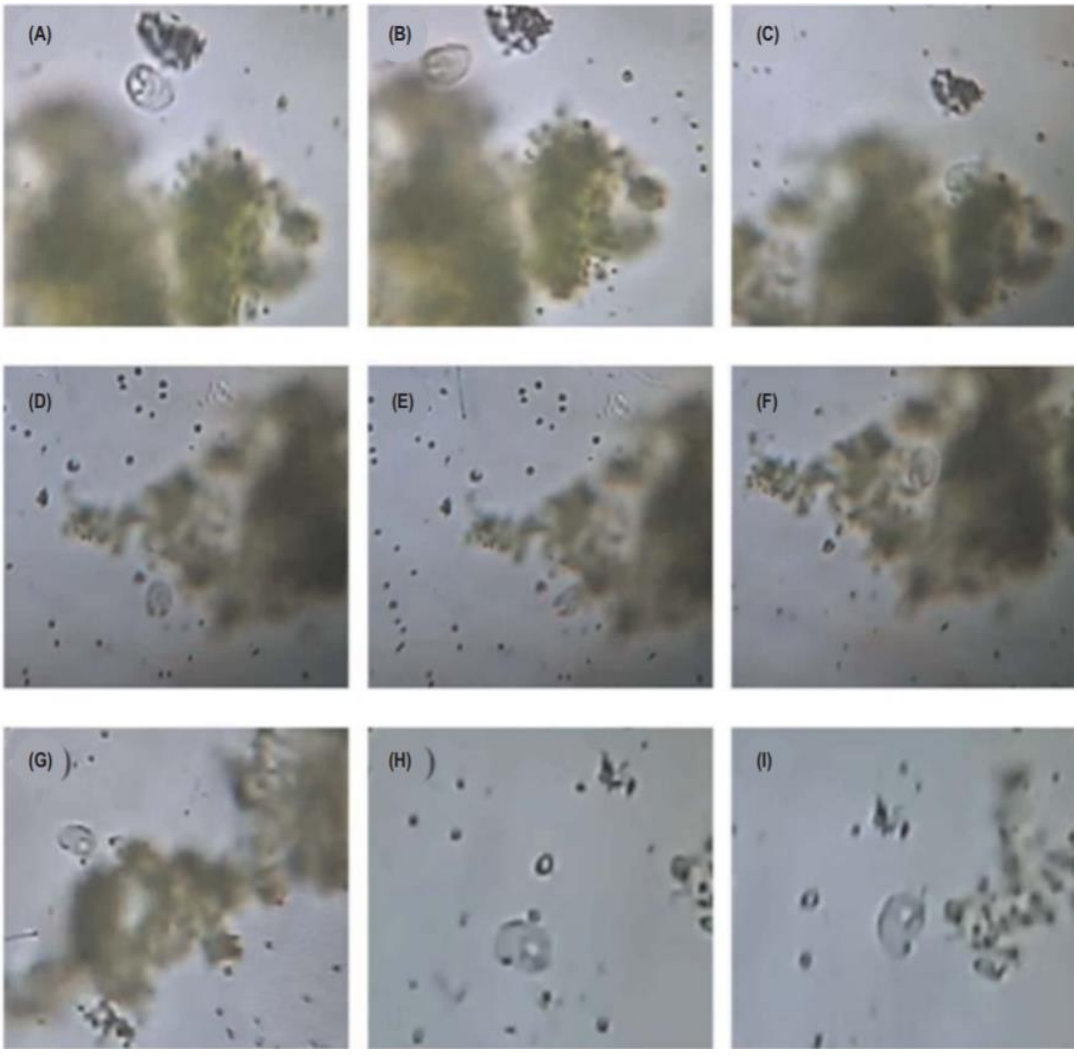

Figure 12: Predatory action of protozoa on *Chlorella vulgaris* (Wahi, 2018)

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CHAPTER 3 - EXPERIMENTAL METHOD

The experimental method for this research is divided into the following sections:

- Preparation of Woods Hole MBL synthetic medium, and initial inoculation phase
- Investigating the parameters affecting the growth rate of *Chlorella vulgaris*
- Enlargement phase
- Total lipid measurement

3.1 PREPARATION OF WOODS HOLE MBL SYNTHETIC MEDIUM, AND INOCULATION PHASE

Chlorella vulgaris strain was obtained from Phycology laboratory, Botany and Microbiology Department, Faculty of Science, Alexandria University. Stock solutions were prepared from chemicals in Table (7). Stock solutions were stored in a refrigerator at 4 ^{o}C.

Table 7: Woods Hole MBL medium recipe
(Nichols, 1973)

Stock solutions	Per litre distilled water	
1. $CaCl_2.2H_2O$	36.76 g	
2. $MgSO_4.7H_2O$	36.97 g	
3. $NaHCO_3$	12.60 g	
4. K_2HPO_4	8.71 g	
5. $NaNO_3$	85.01 g	
6. $Na_2SiO_3.9H_2O$	28.42 g	
7. Na_2EDTA	4.36 g	
8. $FeCl_3.6H_2O$	3.15 g	
9. Metal Mix $CuSO_4.5H_2O$ $ZnSO_4.7H_2O$ $CoCl_2.6H_2O$ $MnCl_2.4H_2O$ $Na_2MoO_4.2H_2O$	 0.01 g 0.022 g 0.01 g 0.18 g 0.006 g	Add each constituent separately to ~750mL of distilled H_2O, fully dissolving between aditions. Finally make up to 1L with distilled H_2O.
10. Vitamin stock Cyanocobalamin (Vitamin B12) Thiamine HCl (Vitamin B1) Biotin	 0.0005 g / L dH_2O 0.10 g / L dH_2O 0.0005 g / L dH_2O	
11. Tris stock	250.0 g / L dH_2O	

To prepare MBL medium, one mL of each stock solution (1–11) was added to one liter of distilled water.

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The axenic unicellular algae (*Chlorella vulgaris)* was initially inoculated in 250 ml flask by adding 5 ml from the *Chlorella vulgaris* aliquot to 100 ml of synthetic medium (MBL) as demonstrated in Figure (13). pH was adjusted to 7.2 using hydrochloric acid, and the medium was sterilized in an autoclave for 15 min (15 PSI, 121.11 ^{o}C). The initial inoculation served as culture stock for further experiments. Inoculation was conducted under controlled laboratory conditions (temperature at 22 (+/- 3) ^{o}C, and light intensity at 80 µ mol m^{-2} S^{-1}) in a culturing chamber. The inoculation was conducted under a regime of 16:8 light to dark cycle.

Figure 13: Initial inoculation of *Chlorella vulgaris*

3.2 INVESTIGATING THE PARAMETERS AFFECTING THE GROWTH RATE OF *CHLORELLA VULGARIS*

The objective from this step was to test different cultivation conditions for *Chlorella vulgaris* using two wastewater streams (Agriculture & Municipal) and benchmark the growth rate with that recorded from cultivation on the synthetic medium (MBL). Agriculture Wastewater was obtained from an agriculture drainage canal in Idku city, Bahira governate while municipal Wastewater was obtained from Katamya Heights Wastewater Treatment Plant in the fifth settlement, Cairo governate. The later was collected after the secondary treatment,

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and before the chlorination step. It is worth to mention that the collected agriculture wastewater was in October during which citric crops were cultivated; a variety in the collected agriculture wastewater may occur due to the difference between crops' need in different seasons.

Fifteen experiments were started simultaneously and continued for 35 days as explained in Table (8). For each experiment, 100 mL of the testing medium was added to a 250 mL flask, and 5 mL of the *Chlorella vulgaris* was added to each flask. pH of the medium was maintained in the range of 7 - 7.5, with a temperature range of 22 (+/- 3) ^{o}C. Some experiments were performed under indirect sunlight (16:8 light to dark cycle) (Cheirsilp & Torpee, 2012), while others were performed under led light by using UTEX RGB-LED Lighting Platform which has 60 total LED Lights (five rows of twelve) with 43,200 millicandela (MCD) output as demonstrated in Figure (14). The UTEX RGB-LED Lighting Platform was obtained from the University of Texas at Austin, USA.

For experiments where the illumination source was led light, the lighting color and illumination duration were examined as well.

Altering the purity of the medium was also tested between sterilized vs. non-sterilized, and pure wastewater medium vs. a mixture of wastewater and a synthetic medium.

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Figure 14: UTEX RGB-Led lighting platform

(The University of Texas at Austin)

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Table 8: Experiments objectives and tested cultivation conditions

Experiment No.	Objective	Cultivation Medium	pH Range	Temperature Range	Light Source	Light Color	Illumination Hours
1	Cultivating *C. vulgaris* on (MBL) synthetic medium to use its growth rate as a benchmark for other experiments	synthetic medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
Cultivation on Agriculture Wastewater							
2	Cultivating *C. vulgaris* on sterilized agriculture wastewater under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in an experiment (1).	sterilized agriculture wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
3	Cultivating *C. vulgaris* on non-sterilized agriculture wastewater under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	non-sterilized agriculture wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
4	Cultivating *C. vulgaris* on sterilized agriculture wastewater in led light with a blue wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	sterilized agriculture wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Led light	Blue	24

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5	Cultivating *C. vulgaris* on sterilized agriculture wastewater in led light with a white wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	sterilized agriculture wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Led light	White	24
6	Cultivating *C. vulgaris* on a mixture between sterilized agriculture wastewater, and synthetic medium (MBL) under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in an experiment (1).	75 ml - sterilized agriculture wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
7	Cultivating *C. vulgaris* on a mixture between non-sterilized agriculture wastewater, and synthetic medium (MBL) under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	75 ml - Non-sterilized agriculture wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle

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Cultivation on Municipal Wastewater							
8	Cultivating *C. vulgaris* on non-sterilized municipal wastewater under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in an experiment (1).	100 ml - Non-sterilized municipal wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
9	Cultivating *C. vulgaris* on non-sterilized municipal wastewater in led light with a white wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in an experiment (1).	100 ml - Non-sterilized municipal wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Led light	White	24
10	Cultivating *C. vulgaris* on a mixture between non-sterilized municipal wastewater, and synthetic medium (MBL) under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	75 ml - Non-sterilized municipal wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle

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11	Cultivating *C. vulgaris* on a mixture between non-sterilized municipal wastewater, and synthetic medium (MBL) in led light with a white wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	75 ml - Non-sterilized municipal wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Led light	White	24
12	Cultivating *C. vulgaris* on sterilized municipal wastewater under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	100 ml - sterilized municipal wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
13	Cultivating *C. vulgaris* on sterilized municipal wastewater in led light with a white wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	100 ml - sterilized municipal wastewater	7 – 7.5	22 (+/- 3) ^{o}C	Led light	White	24

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14	Cultivating *C. vulgaris* on a mixture between sterilized municipal wastewater, and synthetic medium (MBL) under indirect sunlight and compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	75 ml - sterilized municipal wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Sunlight	Sunlight	16:8 light to dark cycle
15	Cultivating *C. vulgaris* on a mixture between sterilized municipal wastewater, and synthetic medium (MBL) in led light with a white wavelength and exposure time of 24 hrs./d. Moreover, compare the growth rate with what is recorded from cultivation on synthetic medium (MBL) in the experiment (1).	75 ml - sterilized municipal wastewater 25 ml - Synthetic Medium (MBL)	7 – 7.5	22 (+/- 3) ^{o}C	Led light	White	24

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3.3 ENLARGEMENT PHASE

Enlargement was performed to the experiments with the cultivation conditions that showed the highest dry weight (g/L) in which 25 ml of *Chlorella vulgaris* was cultivated in 500 ml medium of the chosen conditions which are:

- 75% vol. of non-sterilized agriculture wastewater mixed with 25% vol. of synthetic medium (MBL) in indirect sunlight under 16:8 light to dark cycle.
- Non-sterilized municipal wastewater in indirect sunlight under 16:8 light to dark cycle.

Three replicas were prepared for each chosen cultivation conditions to verify the enlargement results. Air was supplied to each flask through an air pump to generate air bubbles to mix the culture and increased the contact of the culture with air and the medium. Figure (15) represents the enlargement setup.

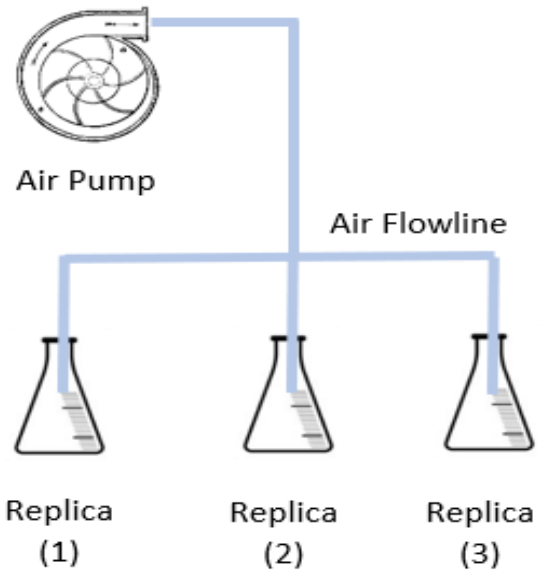

Figure 15: Enlargement phase setup

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3.4 GROWTH MEASUREMENT

The growth of *Chlorella vulgaris* was measured regarding Optical Density (OD) at 680 wavelengths using DR/2000 Spectrophotometer Figure (16) and expressed as Dry Weight (DW). The following equation represents the correlation between the Dry Weight (DW), and the Optical Density (OD) (Hongli Zheng, 2011):

$$DW = 0.560 \times OD_{680} \ (r^2 = 0.986)$$

Figure 16: DR/2000 spectrophotometer

(Hach)

Moreover, calculated Dry Weight was verified by following the below steps:

1. The weight of aluminum weighing dish was recorded
2. 10 mL of the culture was transferred to a 15 mL centrifuge tube
3. *Chlorella vulgaris* cells were pelletized by centrifugation for 3000 rpm for 5 minutes; using Heraeus Labofuge 200 centrifuge.
4. The supernatant was replaced by 10 mL of distilled water
5. Cells were centrifuged again at 1600 rpm for 5 minutes

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6. Supernatant was discarded
7. 1 mL of distilled water was added, and the pellets were suspended by genteelly pipetting them up and down.
8. The concentrated algae were transferred to the aluminum dish
9. The aluminum dish was placed in 50 – 60 ^{o}C oven and the weight was recorded every 0.5h, 1h, 1.5h until it is constant
10. The original weight of the aluminum dish was subtracted from the new weight to calculate the *Chlorella vulgaris* dry weight
11. The growth curve for *Chlorella vulgaris* was plotted.

3.5 TOTAL LIPID MEASUREMENT

Bligh and Dyer method for lipid extraction (Bligh, 1959) was used for the quantification of produced lipid as follows:

1- 8 ml of *Chlorella vulgaris* medium was homogenized in a blender for 2 minutes with a mixture of 10 mL chloroform, and 20 mL methanol to reach 1:2:0.8: parts chloroform: methanol: water (v/v/v).
2- Another 10 mL of chloroform was added, and the mixture was blended again for 30 seconds
3- 10 mL of distilled water was added, and the mixture was blended again for 30 seconds giving a final ratio of 2:2:1.8 chloroform: methanol: water (v/v/v).
4- The homogenate was filtered using Whatman No. 1 filter paper on a Coors No. 3 Buchner funnel with slight suction
5- when the residue became dry, the pressure was applied with the bottom of a beaker to ensure maximum recovery of solvent.

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6- The filtrate was transferred to 50 mL graduated cylinder and allowed few minutes for complete separation in two phases, and the volume of the chloroform layer was recorded (at least 15 ml).
7- The methanol phase was removed by a pipette, with small volume from chloroform to ensure complete removal of methanol phase.
8- Three pre-weighted aluminum dishes were prepared, and to each, a 5 mL from the chloroform layer was added.
9- In the fume hood, the aluminum dish was heated at low heat until the chloroform was evaporated, and only thin lipid layers were left
10- The aluminum dish was dried in a drying oven at 105 ^{o}C for 15 minutes to remove the last trace of chloroform
11- The aluminum dish was cooled in a desiccator for a while
12- The aluminum dish was weighed again to calculate the lipid content
13- The Total Lipid is calculated by the following equation (Pandey, Chisti, Lee, & Soccol, 2013):

$$\text{Total lipid} = \frac{\text{weight of lipid in aliquot} \times \text{volume of chloroform layer}}{\text{volume of aliquot}}.$$

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CHAPTER 4 - RESULTS AND DISCUSSION

As explained in the experimental method chapter, *Chlorella vulgaris* was initially cultivated on a synthetic medium (MBL) for 35 days in Experiment (1), and growth rate was measured in dry weight (g/L). The growth rate measurements as recorded from Experiment (1) was plotted as growth rate curve; this curve was used as a benchmark for other growth rates' curves from other experiments which represent cultivations under different conditions. Figure (17) represents Experiment (1) growth rate's curve in which the cultivation conditions were as follow:

- Cultivation medium: synthetic medium (MBL)
- pH range: 7 – 7.5
- Temp range: 22 (+/- 3) ^{o}C
- Light source: sunlight
- Light hours: 16:8 light to dark cycle

Figure (17) shows that the cell density in the first week is relatively slow due to the low inoculation ratio (small number of cells is added to the new media). In the next three weeks, the growth curve turns to be exponential. *Chlorella vulgaris* growth entered the stationary phase after 28 days of cultivation. Experiment (1) was not intended to depict the full life cycle of *Chlorella vulgaris* but to the benchmark growth rate in synthetic medium (MBL) against growth rates in other cultivation conditions.

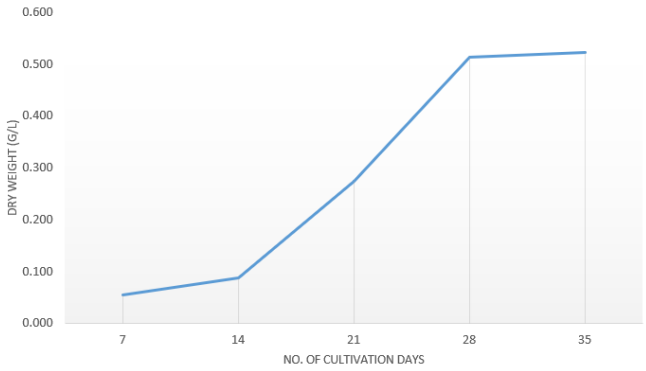

Figure 17: Growth rate curve for *Chlorella vulgaris* in synthetic medium (MBL)

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4.1 STERILIZATION EFFECT ON GROWTH RATE

In studying the sterilization effect on the growth rate, six experiments were conducted on agriculture and municipal wastewaters. The object of those experiments was to investigate whether the existence of other microorganisms in the cultivation medium will suppress the growth of the *Chlorella vulgaris*. Experiments were divided into two groups using sunlight and led light.

4.1.1 STERILIZATION EFFECT IN AGRICULTURE WASTEWATER UNDER INDIRECT SUNLIGHT

In sterilized agriculture wastewater *Chlorella vulgaris* was able to adapt to the new cultivation environment and entered the exponential phase after the first week. After fourteen days of cultivations, *Chlorella vulgaris* achieved growth rate close to the benchmark from Experiment (1). In the third week, *Chlorella vulgaris* did not continue its exponential growth but entered the stationary phase with a big difference from the benchmark. *C. vulgaris* consumed most of the available nutrients in the agriculture wastewater sample in the first fourteen days of cultivation. The remaining nutrients in agriculture wastewater did not support the *C. vulgaris* growth rate to follow the same pattern as in the synthetic media (MBL).

On the other hand, *Chlorella vulgaris* in non-sterilized agriculture wastewater took fourteen days to move from the lag phase to the exponential phase, and after twenty-one days of cultivation, the growth rate started to decline. As agriculture wastewater sample in Experiment (3) was not sterilized, in which other microorganisms exit, *C. vulgaris* took more extended time (fourteen days) to adapt the new environment and to enter the exponential phase. The reason behind this delay is due to the limited amount of nutrients in the agriculture wastewater sample on which other microorganisms in the non-sterilized agriculture wastewater compete with the *C. vulgaris*.

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As demonstrated in Figure (18), the difference between the growth rate in non-sterilized agriculture wastewater in Experiment (3), and the benchmark in Experiment (1) is still significant, and even less than the growth rate recorded from sterilized agriculture wastewater in Experiment (2)

From the observations above we can conclude that sterilization is not a compelling factor on the cultivation of *C. vulgaris* in agriculture wastewater medium using indirect-sun light. As the elimination of other microorganisms from the agriculture wastewater supported only in accelerating the *C. vulgaris* reach to the exponential phase, yet the amount of available nutrients is still the compelling factor *C. vulgaris* growth in agriculture wastewater. Therefore, this cultivation setup was excluded from the enlargement phase.

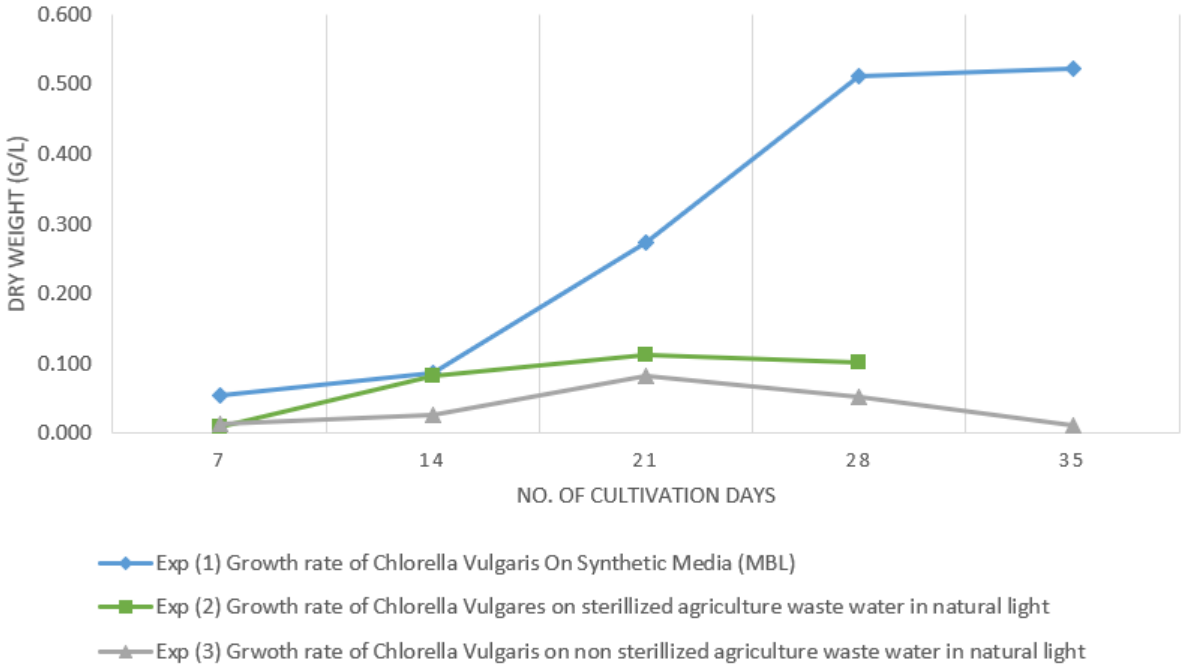

Figure 18: Sterilization effect on growth rate under sunlight in agriculture wastewater

4.1.2 Sterilization effect in municipal wastewater under indirect sunlight

The sterilization effect in municipal wastewater behaved differently than its effect on agriculture wastewater. Sterilizing the municipal wastewater medium caused strange growth

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pattern for *Chlorella vulgaris*. After seven days of cultivation, the growth rate for *Chlorella vulgaris* in sterilized municipal wastewater recorded a significantly high value of 0.332 (g/L), which is higher with 0.277 (g/L) than the benchmark after the same cultivation period. However, after another cultivation week, the growth rate declined with 27% to record a growth rate of 0.244 (g/L). Although this declination was not expected yet the growth rate is still higher than the benchmark after the same cultivation period.

Three weeks after the cultivation start date in sterilized municipal wastewater, the growth rate recorded its maximum value of 0.532 (g/L) which represents a 49% increase in growth rate compared to the benchmark. Unexpectedly, a sharp decline in the growth rate reoccurred as observed after the first week, as demonstrated in Figure (19). The reason behind this unexpected deterioration is that at the beginning of the cultivation C. *vulgaris* growth was supported with available nutrients in the municipal wastewater; however, as wastewater treatment plant, from which the municipal wastewater sample was collected, performs only secondary treatment, other dissolved organic matters (DOM) still exist in the municipal waste which usually is consumed by other organisms in the municipal wastewater. However, sterilization of the municipal wastewater sample had eliminated the referred to organisms which caused inhibition to *C. vulgaris* growth in the second week. As *C. vulgaris* is recognized with its high adaptation capabilities, it entered the exponential phase after the drop-in growth rate from the second week and continued its cycle, yet the existence of other DOMs didn't allow the *C. vulgaris* to stay in the stationary phase, the growth rate declined immediately after twenty-one days of cultivation.

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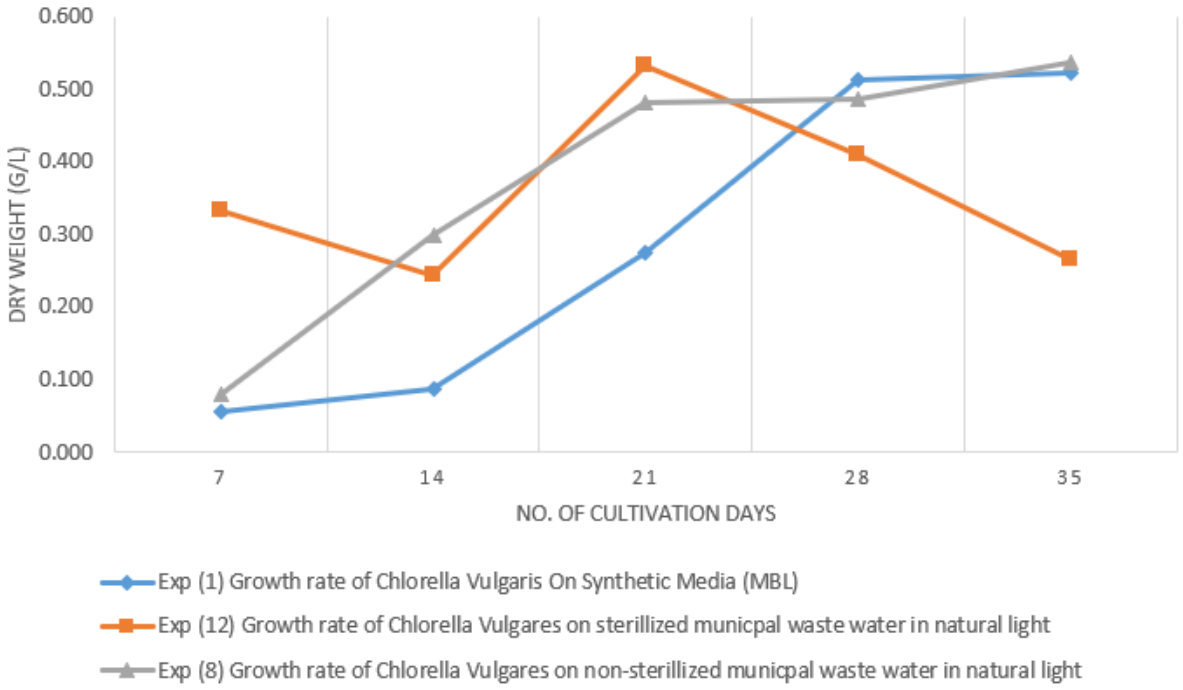

Figure 19: Sterilization effect on growth rate under sunlight in municipal wastewater

On the other hand, non-sterilized municipal wastewater proved to be a very promising medium for cultivation of *C. vulgaris*. After one week from starting the cultivation,

C. vulgaris entered the exponential phase with one week ahead of the growth rate in synthetic medium (MBL). Through the second, and third cultivation weeks, non-sterilized municipal wastewater demonstrated growth rates better than the benchmark (3.4 times higher for the second week, 1.75 times higher for the third week), growth results are presented by the Table (9).

As demonstrated in Figure (19), twenty-eight days after the cultivation start date, the growth rate for *C. vulgaris* in non-sterilized municipal wastewater followed a very similar pattern to the growth rate on synthetic medium (MBL).

Maintaining the municipal wastewater non-sterilized allowed for the existence of other microorganisms such other bacteria which consumed dissolved organic matters (DOM) in the

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wastewater, and remineralize organic compounds (Carbon, Sulphur, Phosphorus) to its inorganic form which are consumed by *C. vulgaris*, thing that allow better growth rate for *C. vulgaris;* this interaction is referred to as microalgae – bacteria symbiosis (Buchan et al., 2014).

As a result, the cultivation on non-sterilized municipal wastewater was nominated for the enlargement phase.

Table 9: Growth rate comparison: synthetic medium and non-sterilized municipal wastewater

# of Days		7	14	21	28	35
Synthetic Medium (MBL)	OD680	0.100	0.158	0.496	0.928	0.946
	DW (g/L)	0.055	0.087	0.274	0.512	0.522
Non-sterilized municipal wastewater	OD680	0.145	0.542	0.872	0.882	0.972
	DW (g/L)	0.080	0.299	0.481	0.487	0.537

4.1.3 Sterilization effect under white LED light with 24 hours illumination

White led light, with 24 hours illumination, stimulated *C. vulgaris* in both sterilized, and non-sterilized municipal wastewater to enter the exponential phase after only seven days from starting the cultivation. However, in the second week, *C. vulgaris* entered the stationary phase while the growth rate in synthetic medium (MBL) continued its exponential growth. In the third week, the growth rate in sterilized medium started to decline, and the growth rate from non-sterilized medium followed it three weeks later as demonstrated in Figure (20).

The continuous exposure to led light empowered *C. vulgaris* with the required energy to adapt to the municipal wastewater medium but caused light saturation to *C. vulgaris* which inhibited the growth rate. Although the existence of bacteria in non-sterilized municipal wastewater supported the growth of *C. vulgaris*; however, the light saturation caused inhibition of the growth rate

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A possible reason for this declination is the continuous stress that the microalgae exposed to by illuminating the cultivation environment continuously for 24 hours (Ogbonna & Tanaka, 2000).

We can conclude that utilizing led light did not support *C. vulgaris* to grow in a pattern neither similar to nor close to the growth rate from the synthetic medium (MBL) whether the municipal wastewater was sterilized or not. Therefore, this cultivation setup was excluded from the enlargement phase.

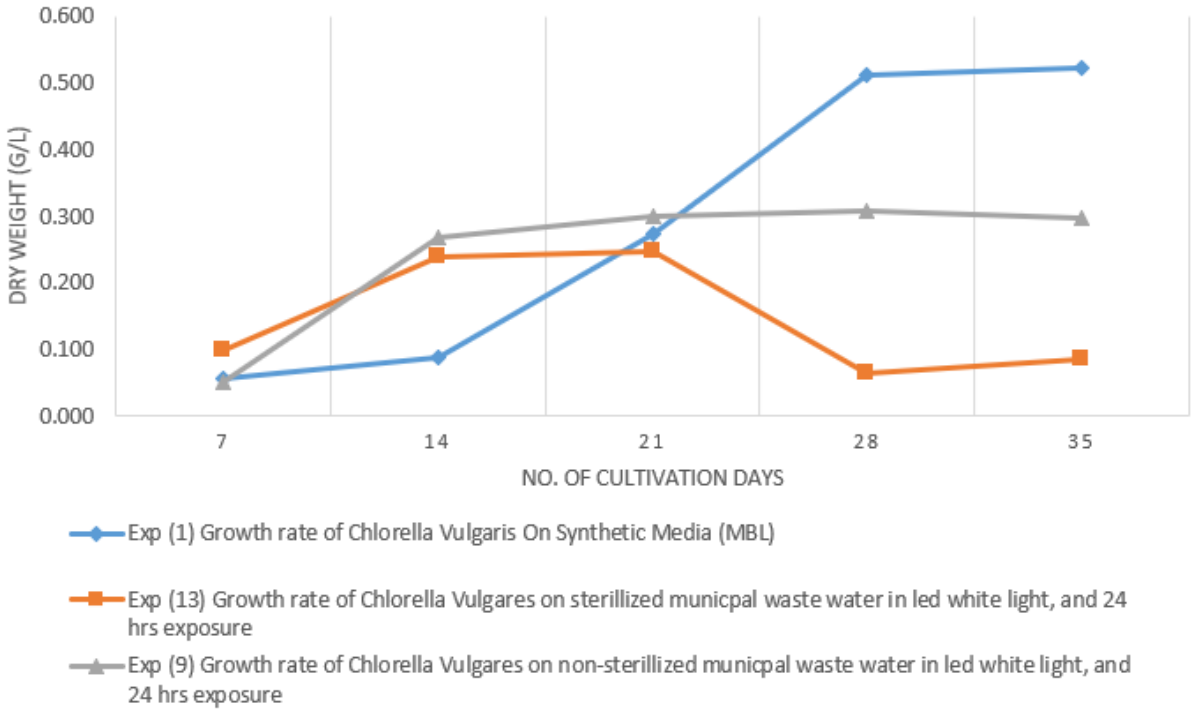

Figure 20: White led light effect on growth rate in municipal wastewater

4.2 LIGHTING COLOR EFFECT ON GROWTH RATE

The growth rate for *Chlorella vulgaris* in blue wavelength of led light with 24 hours illumination behaved similarly with white light regarding entering the exponential phase just after one week of cultivation and declined in the second week, as demonstrated in Figure (21). The continuous illumination has a significant adverse effect on the growth rate for *C. vulgaris*

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(Ogbonna & Tanaka, 2000) regardless of the lighting wavelength. Therefore, this cultivation setup was excluded from the enlargement phase.

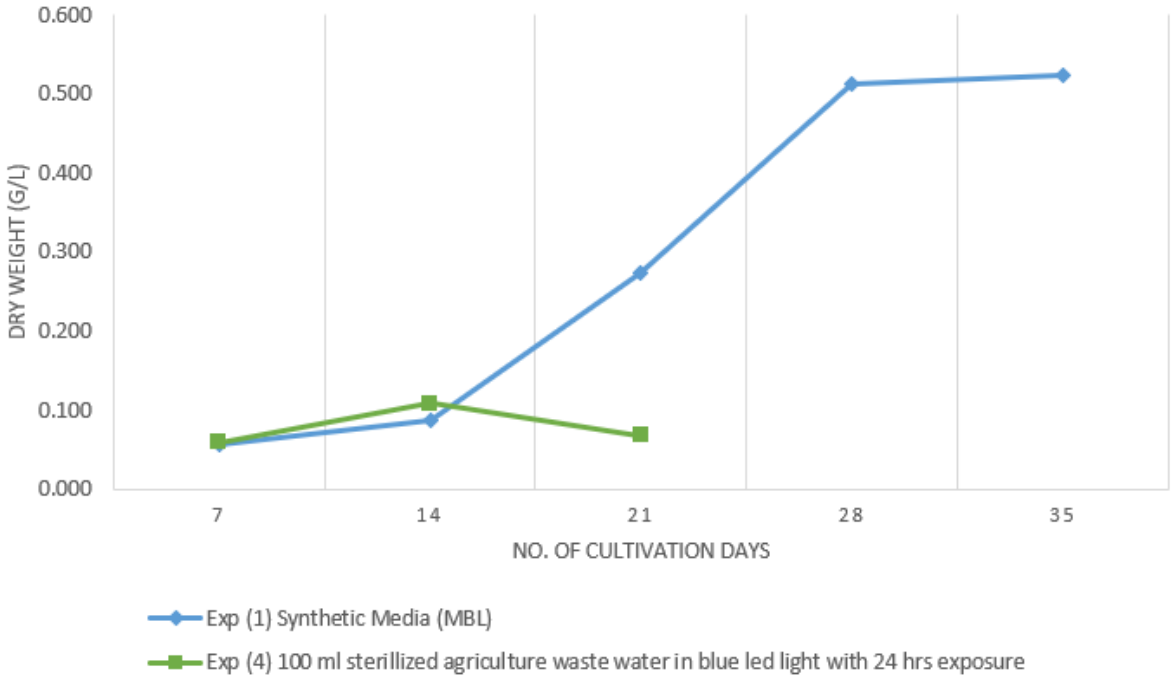

Figure 21: Blue led light effect on growth rate in municipal wastewater

4.3 MEDIA MIXING EFFECT

4.3.1 MIXING EFFECT UNDER LED LIGHT

In experiments to test wither mixing synthetic medium (MBL) with municipal wastewater will stimulate the *Chlorella vulgaris* growth rate or not, two experiments were conducted for each 25 ml of synthetic medium (MBL) were added to 75 ml of non-sterilized municipal wastewater in one experiment, and to 75 ml of sterilized municipal wastewater in another experiment.

Both experiments were conducted under led light with 24 hours illumination. The recorded growth rates from both experiments were very similar to each other in which *Chlorella vulgaris* entered the exponential phase after first cultivation week and continued to

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rise until the third week after which the growth rate started to decline, as demonstrated in Figure (22).

We can observe that led lighting effect enforced the growth rate to follow the same pattern from section 4.1.3. It is becoming evident that putting *Chlorella vulgaris* under continuous stress of illumination suppresses the growth rate (Ogbonna & Tanaka, 2000). Therefore, this cultivation setup was excluded from the enlargement phase.

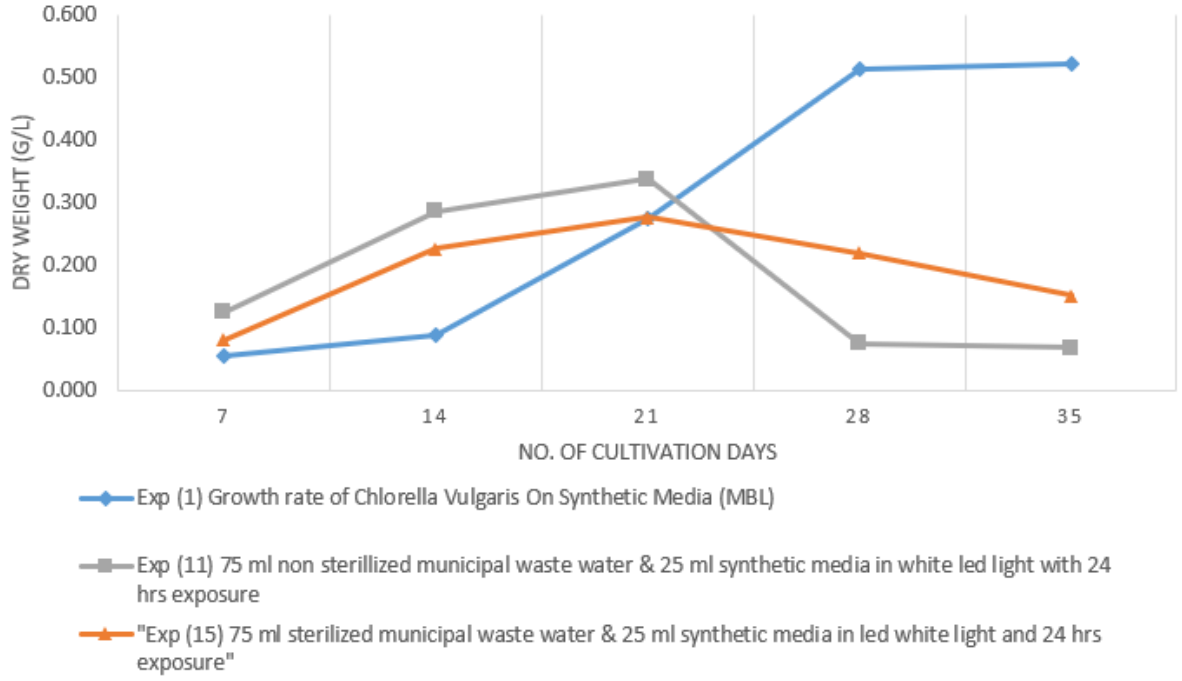

Figure 22: Mixing effect of synthetic medium & municipal wastewater under led light

4.3.2 Mixing effect under indirect sunlight

4.3.2.1 Mixing effect (synthetic medium & agriculture wastewater) under indirect sunlight

It is clear how mixing agriculture wastewater with synthetic medium (MBL) has significantly stimulated the growth rate of *Chlorella vulgaris*. Mixing non-sterilized agriculture wastewater with synthetic medium (MBL) has shown a significant growth rate, even higher

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than the benchmark, as demonstrated in Figure (23). The thing that has strongly nominated that cultivation condition for the enlargement phase. The growth rate from mixing sterilized agriculture wastewater with synthetic medium (MBL) also showed promising results, yet not as high as those observed from mixing with non-sterilized agriculture wastewater. The reasons behind this finding are adding synthetic media (MBL) to the non-sterilized agriculture wastewater supplied *C. vulgaris* with the missing macro and micronutrients which the agriculture wastewater lacks too.

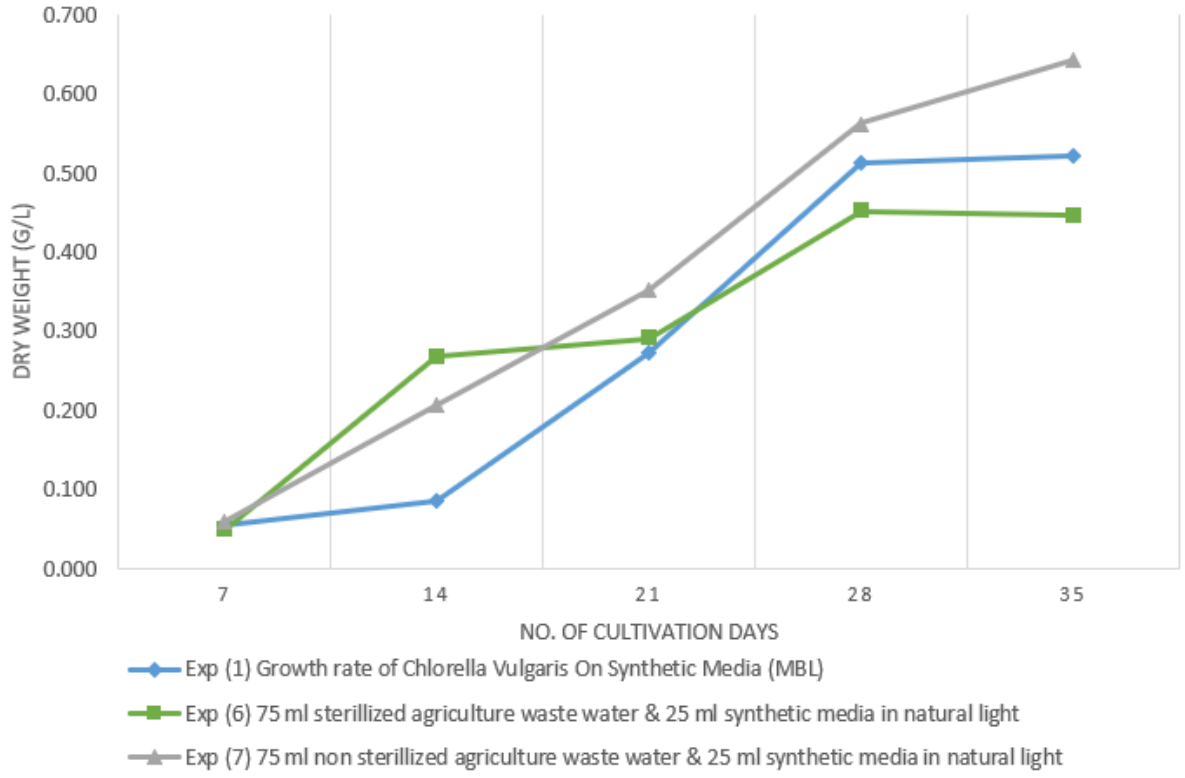

Figure 23: Mixing effect of synthetic medium & agriculture wastewater under sunlight

4.3.2.2 Mixing effect (synthetic medium & municipal wastewater) under indirect sunlight

The first observation we can notice from mixing synthetic medium (MBL) with municipal wastewater, that in both sterilized and non-sterilized municipal wastewater mixture

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the growth rate for *Chlorella vulgaris* started at a higher value compared to that recorded from the benchmark.

Although starting at a very high value, growth rate on non-sterilized municipal wastewater started to decline immediately from the second week. For sterilized medium, the exponential phase continued for three weeks, and declination started in the fourth week, as demonstrated in Figure (24). Although mixing synthetic medium (MBL) with sterilized municipal wastewater in Experiment (14) provided promising results as high as with which was recorded from Experiment (8) (cultivation on pure non-sterilized municipal wastewater) and this is because mixing the municipal wastewater with synthetic medium (MBL) enriched the media with required macro and micronutrients, yet for the objective of reducing the production cost, the setup in Experiment (8) was selected for the enlargement phase as it gave similar promising results without the additional cost of chemicals required for the synthetic medium (MBL) Therefore, this cultivation setup was excluded from the enlargement phase.

The data from Experiment (14) are close to the findings from (Mostafa S. S. M., 2012) research

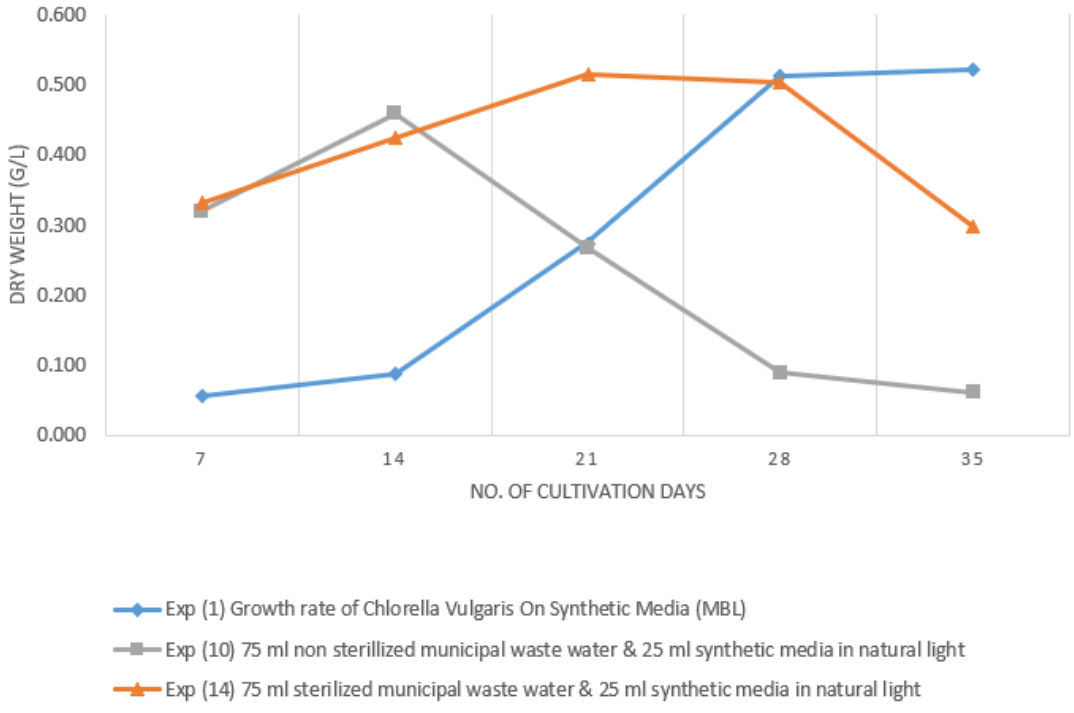

Figure 24: Mixing effect of synthetic medium & municipal wastewater under sunlight

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4.4 ENLARGEMENT PHASE RESULTS

As explained in section 4.3, two cultivation conditions were selected for the enlargement phase which are:

- Non-sterilized agriculture wastewater mixed with the synthetic medium under indirect sunlight
- Non-sterilized municipal wastewater medium under indirect sunlight

So, for each cultivation condition, three replicas were prepared with 500 ml each. The mixture proportion between non-sterilized agriculture wastewater and the synthetic medium (MBL) was 75% to 25% by volume respectively. Moreover, a synthetic medium (MBL) was prepared as well to be used as a benchmark for comparison. The two cultivation groups and the synthetic medium were cultivated under the same conditions, and during the same period to ensure that all media are exposed to the same conditions. On a weekly basis, the growth rate, regarding dry weight, and the total lipid content were measured for each experiment. It was observed that for agriculture wastewater experiments the average growth rate after seven cultivation days was higher than the benchmark which was recorded from the synthetic medium (MBL). On the other hand, for municipal wastewater experiments, the average growth rate was lower than the benchmark. For the remaining of the cultivation period, the growth rates were below the benchmark for all experiments as demonstrated in Figure (25) which illustrates the growth rate comparison.

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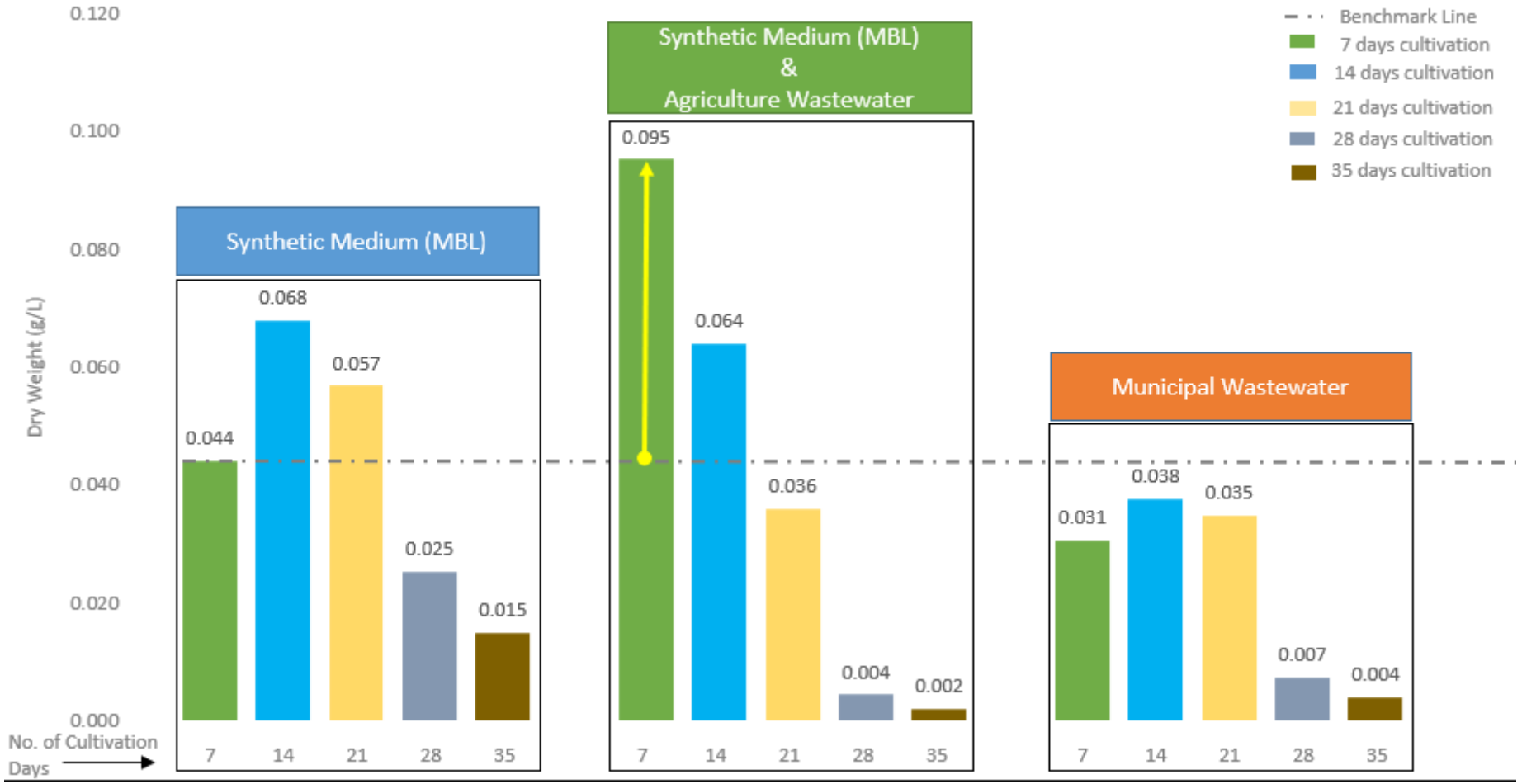

Figure 25: Growth rate comparison between different cultivation media

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For total lipid produced, it is clear as presented in Figure (26) that after 14 days of cultivation, the agriculture wastewater and synthetic medium (MBL) mixture achieved the highest results compared to other media.

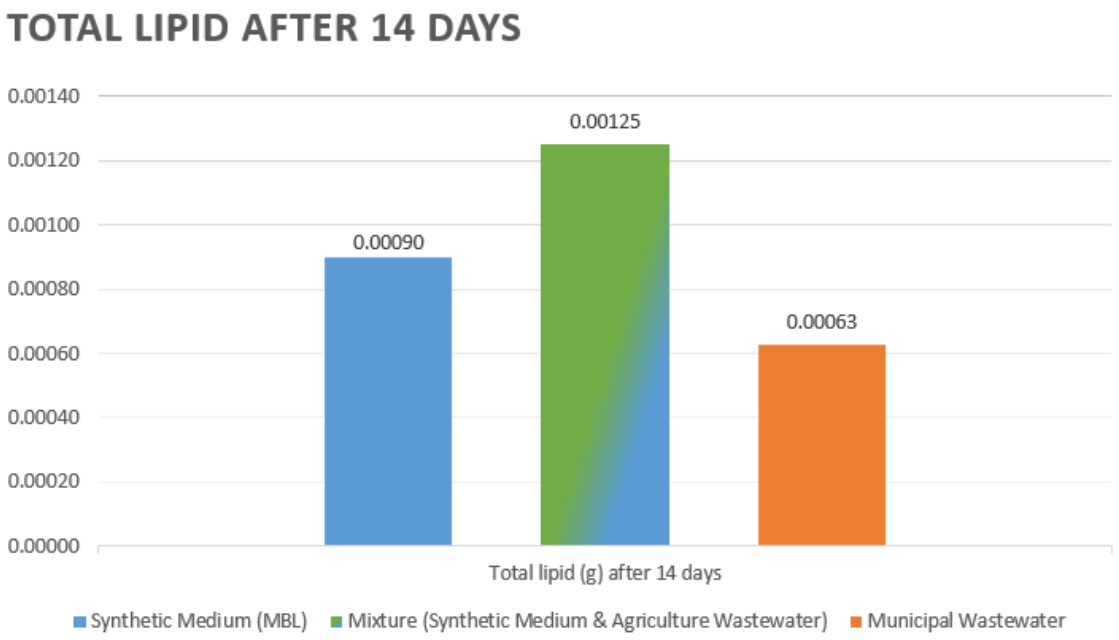

Figure 26: Total lipid comparison after 14 days

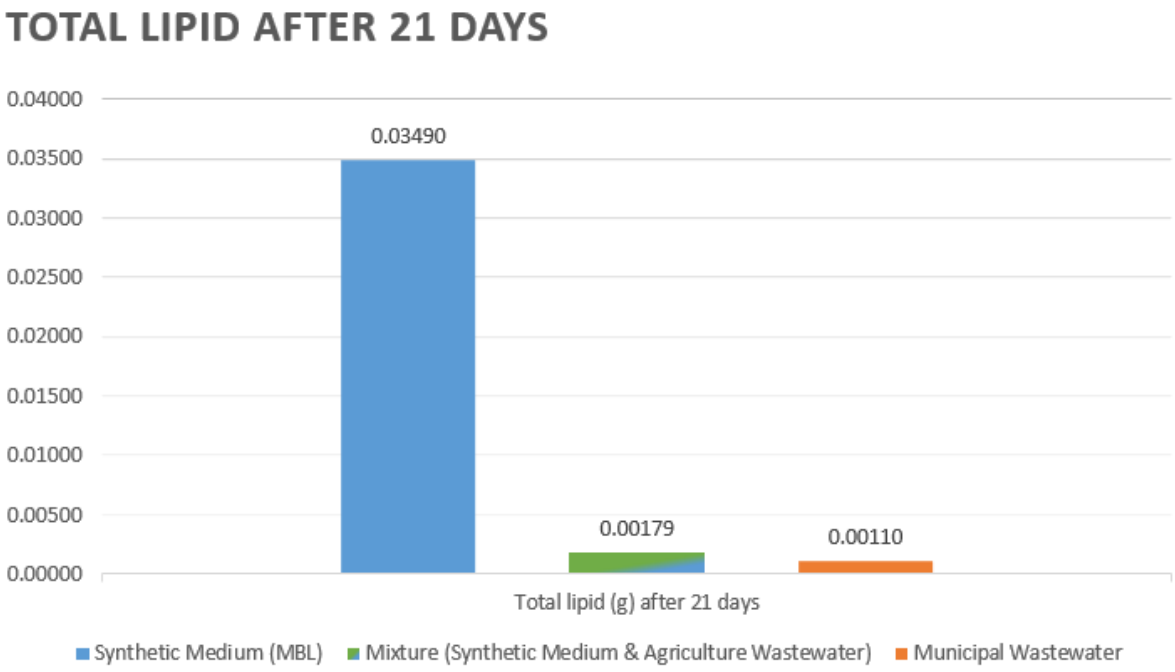

Figure 27: Total lipid comparison after 21 days

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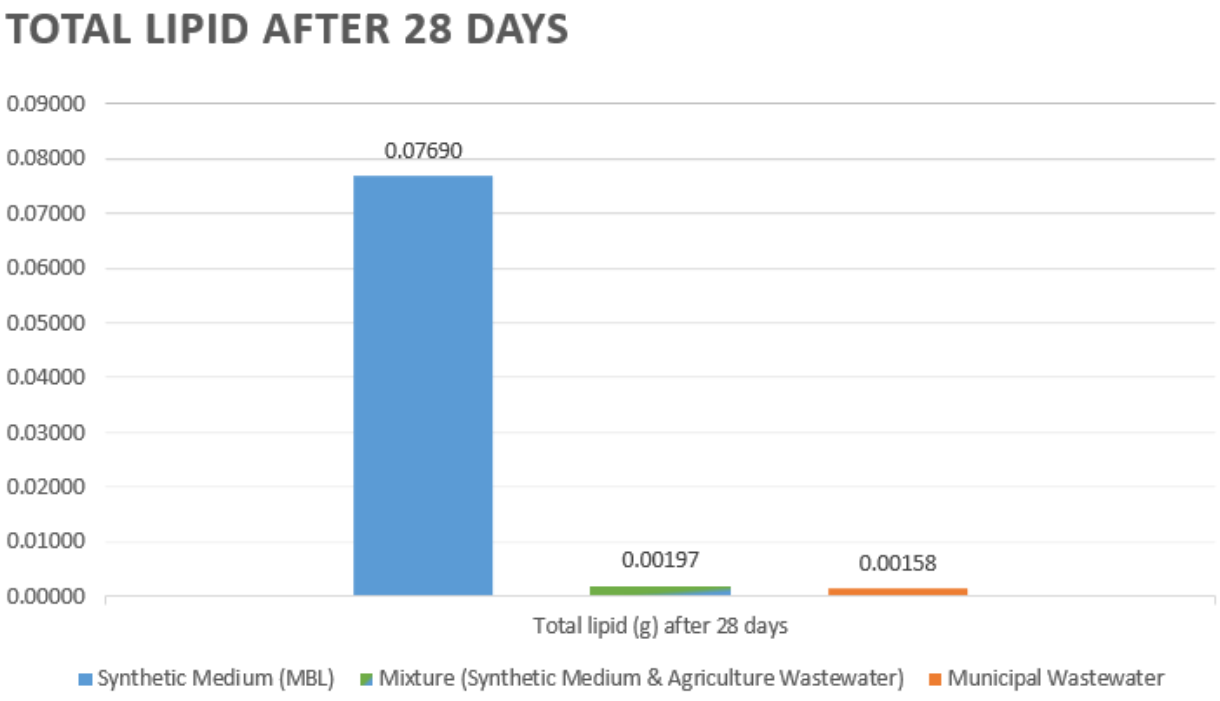

Figure 28: Total lipid comparison after 28 days

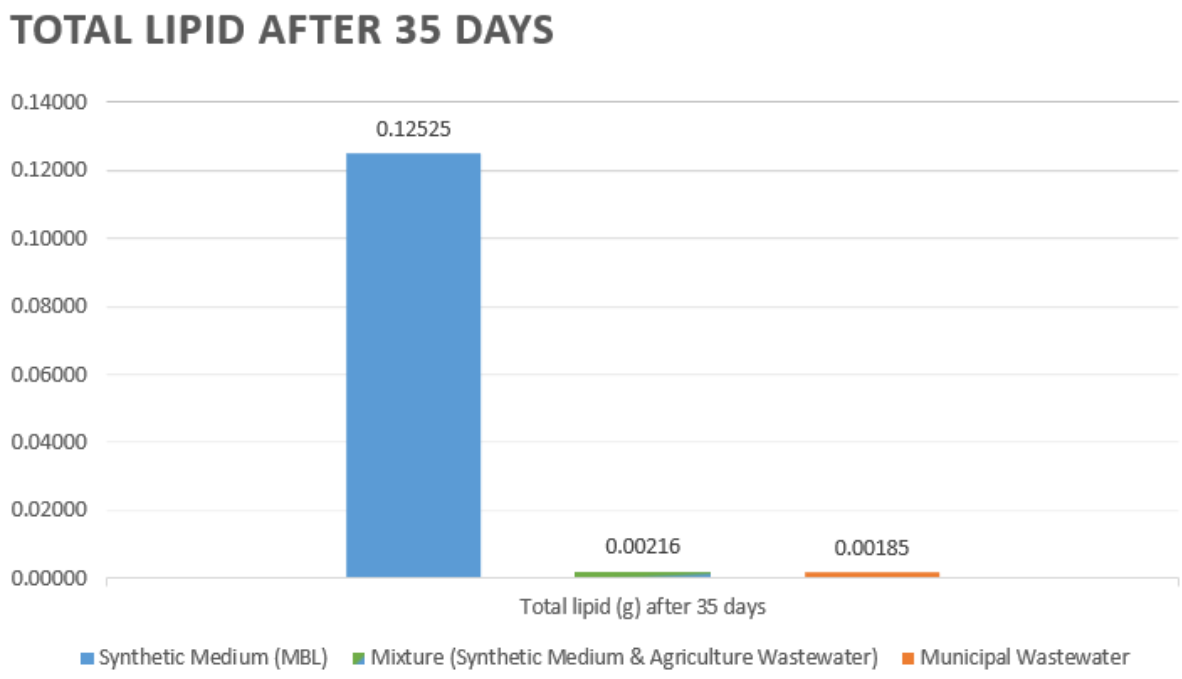

Figure 29: Total lipid comparison after 35 days

The rise of total lipid in agriculture wastewater and synthetic medium (MBL) mixture is due to the $FeCl_3$ in the MBL which can cause an increase in the total lipid by up to 56.6% of the dry biomass weight (Liu, Wang, & Zhou, 2008)

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CHAPTER 5 - CONCLUSION & RECOMMENDATIONS

5.1 CONCLUSION

This research aimed to support the principle of relying on microalgae as an economically feasible renewable source for biodiesel production. Utilizing microalgae for biodiesel production is encountered with the challenge of high production costs. Chemicals in synthetic media that present the nutrients for the microalgae cultivation have a direct relation to the overall production cost, which constitutes an obstacle for replacing traditional diesel with microalgae-based biodiesel.

In a trial to overcome this obstacle, this research investigated the possibility of utilizing nutrients that are available in agriculture, and municipal wastewaters for the cultivation of microalgae. Different cultivation conditions were tested to achieve growth rate as close as possible to the growth rate from the synthetic medium (MBL). *Chlorella vulgaris* was selected for this research due to its availability, and its capability to adapt in different cultivation environments. Agriculture wastewater was collected from an agriculture drainage canal in Idku city, Bahira governorate; while the municipal wastewater was collected from Al Katamya treatment plant in Cairo governorate. From different cultivation parameters that can affect the *Chlorella vulgaris* growth rate, sterilization effect, lighting wavelength, lighting exposure time, and mixing the synthetic medium with wastewater were selected for tests. Other cultivation parameters such as pH, temperature CO_2 ratio were set within the acceptable range. Cultivations were conducted in a closed system to control the cultivation parameters, and to reduce the contamination probability.

From the fifteen different initial cultivation setups, two were selected for the enlargement phase due to their high achieved growth rate. Those two setups are as following:

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- 75% vol. of non-sterilized agriculture wastewater mixed with 25% vol. of synthetic medium (MBL) in indirect sunlight under 16:8 light to dark cycle.
- Non-sterilized municipal wastewater in indirect sunlight under 16:8 light to dark cycle.

Agriculture wastewater group achieved the highest growth rate after seven cultivation days and the highest total lipid production after fourteen cultivation days. On the other hand, recorded results from the municipal wastewater group were below the benchmark.

We can conclude that in order to get the highest total lipid from *C. vulgaris,* when utilizing a mixture of agriculture wastewater and synthetic medium (MBL) extraction should be conducted after fourteen cultivation days.

This finding promotes the agriculture wastewater and synthetic medium (MBL) mixture as a promising alternative for the pure synthetic medium (MBL). Finding a replacement for the pure synthetic medium should support the reduction in total cost for producing biodiesel from microalgae. Applying this work finding on (Juan J. Jaramillo, et al, 2012) feasibility study for producing microalgae oil from *Chlorella vulgaris,* we can conclude that replacing the formulated media in (Juan J. Jaramillo, et al, 2012) model with the agriculture wastewater and synthetic medium (MBL) mixture from this work should reduce the production cost with 12.6% for removing 75% of the required nutrients, and another 29.4% for substituting the artificial light with sunlight. Therefore, the total saving will be 41.7%.

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5.2 RECOMMENDATIONS

The following are recommendations for future researches:

- Different mixing ratios between agriculture wastewater and synthetic medium (MBL) need to be examined to investigate if better total lipid production can be achieved.
- Replacing the synthetic medium, in the agriculture wastewater and synthetic medium mixture, with municipal wastewater needs further investigation which may support further reduction in the overall production cost
- Researching the optimum illumination exposure time that achieves high growth rate and lipid production for cultivating *Chlorella vulgaris* in wastewater.

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